EFFECTS OF ULTRAVIOLET RADIATION ON MICROTUBULE ORGANISATION AND MORPHOGENESIS IN PLANTS

Irina Staxén

Section of Plant Physiology
Lund University, Sweden

Doctoral Dissertation

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Irina Staxén

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Abstract

The involvement of the cytoskeleton in the development of somatic embryos was studied in Larix x eurolepis. Protoplasts were isolated from both somatic embryo-regenerating and non-regenerating cultures and fractionated on a discontinuous Percoll density gradient, whereby a highly embryogenic protoplast fraction could be enriched. Protoplasts of two cell lines of Larix eurolepis, one with regenerating potential and one lacking this potential, were compared. In contrast to the non-regenerating line were a protoplast-like organisation of the cortical microtubules was maintained, re-organisation of this microtubular network occurred in the regenerable line after only three days of culture, indicating that organised growth was occurring. However, this early organisation of cortical microtubules may not always be a valid marker for regenerable and non-regenerable material.

In order to investigate the effect of ultraviolet-B (UV-B, 280-320 nm) radiation on the microtubule cytoskeleton, protoplasts were isolated from leaves of Petunia hybrida and subjected to four different doses of UV-B radiation. The organisation of the microtubules and the progression of the cells through the cell cycle was observed at 0, 24, 48 and 72 h after irradiation. UV-B induced breaks in the cortical microtubules resulting in shorter fragments with increasing amounts of radiation. Also, the division of the protoplasts was delayed, which was related to the absence of an intact microtubule network.

Whole Petunia plants were grown in growth chambers in the presence and absence of UV-B. The plants responded to UV-B with increased rates of CO_2 assimilation, a 60% increase in UV-screening compounds and changes in the morphology of the leaves that were reflected in a 70-100% increase in leaf area and 20% decrease in leaf thickness. The microtubules of the epiderm .l cells was not affected by UV-B, nor was the number of epidermal cells (per unit area). The increase in leaf area in the UV-treated plants appeared due to stimulation of cell division in the leaf meristems.

Key words cytoskeleton; Larix x eurolepis; microtubule; ozone depletion; Petunia hybrida; plant; protoplast; somatic embryogenesis; ultraviolet tadiation; ultrastructure; UV

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Signature I Staxén **Date** 940804

"That is very important," the King said, turning to the jury. They were just beginning to write this down on their slates, when the White Rabbit interrupted: "*Un*important, your Majesty means, of course," he said in a very respectful tone.

"*Un*important, of course, I meant," the King hastily said, and went on to himself in an undertone, "important - unimportant - unimportant - important" as if he were trying which sounded best.

Some of the jury wrote it down 'important' and some 'unimportant.' Alice could see this, as she was near enough to look over their slates; "but it doesn't matter a bit," she thought to herself.

Alice's Adventures in Wonderland

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List of separate publications

This thesis is based on the following publications, which will be referred to by their Roman numerals.

I Staxén I, Klimaszewska K and Bornman CH (1994) Microtubular organization in protoplasts and cells of somatic embryo-regenerating and non-regenerating cultures of *Larix*. Physiol Plant 91: 680-686

II Staxén I, Bergounioux C and Bornman JF (1993) Effect of ultraviolet radiation on cell division and microtubule organization in *Petunia hybrida* protoplasts. Protoplasma 173: 70-76

III Staxén I and Bornman JF (1994) A morphological and cytological study of *Petunia hybrida* exposed to UV-B radiation. Physiol Plant 91: 735-740

IV Staxén I, Bornman JF and Dubé S Responses of *Petunia hybrida* plants exposed to ultraviolet-B radiation. Photosynthetic gas exchange characteristics, growth and ultrastructure. Submitted to Environ Exp Bot.

Abbreviations

ATP	adenosine triphosphate
DAPI	4,6-Diamidino-2-phenylindole
DW	dry weight
HMM	heavy meromyosin
LA	leaf area
PAL	phenylalanine ammonia lyase
PPB	preprophase band of microtubule
SLA	specific leaf area
SLW	specific leaf weight
SOD	superoxide dismutase
UV	ultraviolet
UV-A	ultraviolet-A radiation (320-400 nm)
UV-B	ultraviolet-B radiation (280-320 nm)
$UV\text{-}B_{BE}$	biologically effective UV-B radiation
UV-C	ultraviolet-C radiation (200-280 nm)
UV dose	irradiation x time
WUE	water use efficiency

Preface

The work presented in this thesis is an amalgamate of the involvement of microtubules in plant morphogenesis and the effect of ultraviolet radiation on the cortical microtubules. At the onset of the work it was proposed that changes in the organisation of cortical microtubules in leaf cells could explain some of the changes in leaf morphology that were known to be induced by ultraviolet-B radiation. The involvement of microtubules in morphogenesis and differentiation was examined during the differentiation of somatic embryos in *Larix*. The effect of ultraviolet radiation on microtubules was investigated in *Petunia* protoplasts and *Petunia* leaf epidermal cells.

Today we often hear about the danger related to the decrease in stratospheric ozone and the negative effects it may have on organisms through the increase in short wavelength ultraviolet radiation. However, *Petunia* plants show remarkable tolerance to ultraviolet radiation and respond with mainly morphological changes besides increased biomass accumulation and CO_2 assimilation. Even though exposure of isolated protoplasts to ultraviolet radiation leads to depolymerisation of cortical microtubules and cell cycle arrest, these responses are absent in epidermal tissues from whole plants exposed to even higher ultraviolet doses compared to those used in the irradiation of protoplast suspensions. Therefore, in the section dealing with ultraviolet radiation, the effort has been directed towards explaining how tolerance to ultraviolet radiation is possible.

Microtubules, together with actin filaments, are involved in a multitude of cellular processes in plants and control the development of the plant as well as the physiology of differentiated cells. It has been impossible to name all of those processes and the complex interactions that exist between microfilaments and microtubules in all cells. Instead, only those aspects of microtubular organisation and function that are relevant to the present work have been dealt with.

The number of plant species that have been tested for sensitivity to ultraviolet radiation is in excess of 300. Unfortunately, the variation in radiation doses and irradiation conditions used by the different research groups is just about as large. It is a well accepted fact that the level of background visible light present during the exposure

of plants to ultraviolet radiation is very important for the type of response that will be induced in the plant. In this work only studies conducted under relatively high background visible light have been considered and cited. In the papers cited filters have been used to remove the ultraviolet-C radiation emitted by the fluorescent lamps used to generate UV radiation, so that the experimental protocols are similar to those used in the papers of this thesis. Even so, it can be difficult to compare responses in rice, pine, soybean and *Petunia* subjected to different irradiation regimes and try to extract anything more than a very superficial similarity/dissimilarity in the responses to ultraviolet radiation of plants. For this reason this type of comparison has been kept to a minimum.

Finally, it has been a problem to choose references for citation. They are too few and too many --there are as many opinions as there are readers. When possible, review articles have been cited. Well accepted relationships have been left without reference, even though many years of research and a large amount of effort lie behind these statements. I hereby present my apologies to all the researchers whose work I have not cited, but without which this work would not have been possible.

1 The Cytoskeleton

The term "cytoskeleton" is used to describe cytoplasmic fibrillar cell components. Because the cytoskeleton is composed of several heterogeneous classes of proteins that form dynamic structures in the cells, it has been difficult to provide an all-encompassing definition of the cytoskeleton. One characteristic shared by all components of the cytoskeleton is that they can be separated from the bulk of the cytoplasmic proteins and membranes by extraction with non-ionic detergents. The working definition of the cytoskeleton is therefore as follows: the proteinaceous structure that remains of a cell after extraction with non-ionic detergent. The fact that the cytoskeleton retains its fine structure despite such harsh treatment indicates its intrinsic stability.

Considering biochemical properties of the proteins that build up the filaments and their fine structural appearance (i.e. as seen in electron microscopy sections), three classes of cytoskeletal components can be recognized in both animal and plant cells: intermediate filaments, microfilaments and microtubules.

1.1 Intermediate filaments

Intermediate filaments are a heterogeneous class of proteins which, when polymerised, are very similar in structure and build up filaments of 10 nm diameter (Klymkowsky et al. 1989). The filaments are composed of fibrous polypeptides that have a globular head and a coiled coil rod domain. Several classes are recognized in animal cells: cytokeratins, desmin, vimentin, glial acidic protein and neurofilaments. Lamins, a class of fibrillar proteins that are associated with the nuclear envelope where they probably perform the function of nuclear skeleton, are related to intermediate filaments.

In plant cells, all available data come from studies of cross-reactivity between plant material and antibodies raised against animal intermediate filaments . Dawson et al. (1985) showed, by this method, the existence of intermediate filaments in *Daucus carota*. In that and later studies (Goodbody et al. 1989, Hargreaves et al. 1989) it was shown that intermediate filaments in plants are in close association with microtubules, in that they are found in interphase microtubule arrays, the preprophase band, the spindle and the phragmoplast. The function of intermediate filaments in plant cells is not known,

and electron microscopists have failed, with a few exceptions (Shaw et al. 1991), to show the existence of 10 nm filaments in the cytoplasm of plant cells. This is all the more peculiar considering the fact that intermediate filaments are the most stable of the three classes of cytoskeletal components.

1.2 Microfilaments

Microfilaments are composed of a globular protein called G-actin, which under conditions of high ionic strength and in the presence of ATP polymerizes to fibrillar actin, called F-actin or microfilaments. In cells, actin is in equilibrium between the G-form in a cytoplasmic pool or the F-form. Actin filaments have a diameter of approximately 7 nm, and have been found in all eucaryotic cells. They are generally associated with motility because they were first described in muscle cells where they build up the contractile filaments. Microfilaments are involved in locomotion in other organisms as well, such as amoeboid cells. Initially, the identification of F-actin was based on the property of myosin sub-fragments, the so-called heavy meromyosin (HMM), to bind specifically to F-actin generating an identifiable pattern in electron microscopy sections. Using this method, microfilaments as composed of actin, were first described in the alga *Nitella* by Palevitz et al. (1974). At first they aroused interest because of the body of evidence suggesting that microfilaments rather than microtubules were responsible for the movement of particles in the cortex of plant cells, the phenomenon known as cytoplasmic streaming or cyclosis. However, with the development of immunofluorescence techniques sample preparation times were cut down and the number of studies describing the distribution and organisation of an F-actin network in plant cells increased dramatically. Today, microfilaments are recognized as an essential component of plant cells (Lloyd 1988, Seagull 1989, Steiger and Schliwa 1987). On a general basis it can be said that the microfilaments form an extensive three-dimensional network in the cell cortex and extend from the plasma membrane to the nucleus.

The microfilaments of the cortical network run parallel to the long axis of the cell (Parthasarathy 1985, Parthasarathy et al. 1985, Pesacreta and Parthasarathy 1984). Actin has even been identified in structures formed by microtubules during cellular division (Palevitz 1987, Traas et al. 1987). In these structures actin is in filamentous form, as indicated by the reaction with phalloidin, a drug that binds to F-actin but not to G-actin.

As of today, the function of microfilaments in the context of cell division is not understood, but their presence in the preprophase band, the mitotic spindle and the phragmoplast implies a role for microfilaments during cytokinesis.

Actin is involved in cytoplasmic streaming and this is also the best studied role of actin in plant cells. The model systems for the study of actin in cytoplasmic streaming are the giant algae *Chara* and *Nitella*. Decoration of the actin filaments with HMM has shown that these form bundles with one polarity (Kersey et al. 1976, Palevitz and Hepler 1975), which could explain the unidirectional movement of particles in the cytoplasm. Also, myosin was isolated from the alga *Nitella* by Kato and Tonomura (1977) and the motion of particles along the microfilaments was shown to be ATP and Mg^{2+} dependent and inhibited by concentrations of free Ca^{2+} higher than 0.1 µM (reviewed in Seagull 1989), which is in support of the interpretation that organelle movement in plants is microfilament-based.

Another unequivocal role of the actin filaments is in chloroplast orientation. Plants have to optimize light harvesting for photosynthesis, either by orienting the chloroplast for maximal light absorption if light is sparse or by minimizing light absorption if the incident light is high and could damage the chloroplast. In the alga *Mougeotia*, chloroplasts are located at the endoplasm-ectoplasm interface, in close association with actin filaments. Upon treatment with cytochalasin B, a drug that disrupts the F-actin, movement is inhibited (Haupt and Scheuerlein 1990).

Other roles of actin filaments that are much less understood and seem to be less universal are for example gravity perception (Sievers et al. 1991), cell shape determination and tip growth (fungal hyphae; Levina et al. 1994).

1.3 Microtubules

Microtubules are ubiquitous in eucaryotic but absent in procaryotic cells. The most obvious function for the microtubules in both animal and plant cells is their involvement in the formation of the mitotic apparatus and thereby the separation of chromosomes during cell division. Except for this, both their organisation and function in non-dividing cells are different in plant and animal cells. In plant cells two groups of microtubular structures can be identified from a functional standpoint. First are the interphase microtubular arrays and second the structures formed by microtubules during cell

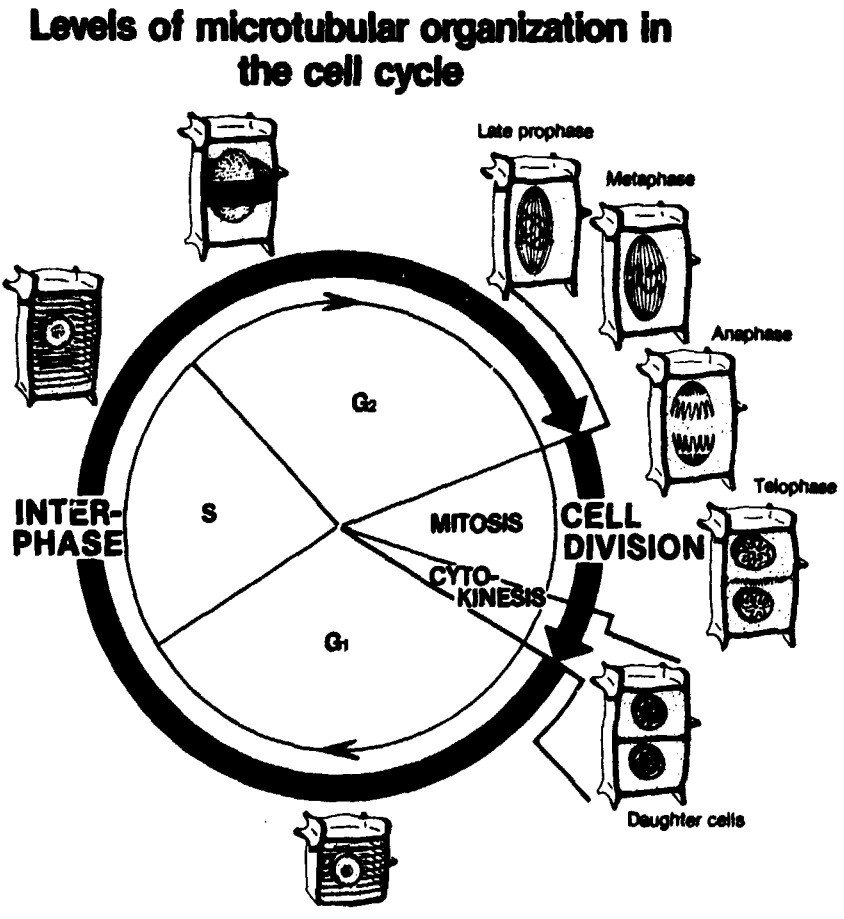


Fig. 1. Levels of microtubular organisation in the cell cycle. During most of the interphase, microtubules form a cortical array but before the onset of mitosis become arranged in a narrow band in close proximity to the plasma membrane, the so called preprophase band (PPB). The PPB is positioned in the equatorial plane of the nucleus. During mitosis, microtubules occur in the form of a spindle but disappear with the separation and movement of chromatids to opposite poles of the cell. New microtubules give rise to the phragmoplast, at the equator of which the cell plate forms. The cell plate differentiates towards, and eventually joins the wall of the dividing cell.

division: the preprophase band of microtubules, the mitotic spindle and the phragmoplast.

As the cell progresses through the cell cycle, the cortical microtubules disappear, the preprophase band forms transiently and gives way to the mitotic spindle. Eventually the phragmoplast forms. It has long been disputed whether the transition between interphase and mitosis necessitates new tubulin synthesis or if the tubulin of the interphase cortical arrays is re-used. New evidence based on microinjection of fluorescent labelled tubulin into *Tradescantia* stamen hairs favour the latter hypothesis (Zhang et al. 1993). The sequence of microtubular structures formed in plant cells and their relation to the cell cycle are shown in Fig. 1.

1.3.1 Interphase microtubule arrays

In animal cells at interphase, the microtubules are organised by distinct structures in the cytoplasm, the centrosomes. From these, the microtubules radiate out into the cytoplasm. Drug studies in which the cortical microtubules are depolymerized, indicate that these radial microtubule arrays are directly or indirectly responsible for the localization of various cytoplasmic organelles such as Golgi bodies (Sandoval et al. 1984, Thyberg and Moskalewski 1985), mitochondria (Ball and Singer 1982) and endoplasmic reticulum (Dabora and Sheetz 1988, Lee and Chen 1988).

The situation is different in plant cells. First, organelles such as endoplasmic reticulum and chloroplasts are anchored and move along the actin-based microfilament system. Second, plant cells at interphase lack distinctive microtubule organizing centres such as the centrosomes of animal cells. Instead, microtubules are organised in an ordered network in the cortical cytoplasm, close to the plasma membrane. The nature of the connection of the cortical microtubules to the plasma membrane is not known even if it is generally accepted that it exists and that the plasma membrane together with the nuclear envelope (Fig. 2) function as microtubule organizing centres. Evidence for the connection of the cortical microtubules to the plasma membrane is mostly indirect. Membrane fragments have been shown to contain fragments of microtubules after staining with antibodies specific for tubulin. The membrane ghosts lose their ability to nucleate microtubules after treatment with high concentration of salt (Shibaoka 1993). The existence of a transmembrane protein has been postulated for the anchorage of the cortical microtubules to the plasma membrane (Akashi and Shibaoka 1991).

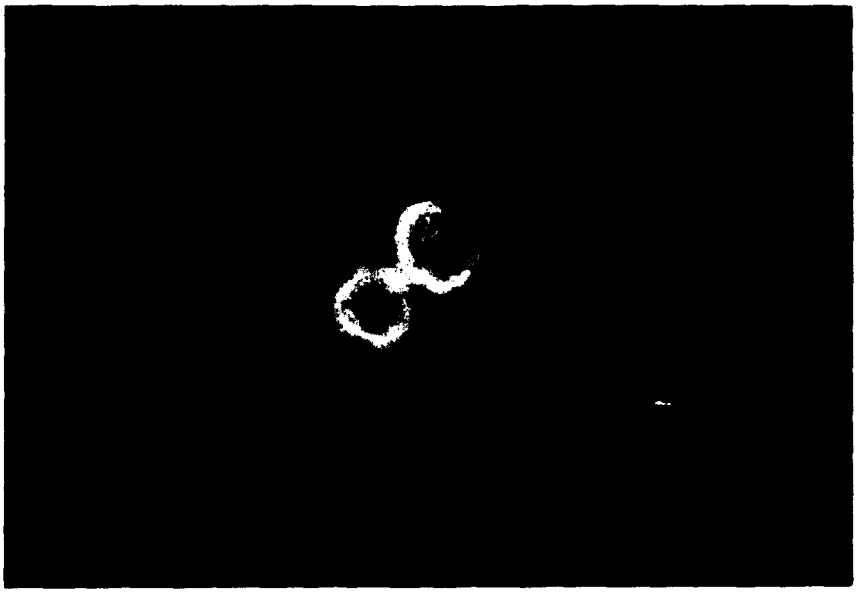


Fig. 2. After cell division, microtubules are formed on the nuclear envelope, which together with the plasmalemma function as microtubule organizing centers in plant cells.

Anchoring of the microtubules to the membrane confers them stability and ensures their proximity to the membrane, which is necessary for the only known function of cortical microtubules in plant cells: the orientation of cellulose microfibrils in the cell wall through directing the cellulose synthase complex in the plasma membrane (Ledbetter and Porter 1963, Palevitz 1982, Robinson and Quader 1982).

Except for the above mentioned function, the role of the cortical microtubuli in interphase plant cells is uncertain. The endoplasmic reticulum is connected to the actin microfilament system as shown by ultrastructural studies and drug induced depolymerisation of microfilaments in living cells (Lichtschield et al. 1990, Quader 1990). Chloroplasts are also anchored to and moved along microfilaments (see section on actin). With regard to Golgi bodies, mitochondria, nucleus and other organelles there is ambiguous evidence suggesting both action of the microtubules and microfilaments in the distribution of the organelles inside the cell. Myosin, the microfilament based motor, has been isolated from plant cells (*Arabidopsis*, Knight and Kendrick-Jones 1993; tomato, Vahey et al. 1982) and shown by immunofluorescence to localise with mitochondria, amyloplasts and vegetative nuclei of grass pollen (Heslop-Harrison and

Heslop-Harrison 1989), the nucleus of tobacco pollen (Tang et al. 1989) and endoplasmic reticulum (Higashi-Fujime 1988), suggesting that these organelles are connected to the microfilaments. The role of the cortical microtubules could be indirect through stabilisation and/or orientation of the cortical microfilament network, as microfilaments are not structurally as stable as the microtubules. Very often, the two cytoskeletal components co-distribute when plant cells are double-labelled for immunofluorescence. Actin filaments and microtubules have been shown to co-localise in all structures formed by the microtubule: the interphase cortical network, the preprophase band, the mitotic spindle and the phragmoplast. One line of evidence for the existence of a dynamic interaction between the microtubuli and the microfilaments comes from experiments in which depolymerisation of the microfilaments by cytochalasin treatment leads to the broadening of the preprophase band (Mineyuki and Palevitz 1990) even though actin is not necessary for the formation of the preprophase band. The proteins connecting the actin filaments to the microtubuli are not known. The role of the actin filaments in the phragmoplast seems to be the transport of vesicles containing the cell wall material to the cell plate that is deposited in the mid-region of the phragmoplast. The function of the actin filaments found in the mitotic spindle is more uncertain, but it has been proposed that they are involved in the poleward movement of the chromosomes (Czaban and Forer 1994). Microfilaments could also be active in distribution and organisation of the membranes (e.g. endoplasmic reticulum) that are found on the polar caps of the mitotic spindles and have also been shown to enclose the kinetochore-to-pole microtubules in mitotic cells (Hepler 1980, Ryan 1984, H Quader --personal communication). Mitotic spindles in plant cells are dynamic structures in themselves, aside from the chromosome movement (Palevitz 1993). The spindles are broad at metaphase (Fig. 6 in paper I) and undergo various degrees of contraction as the cell progresses through anaphase. An extreme case of contraction to a well focused point at the pole is found in conifers (Fig. 7 in paper I; Fowke et al. 1990; Paper I) but is by no means restricted to them. This focusing of the kinetochore fibres can also be found in, for example, onion root cells (Palevitz 1988) and in *Petunia* cells (Fig. 3). An actin based motility system could account for the contraction of the mitotic spindle.

Fig. 3. The poles of mitotic spindles of plant cells often undergo various degrees of contraction as the cell progresses through anaphase. This phenomenon has been described for conifers but can be found in other species as well. In this figure, the mitotic spindle of a *Petunia* cell is shown.

1.3.2 Microtubules in morphogenesis and cell polarity

The topic of the development of structural features of an organism or part of an organism (morphogenesis) and thereby of the establishment of a main morphological axis with structurally or physiologically distinct poles (cell polarity) is central to cell biology.

In plant cells, due to the presence of the cell wall, the determination of the final shape of an organ takes place at the time of cell division in the meristems, when the division plane of the meristematic cells is determined to either an anticlinal or a periclinal orientation. After the cell has divided, its position within the tissue is fixed. The next step in the determination of the final shape of the organ is at the level of control of the direction and extent of cell expansion. The exact mechanisms that determine the above mentioned processes is not understood but it most certainly involves signal transduction and gene expression as exemplified by the action of phytohormones (Shibaoka 1991) and of light (Zandomeni and Schopfer 1993) on the

orientation of cortical microtubules. The microtubules reflect, and perhaps even influence to some extent, the course of development of a cell concerning both the determination of the division plane and the direction of cell expansion.

In many of the systems studied, the cortical microtubules are often seen in parallel alignment to each other and in transverse orientation to the long axis of the cell. This is the case, for example, in epidermal cells of pea seedlings (Sakiyama and Shibaoka 1990), in the epidermal and cortical cells of mung bean (Roberts et al. 1985), as well as in elongating protoplast-derived cells (Hasezawa et al. 1988, Lloyd et al. 1980, Simmonds and Setterfield 1983, paper I). Microtubules are often seen to co-align with the cellulose microfibrils in the cell walls, and even if their involvement in the deposition of cell wall material has been debated by Emons et al. (1992), the widely accepted theory is that the cortical microtubules are involved in determining cell shape at the early stages of cell differentiation. This is indicated by the fact that depolymerisation of microtubules by colchicine in elongated, protoplast-derived cells eventually results in spherical cells, even though the cell wall is still present (Lloyd et al. 1980). The importance of the cortical microtubules in the determination of an axis of the cell and cell polarity is also illustrated by the fact that regenerating protoplasts can establish transverse cortical microtubular arrays faster than non-regenerating protoplasts. The relationship between the organisation of cortical microtubules and differentiation has been observed in a few systems. When microcalli derived from *Petunia hybrida* protoplasts were cultured on two different media, the calli either regenerated shoots, or remained in an undifferentiated state. The cortical microtubules of the regenerating calli had a transverse orientation while those in the non-regenerating calli had a random organisation (Traas et al. 1990). The same relationship between the organisation of microtubules and regenerating potential was observed when protoplasts of two cell lines of *Larix x eurolepis*, one with embryo-regenerating potential and one lacking regenerating potential were compared (paper I). In the protoplasts of the regenerating cell line the re-organisation of the cortical microtubule network was accomplished after 3 days of culture and accompanied by an elongation of the cells (Fig 11 in paper I). On the other hand, dividing cells of the non-regenerating line maintained a protoplast-like organisation of the cortical microtubules. The individual cells of the embryo-like structure in the regenerating cell lines had a well established network of cortical microtubules, in parallel orientation (see Figs. 13, 14 in paper I, see also Fig. 4, 20 in

Fowke et al. 1990). In contrast, Dijak and Simmonds (1988) studied the induction of embryogenesis by electrical field stimulation of mesophyll protoplasts from *Medicago sativa*, and showed that regenerating protoplasts maintained the random organisation of cortical microtubules, while the non-regenerating protoplasts attained parallel arrays of cortical microtubules after two days of culture. Even if this type of comparison between a regenerating and a non-regenerating system can give us clues as to the role of the microtubules in the establishment of cell axis and polarity, it does not distinguish between cause and effect. The question is whether the more rapid re-organisation of the cortical cytoskeleton merely reflects the new developmental pathway taken by the cell or if it is a precondition for cellular differentiation.

The involvement of the cytoskeleton in cell differentiation has been studied in connection with wound reaction. This system is interesting because wounding induces a similar reaction in the cells surrounding the site of injury as does protoplast isolation. Upon wounding there is a stimulation of cell division and a change in the polarity of the cells surrounding the wound. In a similar manner, protoplast isolation also induces cell division and, in the case in which organs regenerate from the protoplast derived cells a change in polarity (as discussed previously). Drug studies of wounded tissues using microfilament or microtubule disrupting agents (Hush et al. 1990, Hush and Overall 1992) have shown that while the microfilaments are necessary for the normal progression of the cells through the cell cycle phases and in particular for cytokinesis (i.e. they observed an accumulation of cells with phragmoplasts in cytochalasin B treated samples), it is the microtubule system that plays the critical role in the establishment of cell polarity. Oryzalin treatment of the tissue prior to wounding prevented the change of cell shape normally observed in the tissue surrounding the wound. A washing out of the drug returned the normal response to the tissue. These results favour the interpretation of a more direct role for the microtubules in morphogenesis: for example in the differentiation of organs in *Petunia* and *Larix*, the ordered microtubular arrays are necessary for the establishment of cell polarity and therefore occur at an early stage after protoplast isolation. In the case of *Petunia* protoplasts exposed to UV-B radiation (paper II), the lack of a functional cortical microtubule-cytoskeleton is related to the arrest of the division of the cells.

1.3.3 Microtubules in epidermal cell development

In all organisms, growth is the result of cell division and cell expansion. In plants, the final shape of an organ is determined by both genetic and environmental factors. The interplay between them enables the plants, which are sessile organisms, to show an extremely plastic development. Thereby, plants can adapt to the natural variation of the habitat. For example, leaf morphology varies greatly and is dependent on the light quality and intensity during development. Even if the main outline of the final leaf shape is genetically controlled (a pine tree will under no circumstances develop palm leaves), final cell size and cell number are influenced by environmental factors prevailing during the development of the leaf. In extreme cases more than 90% of the cells in a leaf arise from divisions that take place after the unfolding of the leaf primordium (Dale 1988).

Microtubules are important for the final shape of the epidermal cell and they might influence it by controlling the pattern in which microfibrils are deposited. The most striking example of this is in cells that have an elaborate lobed shape when they are fully differentiated. In this type of cell the microtubules form intricate patterns that fully coincide with the pattern of secondary cell wall deposition. The coincidence between microtubule bundles and wall thickening was observed during the differentiation of epithem cells in *Pilea cadierei* (Galatis 1988). Bundling of microtubules in the proximity of the plasma membrane was observed before the cells were fully expanded. As the cells underwent turgor-driven expansion, swelling was restricted by the cell wall thickenings adjacent to the microtubule bundles, resulting in lobed cells.

Bundles of microtubules underlying cell wall thickenings during mesophyll cell differentiation have also been observed in maize (Apostolakos et al. 1991), where they also resulted in lobed mesophyll cells. When the tissue was treated with oryzalin during the differentiation, the anatomy of the leaf was seriously altered, resulting in isodiametric mesophyll cells, no cell wall thickenings and no intracellular space formation. Microtubule organisation was studied during the development of epidermal cells of *Vigna sinensis*, a species that has a wavy, paracytic epidermal pattern (Esau 1977), as has *Petunia hybrida*. Again, a direct relationship between microtubular organisation and intercellular space formation and epidermal cell differentiation was noted (Panteris et al. 1993). At an early stage in the development of the leaf, the epidermal cells had even cell walls and the cortical microtubules were evenly distributed. As the cells differentiated, the microtubules in the cells became bundled, cell

wall thickening occurred at the places were the microtubules bundled and eventually the whole epidermal cell became wavy. The conclusion from these and related studies would be that the microtubules are essential for the morphogenesis of mesophyll cells by determining the deposition of cell wall thickenings and the subsequent determination of cell shape upon cell enlargement by turgor forces (cell swelling).

2 The quest for UV-B effects

In the biological sciences, UV radiation is arbitrarily divided into: UV-C (200-280 nm), UV-B (280-320 nm) and UV-A (320-400 nm). All wavelengths are present in the solar spectrum. However, due to differential absorption in the atmosphere, significant levels of radiation at the earth surface can be measured only above 295 nm. A decrease in stratospheric ozone concentration will alter the spectral irradiance in the interval between 295 and 310 nm. Radiation below 295 nm will increase only at ozone concentrations 90% below those existing today. Above 310 nm, absorption by ozone is so low that it will not influence the amount of radiation reaching the ground.

In 1961 Lockhart and Brodführer Franzgrote wrote: "Interest in the effect of ultraviolet on plant growth began when the climate of the Alps was found to be beneficial to human health". The work carried out in the beginning of the 20th century was concerned with practical aspects of greenhouse climate. The authors continue to write that "a number of early investigations ... report stimulation of plant growth" and also point out that "injurious effects could be elicited with radiation of wavelengths longer than 289 [nm] only at energy levels much higher than could be expected from solar radiation".

The renewed interest in the biological effects of short wavelength UV radiation comes from the observation in the mid-seventies of a decrease in the level of stratospheric ozone. First observed above the Antarctic and denoted by what has become known as "the ozone hole", decreases of ozone above densely populated, mid-latitude regions of the Northern hemisphere have now been reported.

On the other hand, relating the observed decreases in stratospheric ozone to actual

increases in ground-level UV-B irradiance has proven to be much more difficult, the main reason being that the short-wavelength radiation is efficiently absorbed by ozone and other particles in the troposphere. Predicted increases in UV-B radiation rely on the assumption that aerosol particles, cloud cover and other atmospheric conditions will remain the same. This part is meant to give a summary of our present level of understanding of some of the effects of UV-B radiation on plants. At the same time, some of the uncertainties related to the determination of the impact of a potential increase in UV-B radiation will be considered. It is hoped that further consideration of the points taken up in the following sections will aid in the efforts of interpreting how UV-B radiation influences plant growth and not only the consideration of growth reduction (many times described as damage) that no doubt is a result of the presence of short wavelength radiation in the solar spectrum.

2.1 The concept of biologically effective radiation

It is not possible to discuss modern UV research without defining the concept of *biologically effective radiation* (UV-B_{BE}). In order to produce a biological effect, a photon must be absorbed by a molecule of the target organism. The probability of a photon being absorbed is a function of the electronic configuration of the target molecule and of the wavelength of the photon. The energy absorbed by the molecules can be either lost as heat or cause a biological effect. A plot of the relative efficiency of a photon versus their wavelength for causing a given biological effect is called an action spectrum. If the action spectrum and the absorption spectrum of a molecule coincide, the possibility exists that the molecule under investigation is responsible for the biological effect. The determination of target molecules for UV-B action, indeed for the whole short-wavelength part of the spectrum, is immensely difficult because these photons are readily absorbed by an innumerable variety of molecules that are ubiquitous in all living organisms. Photons of wavelengths below 400 nm will be absorbed by molecules such as nucleic acids (both DNA and RNA), phenolic compounds in plants (e.g. lignin, tannins, flavonoids), and all proteins that contain the amino acids tyrosine, tryptophane and phenylalanine. Because all proteins in differentiated tissues contain these amino acids to some extent, they are all potential mediators of UV effects and it has so far not been possible to limit the range of molecules involved in UV-B responses.

Tab. 1. Comparison of the action spectra AΣ-9 and AΣ-21. The data were extracted from Vu et al. (1981).

unweighted irradiance	AΣ–9		AΣ–21	
(mW m^{-2})	weighted (mW m^{-2})	O_3 reduction (%)	weighted (mW m^{-2})	O_3 reduction (%)
1.138	8.49	32	17.46	6
1.448	10.45	38	21.69	21
2.028	13.78	47	29.23	36

Multiplying the effect of a photon at any given wavelength on the action spectrum by the spectral irradiance of the sun will give a value that reflects both the relative effectiveness of the photon and the number of photons at that wavelength that reaches the earth. The integral of the products is the biologically effective radiation for a given stratospheric ozone concentration, and is called *biologically effective UV-B radiation.* This value has no physical equivalent, it is not something we can measure, but will vary considerably according to the action spectrum that we choose for weighting. The implication is that if the choice of action spectrum is incorrect, so also will be the interpretation of the results of an experiment. Different action spectra are used by different research groups, which means that one should be careful when comparing results from different laboratories, and when trying to draw global conclusions as to the impact of a reduction in stratospheric ozone. An illustration of the fact that the usage of different action spectra give large variations in the amount of UV-B_{BE} and ozone reduction can be found in Tab. 1. Using three different unweighted irradiances, the plant action spectrum of Caldwell (AΣ–21) and an action spectrum designated AΣ–9 (Cams et al. 1977, Thimijan et al. 1978) are compared. For example, for the same unweighted irradiance, AΣ–21 results is a weighted irradiance of 17.46 mW m^{-2} which corresponds to a 6% ozone reduction while AΣ–9 results in a weighted irradiance of 8.49 mW m^{-2} which corresponds to a 32% ozone reduction.

A large number of action spectra have been constructed during the past 20 years. They can be classified according to the material they have been developed on, for example whole plants, plant parts or organelle sub-fractions. The other way of classifying action spectra that will be relevant for the discussion is according to the

shape of the action spectrum: one class of action spectra has the maximum response in the short-wavelength UV-B region (at 280 nm) and the response declines exponentially (by a factor of 10^5) as the wavelength increases, so that the response at 320 nm is close to zero. Examples of this are the action spectra for induction of pyrimidine dimers in naked DNA (Setlow 1974) and the generalised plant action spectrum proposed by Caldwell (1968, 1971) that is widely used today in plant research. Caldwell did not mathematically describe the weighting function of the action spectrum. Instead, at a workshop held in Beltsville in 1976, the use of the following weighting function derived from the DNA action spectra in bacteria was proposed:

$$y = \exp -[(265-\lambda)/21]^2$$

This weighting function also describes the generalised plant action spectrum, and is found under the designation AΣ–21 (Thimijan et al. 1978). It should be noticed that both this equation and another equation (Green et al. 1974) that fits the spectrum proposed in graphic form by Caldwell (1968, 1971) is used today for calculating UV-B_{BE}.

The action spectra for whole organisms and organelle sub-fractions differ in two essential respects from the action spectra based on DNA. For the former two, the action at 280 nm is lower and there is considerable effectiveness throughout the UV-A region (Bornman et al. 1984, Cen and Björn 1994, Quaite et al. 1992, Steinmetz and Wellmann 1986). It is noteworthy that when the induction of pyrimidine dimers in whole plants was studied, there was a considerable effect in the UV-A region (Quaite et al. 1992). The effectiveness of photons at 330 nm decreased by a factor of 100 compared to photons at 290 nm, while in the solar spectrum there is 1000 times more radiation at 330 nm compared to 290 nm (Quaite et al. 1992). This has serious implications for the assessment of the consequences of ozone depletion and the implicit increase in UV-B_{BE}. First, using the action spectrum described by the equation AΣ–21, the importance of short wavelength radiation (i.e. UV-B) is overestimated, while the effectiveness of long wavelength radiation (i.e. UV-A) is underestimated. The overall result is that the impact of ozone depletion assessed by using this equation might be greater than will actually be the case. Secondly, due to the absorption spectrum of ozone, the increase in radiation reaching the biosphere will be greatest at wavelengths between 295-310 nm and zero at wavelengths above, as has been demonstrated by *de facto* measurements of irradiance at ground level (Kerr and McElroy 1993, but see also Michaelis et al. 1994). Plants growing in natural environments are exposed to doses of UV-A radiation that are three

orders of magnitude higher compared to the irradiance in the UV-B region. Plants have developed mechanisms to cope with the stress and even use that part of the spectrum to regulate growth, (see section 2.3.1). In order for an increase in the UV-B region to have a measurable effect on plant growth, a given process has to be affected only by UV-B with little effect in the UV-A region. Such a process has yet to be described, and it is doubtful if it exists. On the other hand, action spectra that have a tail in the UV-A region describe processes that will not be influenced by an increase in UV-B radiation to any noteworthy extent, either because the action is masked by the effect of the radiation in the UV-A region, or because the "damage" is efficiently repaired by mechanisms that are already operating.

2.2 Protective mechanisms

2.2.1 Avoidance. Some organisms live in environments where the sun rays never reach them, such as caves, underground or in the depths of the sea. Humans can choose to stay indoors, change clothing habits or use sunscreen compounds to protect themselves. Because plants are dependent on sunlight for photosynthesis, avoidance is not an option available to them. Even so, plants can be found growing in habitats where the levels of UV-B radiation are higher than any realistic decrease in ozone will give at mid-latitude regions, e.g. in the open savannahs or on the slopes of the mountains in the equatorial regions. Obviously, other protective mechanisms exist.

2.2.2 Screening of UV-B radiation. This class of protective mechanisms can be divided into external protection and intracellular stored compounds. Fur, feathers, hair, callous layers of skin, and in the case of plants cuticular waxes and bark protect against UV-B radiation. Melanin in the human skin and the flavonoids stored in the vacuoles of the plant epidermal cells are efficient at absorbing UV-B radiation. Flavonoid deficient mutants are extremely sensitive to UV-B induced damage (Li et al. 1993). Screening of UV radiation is also important in protecting apical meristems, which are shaded from UV-B by the young, expanding leaves.

2.2.3 Penetration of UV-B radiation inside living tissues. As a consequence of the presence of UV-screening pigments in the epidermal tissue, the amount of UV-B radiation at deeper levels is greatly reduced, usually to less than 1% of incident radiation. First proposed by Metzner (1930), the role of the flavonoids in reducing UV-B

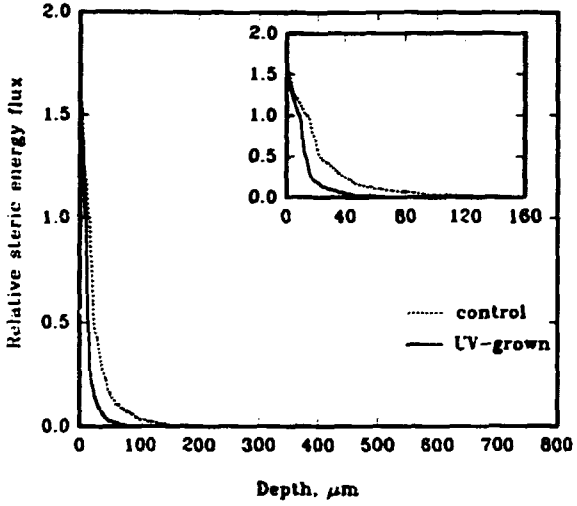


Fig. 4. Penetration of 310 nm radiation into leaves of *Petunia hybrida*. Only collimated radiation was measured in order to estimate the differences in penetration between plants grown with or without UV-B radiation. Six measurements were done with the adaxial leaf surface towards the light source. The values were normalised to incident radiation. By expanding the y-axis the insert shows more clearly the differences between the two curves.

radiation levels inside plant tissues has been demonstrated using fibre optic microprobes inserted at different depths in leaves (Cen and Bornman 1993, Day et al. 1992, DeLucia et al. 1992). As shown in Fig. 4 for *Petunia*, the 60% increase in UV-screening pigments due to the exposure to UV-B radiation (paper III, IV) was reflected in a 93% attenuation of the amount of 310 nm radiation at 30 μm inside the leaf compared to 75% attenuation in the control plants. The incident radiation used for this type of measurement is ca 10 times higher (at 310 nm) compared to greenhouse chamber conditions. This means that the possibility exits that *in nature* the amount of radiation within leaves at this wavelength might be lower than that experimentally determined.

2.2.4 DNA repair. Even under natural conditions, cellular DNA is repeatedly damaged. The modifications induced in the DNA structure are either a direct result of the absorption of photons by DNA (exemplified by the formation of pyrimidine dimers, the major form of UV lesion) or the result of the formation of oxidising metabolites.

Only the former case will be discussed in detail.

If left unrepared, pyrimidine dimers will eventually lead to cell death because they may block DNA transcription and thereby impair protein synthesis, or DNA replication which will in turn prevent cell division. Originally, DNA repair was studied using *Escherichia coli* and this is still the best understood system for mutagenesis mechanisms and DNA repair (Livneh et al. 1993). Even if postulated, the existence of DNA repair mechanisms in plants was for some time a matter of debate and because many early studies had failed to identify a repair mechanism in plants, it was suggested that it did not exist. However, we know today of the existence of repair systems in phytoplankton (Karentz et al. 1991), higher plants (McLennan 1987) and photolyase activity has been observed in *Arabidopsis* (Pang and Hays 1991). Of the plenitude of repair mechanisms that are operating in *E. coli*, only two have been demonstrated for plants: excision repair and light activated repair enzymes (photolyases). Both are important for the tolerance of plants to environmental UV radiation (NB both UV-B and UV-A) as suggested by a study on *Arabidopsis* (Britt et al. 1993).

2.2.5 Redundancy of the genetic information. There are two important aspects to be considered here. First, most proteins in higher organisms have been shown to be coded by multigene families. In all cases studied so far, the isoforms can replace each other in cellular functions, even if the genes coding for these proteins are tissue specific or developmentally regulated. This means that the inactivation of one gene as a consequence of a UV induced lesion could be compensated by the transcription of another gene from the same family. Second, only a minor part of the DNA codes for proteins. Mutation in non-coding regions will be of no importance for the survival of the organism. As a consequence, the majority of the pyrimidine dimers induced by UV can be regarded as background and of no importance for cell survival. A non-critical measurement of pyrimidine dimers is questionable as an indication of UV induced "damage" because a site-specific repair mechanism seems to be operating, leading to the transcribed regions of the DNA being preferentially repaired (Bohr et al. 1985, Thomas et al. 1989). These observations have been made in mammalian systems, but a similar mechanism could well exist in plants, as indicated by the fact that *Arabidopsis* plants can tolerate a relatively high level of dimers (1/42,000 bp; Britt et al. 1993).

2.3 Plant responses to UV-B radiation

When considering plant responses to UV-B radiation, a question of paramount importance has to be answered: is all UV-B radiation harmful, i.e. are even current levels of UV-B radiation reducing plant growth, or do plants tolerate a certain amount of radiation in the environment? So far, there is considerable evidence suggesting that even if this type of radiation is harmful to life if present in sufficiently high amounts, it is apparently well tolerated at present solar irradiance. Plants have adapted to the presence of UV-B in the environment, and can tolerate even higher doses of UV-B than those presently occurring. This tolerance is not unreasonable, considering that plants have evolved in an environment in which UV-B radiation (above 295 nm) is a natural component of the solar spectrum. However, repair of damaged molecules will be at the expense of energy and the diversion of energy towards repair would be reflected in a decrease in biomass production. In this case we would expect the growth reduction to be proportional to the increase in $UV\text{-}B_{BE}$. From the studies conducted so far this seems not to be the case. A dose-response relationship has only been observed for the induction of flavonoid synthesis (Wellmann 1983).

The data in Tab. 2 represent a small selection of the studies conducted so far in the UV-B field. In a review by Krupa and Kickert (1989) about 200 species are covered, with the drawback that there is no mention of the UV-B doses used in the different investigations, or irradiation protocols (e.g. background visible light, presence/absence of UV-C radiation in the treatments). However, the results presented in Tab. 2 are typical of plant responses to UV-B radiation, and point towards the following important relationships. Plant responses to UV-B radiation can be classified into physiological parameters and morphological responses. The morphological and physiological response classes do not always occur together. Some plants show reduction of biomass without decreases in net CO_2 assimilation rates. The reverse also occurs. *Petunia* plants respond to UV-B radiation by increased biomass and increased CO_2 assimilation rates (paper IV). The most consistent response to increased UV-B radiation is an increase in flavonoids. Another point that is obvious from Tab. 2 is that the doses of UV-B radiation are as variable as are the species investigated. This fact makes generalisations of plant responses to UV-B radiation and comparisons between species difficult and uncertain.

Tab. 2. Response of plants to UV-B radiation. 1 = UV-B_{BE} weighted using the equation AΣ21 normalised at 300 nm, kJ m^{-2} day^{-1}. 2 = unweighted UV-B kJ m^{-2} day^{-1}. Abbreviations: DW, dry weight; LA, leaf area; PAL, phenylalanine ammonia lyase; SLA, specific leaf area; SLW, specific leaf weight; SOD, superoxide dismutase; WUE, water use efficiency; wt, weight. Symbols: - decreased; ± unchanged; + increased.

Plant	UV Dose	Response effect	Reference
Rumex patientia	(0, 9.7, 24.5)[1]	- leaf expansion + anthocyanin	Lindoo and Caldwell 1977
Cucumis sativus cv Ashley and Pointsett	(0, 18.2)[1]	- plant height - LA ± leaf DW ± PAL activity ± SOD activity	Krizek et al. 1993
Triticum aestivum *Avena sativa* *Zea mays*	(0, 9.6)[2]	- LA - SLW - shoot height - leaf length + tillering + no. of leaves	Barnes et al. 1990
Erica curviostris *E. fairii* Boles *E. nudiflora*	(7.8, 9.4, 11.4)[1]	- pollen germination - net CO_2 assimilation (not *E. curviostris*) ± apparent quantum yield ± WUE ± carboxylation efficiency	Musil and Wand 1993
Picea abies	(0, 6.2)[1]	± Chl content ± DW of needles ± Chl *a/b* ratio ± net CO_2 assimilation	Dubé and Bornman 1992
Oryza sativa cv IR-36	(8.8, 13.8)[1]	- LA - leaf wt - biomass - seed yield ± SLW ± stem wt ± root wt	Ziska and Teramura 1992
cv Fujijama-5	(8.8, 13.8)[1]	- leaf wt - stem wt - biomass - seed yield ± SLW ± root wt + LA	

Tab. 2. continued

Plant	UV dose	Response effect	Reference
Phaseolus vulgaris	(0, 6.2)[1]	- LA ± leaf DW ± Chl content ± carotenoid + plant height + leaf thickness	Cen and Bornman 1990
Beta vulgaris	(0, 6.9)[1]	+ DW of leaf lamina ± DW of petioles ± DW of storage root ± chl content	Panagopoulos et al. 1992
Helianthus annusu cv Uniflorus	(0, 6.3, 21.0)[1]	- stomatal resistence ± DW of shoot ± DW of leaf lamina ± hypocotyl length ± Chl content ± IAA content + no. of leaves + carotenoid content + SLA	Panagopoulos 1992
Petunia hybrida	(0, 8.9)[1]	- leaf thickness ± Chl content ± protein content + leaf area + no. of axillary shoots + net CO_2 assimilation + UV screening pigments + shoot DW	Paper IV

2.3.1 Morphogenic responses to UV-B radiation

To date, we know of the existence of three classes of photoreceptors in higher plants: phytochrome, a blue light receptor and a UV-B receptor. The definition of the UV-B receptor is that it has the peak of action at 290 nm, and no action at wavelengths longer than 350 nm (Yatsuhashi et al. 1982). The three pigments individually or a combination of them act to create a biological response and regulate plant growth (Mohr 1986, Wellmann 1983).

The induction of anthocyanin and flavonoids is a result of the co-action of phytochrome and of the blue/UV-A photoreceptor (with an UV-B component). In

parsley, the action is sequential, meaning that either UV or red light alone are not effective at inducing the response. In order for phytochrome to be active, the tissue has to be pre-treated with UV (Ensminger and Schäfer 1992, Wellmann 1983).

The inhibition of hypocotyl elongation is an often observed response of seedlings to UV-B radiation. Using an *au* mutant of tomato (which is severely deficient in spectrophotometrically and immunologically detectable phytochrome) and its isogenic wildtype line, Lercari et al. (1990) showed that the control of hypocotyl elongation was independently controlled by phytochrome and by the UV photoreceptor. Hypocotyl elongation was inhibited by red light and by UV in the wild type. When both light treatments were applied simultaneously, the inhibition was greater. In the *au* mutant, hypocotyl elongation was only inhibited by treatment with UV radiation. From these experiments it was suggested that the action of an UV photoreceptor is sufficient for the inhibition of hypocotyl elongation (at least in tomato). Even though in the wildtype phenotype the action of phytochrome is predominant under high visible light irradiances, there is an action of UV at high UV irradiance.

Although this type of experiment has not been conducted on other plants, it is reasonable to assume that tomato is not an exception, and that similar mechanisms are operating in other plants as well. In particular, leaf morphology has often been shown to be altered by UV radiation (paper III, IV and references therein; Bornman and Teramura 1993). The regulation of growth by an UV photoreceptor is the most plausible explanation for the cases in which the morphology of the plants is altered, with little or no physiological changes (under reasonable UV-B doses).

3 UV radiation and cellular functions

Knowledge of the effect of ultraviolet radiation on the cytoskeleton comes from studies in mammalian systems. In the early seventies it was proposed that the ultraviolet component of sunlight was the major cause of skin cancer. Also, the observation was made that chronical exposure of skin to ultraviolet radiation led to premature skin ageing, for example loss of elasticity of the skin. Great effort has therefore been put into elucidating the mechanisms by which UV radiation alters cellular functions. Investigations have followed two directions, namely the effect of UV radiation on the organisation of the cytoskeleton and the effect of UV radiation on cell division.

3.1 UV radiation and the cytoskeleton

The stability of the three classes of cytoskeletal components (microfilament., microtubules and intermediate filaments) has been studied in animal cells. These studies were carried out on *in vitro* cultured fibroblasts of different origin (Zamansky et al. 1985, 1991, 1992; Zamansky and Chou 1987, 1990). Cell survival was not affected by UV-B or UV-A levels corresponding to present day solar values, and a similar relative insensitivity of cell survival to UV radiation was observed in *Petunia* protoplasts as well (paper II). In fibroblasts, no consistent dose-response relationship was observed when chromosomal and chromatid aberrations were measured, and the levels of injury were only slightly higher compared to spontaneous levels. In these cells, UV radiation induced disruption of the normal organisation of the cytoskeleton. The sensitivity of the *microfilaments, microtubules and keratin intermediate filaments to UV-C, UV-B and* UV-A was tested separately, and the relative sensitivity of fibroblast cytoskeleton is summarised in Tab. 3. For all three classes of filaments higher radiation doses lead to increasing loss of filament structures. Considering UV-C radiation, the microtubules and actin filaments are stable, while keratin intermediate filaments are equally sensitive to UV-C and UV-B radiation. Irradiation with either UV-C or UV-B usually results in the condensation of the keratin intermediate filaments into the perinuclear regions and a loss of the alignment of the filaments. Also, the re-organisation of keratin filaments that is induced *in vitro* by mM Ca^{2+} in the medium is inhibited in the irradiated cells.

Tab. 3. Relative sensitivity to UV radiation of the three cytoskeletal structures of cultured fibroblasts.

Radiation	Microfilaments	Microtubules	Intermediate filaments
UV-C	stable	stable	sensitive
UV-B	stable	sensitive	sensitive
UV-A	stable	sensitive	stable

Microtubules are only depolymerized by UV-B radiation, while they are insensitive to irradiation with either UV-C or UV-A. Increasing doses of UV-B radiation lead to loss of much of the anti-tubulin staining in the cytoplasm, although in these cells some microtubules remained associated with the microtubule organizing centres. Actin filaments are stable under the UV doses which lead to disruption of the keratin intermediate filaments and microtubules.

Even plant cortical microtubules are sensitive to UV-B radiation *in vitro* (paper II). Increasing doses of UV radiation result in shorter fragments of cortical microtubules. However, these are not lost but persist in the proximity of the plasma membrane. As mentioned earlier, plant cells lack distinct microtubule organising centres in the cytoplasm. Instead, regions in the plasma membrane are supposed to fulfil the function of nucleating cortical microtubules. This could have as an effect that, even though severely fragmented, the microtubule parts remain attached to the plasma membrane. Interestingly, the time taken for the cortical microtubules to regain their original organisation is relatively long in both systems: more than 9 h in the fibroblasts, and between 24-72 h (depending on the UV dose) for the *Petunia* protoplasts (paper II). This can be compared to the 15 min to a few hours necessary for the reappearance of a cortical microtubular network after the washing out of microtubule depolymerising drugs. The most reasonable explanation for this observation is that the cytoplasmic tubulin pool is damaged as well as the microtubular polymers, and the cytoplasmic tubulin cannot be used in the polymerisation of new filaments during the recovery period.

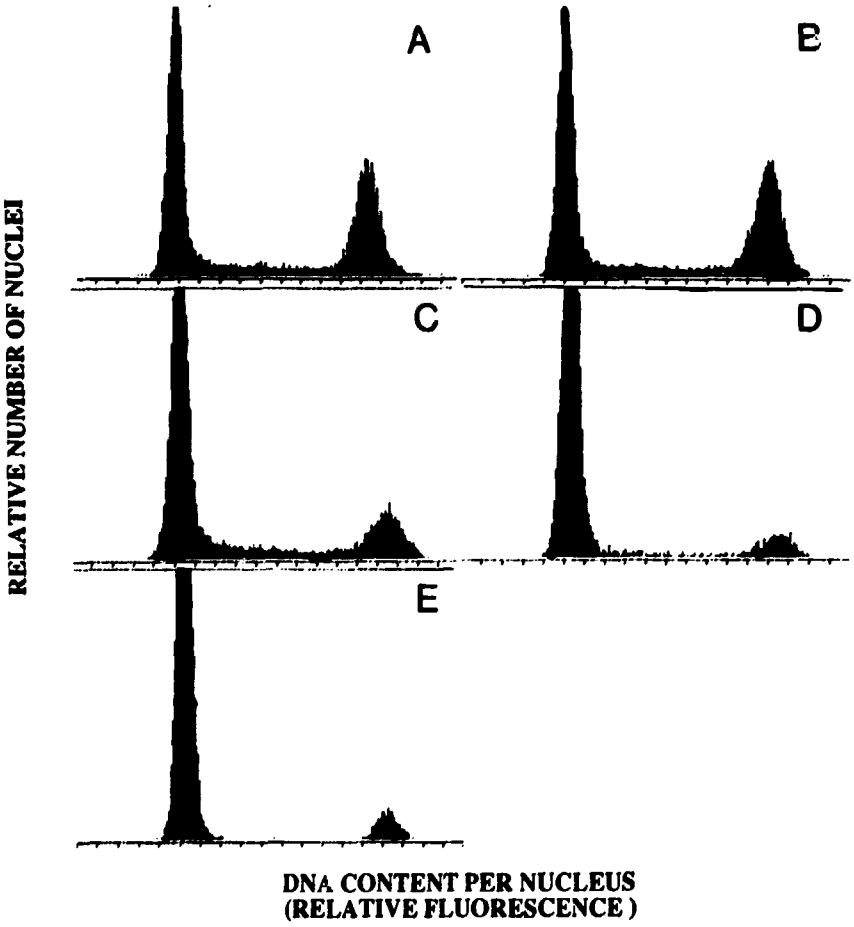


Fig 5. Flow-cytometric estimation of fluorescence distribution in *Petunia hybrida* nuclei stained with DAPI. Protoplasts were irradiated 30 min after isolation with 0 (A), 4 (B), 8 (C), 12 (D) or 24 (E) mmol m^{-2} UV-B. The nuclei were analysed 48 h after irradiation.

3.2 UV radiation and cell division

When cells are exposed to UV radiation *in vitro*, the first response is an inhibition of DNA synthesis. As mentioned in section 2.2.4, UV radiation induces lesions of various kinds in the DNA of exposed organisms. In plant cells, 90% of the injury is in the form of thymidine dimers and the remaining 10% are (6-4) photoproducts. It is not clear whether all stages of the cell cycle are affected by UV. However, there are indications that G0/G1, S and G2 are relatively sensitive, while the progression of the cells through mitosis is relatively insensitive (see references in paper II).

In *Petunia* protoplasts irradiated with UV-B, only the effect on the G1 phase was clearly demonstrated. The time required for the protoplasts to resume DNA synthesis increased with the UV dose as can be seen in the histograms presented in Fig. 5 (see also Fig. 5 in paper II). In the protoplasts cultured for 48 h after irradiation with UV-B, the distribution of the nuclei of the protoplasts irradiated with 4 mmol UV-B photons is similar to that in the control cells. In the sample treated with 8 mmol UV-B photons, the cells have started to divide, while no cell division can be detected in the samples treated with 12 or 24 mmol UV-B photons.

The reasons behind the block in cell division after UV irradiation are complex, and different for cells that are in G0/G1, S or G2 at the time of irradiation. For G0/G1 and S cells, the most plausible explanation is that time is required for the repair of the lesions before DNA synthesis resumes. This explanation is not valid for cells in G2, since DNA replication is completed in these cells. A few proteins involved in the control of cell division are synthesised in G2, particularly the class of proteins known as cyclins (Francis 1992, Nurse 1990). In G2, the newly synthesised cyclin associates with another protein called p34, and after dephosphorylation of p34, this complex is an active protein kinase that phosphorylates various proteins that in turn determine the onset of mitosis. These could be potential targets of UV action in G2 cells.

4 Concluding remarks

The cytoskeleton, and in particular the microtubules, are involved in plant morphogenesis. Protoplasts from cultures that have the potential to regenerate organs, usually also show a faster re-arrangement of the cortical microtubules into parallel arrays, as was shown to be the case in *Larix*. In paper I protoplasts were isolated from both regenerating and non-regenerating cultures of hybrid larch. Immunofluorescence studies of highly embryogenic protoplast fractions showed that microtubules became organised into parallel arrays after 3 days of culture, at which time the protoplast-derived cells also became elongated, indicating the establishment of cell polarity. In most of the protoplasts of the non-regenerating tissue, microtubules retained a random orientation and a spherical shape for a longer period. It is possible that the re-arrangement of the cortical cytoskeleton is necessary for the establishment of cell polarity.

Because of the importance of the microtubular cytoskeleton in plant morphogenesis, it was expected that the changes in leaf anatomy due to UV-B radiation were the result of the impaired function of the microtubules due to UV-B radiation. Indeed, when mesophyll protoplasts of *Petunia hybrida* were subjected to UV-B radiation (paper II), UV radiation induced breakes in the cortical microtubules resulting in shorter fragments with increasing doses. Also, the protoplasts were delayed in their progression through the cell cycle. The commencement of DNA synthesis in the irradiated protoplasts followed the re-establishment of a microtubule network. However, when *Petunia* plants were exposed to UV-B radiation, the cortical microtubules of epidermal cells were not affected (paper III). Also, the observed increase in leaf area appeared due to an increase in the divisions of the leaf meristems rather than an alteration in the expansion of the epidermal cells. Stomatal frequency was not altered, even though the formation of stomatal complexes occurred at a time when the leaf was already exposed to UV radiation. In a later study it was shown that the changes in morphology induced by UV radiation in *Petunia* plants were coupled to higher biomass accumulation and higher rates of CO_2 assimilation (paper IV).

Petunia is not an exception, since stimulation of growth by ultraviolet radiation has also been observed in other species as well. Plants have been classified into

"tolerant" and "sensitive" species, and ultraviolet radiation into "damaging", "inhibitory" and "stimulatory" regions.

Explaining the stimulatory effects of UV radiation is much more difficult than explaining effects that have been classed as damaging or inhibitory, and that usually occur at wavelengths lower than 290 nm. Usually, plants that are "tolerant" to UV radiation also show stimulation of growth by low/middle doses of radiation, when they are compared to control plants that have been grown in the absence of UV radiation. These are also the species in which a change in flowering induction has been observed. *Petunia* is one of these species, and shows the typical response: increased biomass accumulation, larger leaves, increased branching, deeper green-colouring, shortening of the time to flowering. It is plausible that these plants possess a UV receptor that regulates growth, of the type that has been proposed for tomato. This could account for the apparent requirement for low doses of UV for the optimal growth of the plants. Most certainly protective mechanisms such as increased flavonoid production and DNA repair mechanisms are important for the survival of the organism.

Acknowledgements

This is, without doubt, the part of the thesis that is most difficult to write. Not less so considering that it is the only chapter that everyone reads, or at least that all start by reading. While the work proper either produces a yawn: "I've-heard-it-all-before" reaction, or leaves the reader untouched: "not-my-field" response, the acknowledgements are different. Here, we expose our thoughts and feelings, and pay reverence to the people who have aided us in our efforts to make a difference.

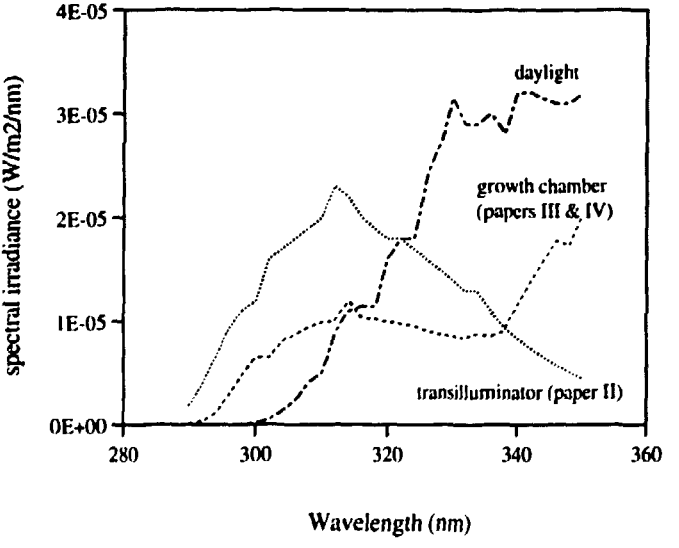
Our lives are enriched by the small gifts of trust that we are given by people every day. They open new horizons and give us the power we need to make things happening by teaching us to trust ourselves. So, to all the people within the Department as well as in the "outside" world, who have encouraged and trusted me, from the bottom of my heart -thank you!

Like the King of Hearts, we are often left wondering about what is significant and what represents futile side-tracks. We need the wisdom, experience and guidance of senior scientists to teach us how to tell them apart. I was fortunate to meet some of these scientists, and I am thankful to them.

Sometimes we meet people who are our intellectual or spiritual twins. They have a silver key to an unoccupied room in our soul. With them we discover and explore new sides of ourselves and our lives are never the same afterward. They come into our lives to stay forever. These people we can only thank by giving them our unconditioned love and trust.

Appendix

Emission spectra of the lamps used in experiments in papers II-IV and how they compare to the daylight spectrum.



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