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Biochemical and Molecular Control of Degreening in Microspore-derived Embryos of *Brassica napus* cv Topas.

By

© JAY WILLIAM KENNEDY

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE.

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Biochemical and Molecular Control of Degreening in Microspore-derived Embryos of *Brassica napus* cv Topas** submitted by **Jay William Kennedy** in partial fulfilment of the requirements for the degree of **MASTER OF SCIENCE in PLANT MOLECULAR BIOLOGY**.

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DATE: October 29, 1993

ABSTRACT

The focus of this research was to examine molecular aspects of degreening during canola embryogenesis. The effect of abscisic acid (ABA) and 6–benzylaminopurine (BAP) on chlorophyll, chlorophyll a/b binding (Cab) gene expression, and the abundance of chlorophyll a/b binding (CAB) proteins was investigated. Microspore-derived (MD) embryos of *Brassica napus* were employed as a model for canola seed development.

Western blot analysis of light-harvesting polypeptides showed that MD embryos cultured under constant illumination accumulate CAB polypeptides (photosystem I and photosystem II components) in a time-dependent manner. Illuminated green MD embryos cultured in the presence of ABA (50 µM) rapidly lose chlorophyll and CAB polypeptides. The elimination of these photosystem components is more effectively stimulated by abscisic acid in the light than it is by light-deprivation. Northern blot analysis of total RNA from MD embryos cultured in the presence of light and ABA (50 µM) established that the LHC IIb Type I Cab transcript level is reduced almost below detection within 6 h of treatment. In the absence of light, ABA (50 µM) directs a decline in the Cab transcript but the effect is less pronounced.

Incubation of greened MD embryos with BAP (5 µM) significantly reduces the level of embryonic chlorophyll and fails to enhance the accumulation of CAB polypeptides by these tissues. Comparison of total RNA from illuminated control and illuminated BAP (5 µM) treated MD embryos revealed a diminished level of Cab transcript in the latter.

Greened MD embryos deprived of light exhibit reduced chlorophyll and CAB polypeptide levels compared to their illuminated counterparts. However, unlike the light grown tissue, these embryos undergo massive and immediate accumulation of the LHC IIb Type I Cab transcript within the first 6 h of light deprivation.

Although the [illegible] that ABA mediates photosystem disassembly in MD embryos of *B* [illegible] required to establish how these events relate to canola seed matura[illegible]

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LIST OF ABBREVIATIONS

A	ampere
ABA	abscisic acid
ALA	δ–aminolevulenic acid
BAP	6–benzylaminopurine
bp	base pair
Cab	chlorophyll a/b binding (gene)
CAB	chlorophyll a/b binding (protein)
cc	cubic centimeter
cDNA	complementary deoxyribonucleic acid
chl	chlorophyll
cv	cultivated variety
DAF	days after flowering
DAP	days after planting
DPA	days post-anthesis
dCTP	deoxycytidine 5'–triphosphate
DEPC	pyrocarbonic acid diethyl ester
DGAT	sn 1,2–diacylglycerol acyltransferase [E.C. 2.3.1.20]
E	einstein
EDTA	ethylenediaminetetraacetate
JA	jasmonic acid
hnRNA	heteronuclear ribonucleic acid
kDa	kilodalton
LEA	late embryogenesis abundant
LHC	light-harvesting complex
MD	microspore–derived

MOPS	3–[N–morpholino]propane–sulfonic acid
NaAc	sodium acetate
NaCit	trisodium citrate
$NADP^+$	nicotinamide–adenine dinucleotide phosphate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pLHCP	precursory light-harvesting complex protein
PS	photosystem
SDS	sodium dodecyl sulfate
sn	stereochemical number
TAG	triacylglycerol
TE	TRIS EDTA
TRIS	tris(hydroxymethyl)methylamine
v/v	percent "volume in volume"
V	volt
VLCFA	very long chain fatty acid
w/v	percent "weight in volume"
W	watt

1. INTRODUCTION

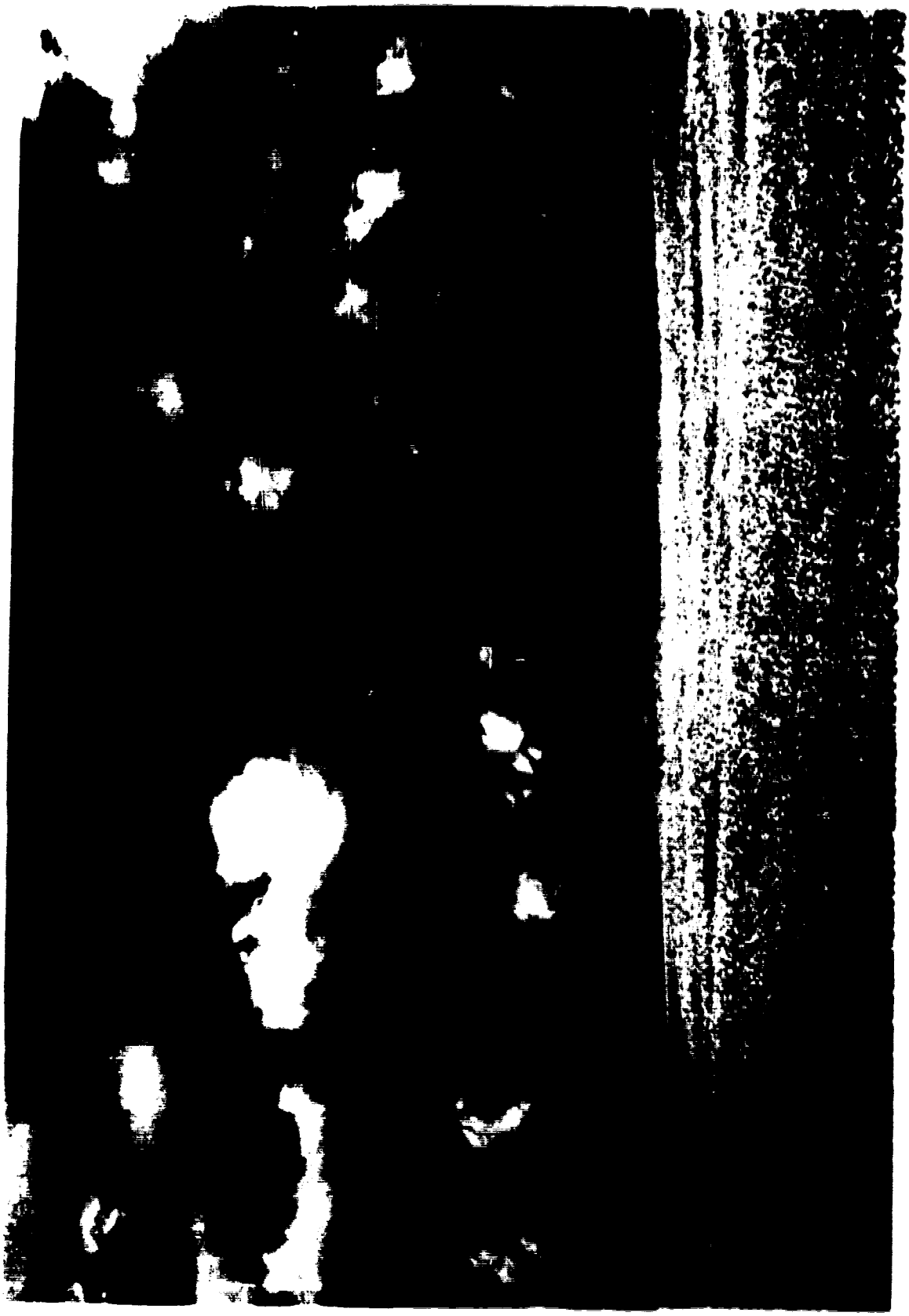
The term 'canola' is used to describe cultivated varieties of *Brassica napus* and *Brassica campestris* (Cruciferae family) that produce seeds bearing low levels of erucic acid and glucosinolates (permissable quantities defined in Thomas 1984). Canola varieties are grown extensively in Canada (Figure 1) as a source of edible plant oils. The value of the canola crop, however, is markedly reduced if chlorophyll and its pigmented derivatives are present in the embryo at seed maturity. This condition, termed 'green seed', is promoted when the crop is exposed to a sub-lethal frost prior to the completion of normal seed maturation. The improper retention of chlorophyll by the embryo results in the co-purification of pigment and oil during the crushing process. Oil contaminated in this manner is economically undesirable and, consequently, chlorophyll must be removed, resulting in a loss of profit to the grower.

We believe that the consistent production of chlorophyll-free canola oil is attainable through the strategies of plant biotechnology and genetic engineering (not through costly methods of oil purification). Prior to the successful implementation of such strategies it is imperative that a fundamental biological understanding of the molecular processes involved in embryonic degreening be obtained.

The temporally aberrant appearance of chlorophyll a/b binding (CAB) polypeptides late in embryo development may be a major contributing factor to the production and/or stabilization of green pigments in frost-stressed canola. The phytohormones ABA and cytokinins are known to affect the levels of CAB proteins and chlorophyll in other plant species. Therefore, a molecular analysis was undertaken to determine the effect of these hormones on the levels of embryonic chlorophyll and CAB proteins during canola embryogenesis. Microspore-derived embryos, a model for seed development in the *Brassicas*, were employed to obtain this information.

Figure 1. A flowering canola field in Southern Alberta[1].

[1] Photograph by David H. Wirzba - used with permission.



2. LITERATURE REVIEW

2.1 PHOTOSYNTHESIS

2.1.1 Conversion of energy - electromagnetic to chemical

Although a review of photosynthesis is not within the scope of this work, certain components of the photosynthetic machinery must be identified to facilitate an interpretation of the green seed problem at the molecular level. Oxygenic photosynthesis in the Plant Kingdom involves a complex set of chemical and photochemical reactions that occur within the chloroplast. This process is accomplished on the thylakoid membrane of the plant chloroplast which provides the framework for the organization of molecular players involved in photon utilization. Photosystems one and two (PSI and PSII) are multi-component integral membrane complexes that move excited electrons against a potential gradient upon photon reception by internalized chlorophyll molecules (Golbeck 1992; Mattoo *et al.* 1989). In a chain of redox reactions on the thylakoid membrane, the electron(s) is passed in step-wise fashion through a series of electron carriers from water to $NADP^+$ thereby generating potential energy. Coupled to this process is the translocation of protons across the lipid bilayer into the lumenal space by a large multi-component membrane assembly termed the b_6f complex (Anderson 1992). The vectorial movement of protons into the lumen produces an electrochemical gradient across the thylakoid membrane that is exploited by yet another chloroplast multi-subunit complex to produce cellular energy in the form of ATP (Ort and Oxborough 1992). Cellular energy and reducing equivalents generated by photosynthetic electron transport fuel the thermodynamically unfavorable assimilation of CO_2 into carbohydrate thereby conferring autotrophism to the plant. Although all chlorophyll is held within protein assemblies (Markwell *et al.* 1979), only a small percent of the pigment is bound to polypeptides involved in the photochemical reactions described above. Instead,

the vast majority of chlorophyll has a more intermediate role in the process of photosynthesis and is, as a consequence, associated with polypeptides of a distinct nature (Bennett 1979). These pigment-protein assemblies are discussed next.

2.1.2 The chlorophyll a/b binding apoproteins

The polypeptides with which the bulk of chlorophyll associates are, like the chlorophyll complexes of the reaction centre, integral membrane apoproteins. These pigment-protein complexes are associated with, but peripheral to, the reaction centres of PSI and II. Polypeptides in these complexes are called the chlorophyll a/b binding (CAB) apoproteins because they bind chlorophyll b in addition to chlorophyll a. Although they too act as photon receptors, the CAB complexes are not required for core photochemical reactions (Thornber 1975) and do not contribute electrons to the thylakoid redox chain (Bennett 1979). Instead, these pigment-protein complexes increase photosynthetic efficiency by funnelling photons into the reaction cores where the energy promotes PSI and II core pigment oxidation and electron transport. Because these membrane assemblies only enhance energy absorbtion and utilization by the plant, they are either referred to as the light-harvesting complexes (LHCs) or the photosystem antennae (Green *et al.* 1991). There are nine distinct LHCs, of which four (collectively known as LHC I) exclusively service PSI and the remainder harvest light for PSII (Thornber *et al.* 1993; Preiss *et al.* 1993).

LHC II, the light-harvesting antenna for PSII, is the most abundant chlorophyll a/b binding complex in higher plants (Bennett 1983). The detailed intermolecular organization of LHC II includes four (Chitnis and Thornber 1988) or five (Thornber *et al.* 1993) distinct CAB components - LHC IIa (CP 29), LHC IIb (CPII), LHC IIc (CP 26), LHC IId (CP 24), and LHC IIe. Of these, LHC IIb has been the best characterized and it is composed of one minor and two major polypeptides of molecular masses 25 to 28 kDa (Green *et al.* 1991). It is worth noting that the molecular techniques employed to study canola LHC IIb in this thesis report

do not permit the identification or characterization of the three LHC IIb component apoproteins. Therefore, from this point forward, these polypeptides will collectively be referred to as LHC IIb. However, the reader should not be confused by distinctions that are made between LHC IIb and other CAB complexes. Depending on the antibody probe used in Western blot analyses, light-harvesting proteins from both canola photosystems are readily distinguishable from LHC IIb.

Although the nine CAB entities are biochemically distinct, their commonalities include antigenic relatedness (White and Green 1987a), appreciable genetic homology (Green *et al.* 1991), and analogous trans-bilayer organization (Thornber *et al.* 1993). CAB proteins are encoded by a nuclear gene family (Buetow *et al.* 1988; Dunsmuir 1985) that evolved via duplication and mutation of an ancestral gene sequence (Green *et al.* 1991). The properties of Cab genes and their products are under investigation by many laboratories and, consequently, CAB nomenclature has become difficult to follow. The confused or interested reader is referred to recent articles that specifically address this problem (Green 1988; Jansson *et al.* 1992).

Expression of the genes encoding the major light-harvesting protein, LHC IIb, is responsive to light (Apel and Kloppstech 1978; Cuming and Bennett 1981; Tobin 1981) with transcript abundance generally mediated by the light receptor phytochrome (Apel 1979). Translation occurs on free cytoplasmic polysomes, a precursory apoprotein (pLHCP) is generated (Apel and Kloppstech 1978; Cuming and Bennett 1981; Tobin 1981) and by virtue of a transit sequence the pLHCP is directed to the chloroplast and across the envelope in an energy dependent manner (Grossman *et al.* 1980). Processing of the LHC IIb apoprotein occurs on the thylakoid membrane (Chitnis *et al.* 1988) and involves the proteolytic removal of the transit sequence leaving behind the mature (lower molecular mass) light-harvesting polypeptide (Apel and Kloppstech 1978).

An examination of the transbilayer organization of the LHC IIb polypeptide demonstrated a stromally situated amino terminus, a lumenally situated carboxy terminus, and three membrane spanning α helices (Burgi *et al.* 1987). The above findings have since been confirmed with the recent elucidation of the three dimensional structure of this polypeptide by electron crystallography (Kuhlbrandt and Wang 1991). *In planta*, LHC IIb exists as a homo-trimer with each subunit binding 15 chlorophyll molecules. From the analysis, Kuhlbrandt and Wang submit that the primary role of LHC IIb is to efficiently pack photosynthetic pigments into an orientation that optimizes energy transfer from the chromophores to the reaction centres. The potential for involvement of LHC IIb in the promotion of green pigment retention by rapeseed embryos becomes apparent with the realization that, *in planta*, over half of all chlorophyll is incorporated into this pigment-protein assembly (Bennett 1983; Green 1988).

2.2 PHYTOHORMONES AND PHOTOSYSTEM COMPONENTS

The biological regulation of LHC IIb apoprotein abundance is a complex process that is mediated by light, phytohormones, pigment availability, and the state of tissue development. Both the cytokinin class of phytohormones and abscisic acid have been identified by our group as potential mediators of the green seed problem in canola. Because these compounds are known to influence many aspects of plant chloroplast and photosystem development and because seeds are a rich source of these compounds (van Staden *et al.* 1982; Zeevaart and Creelman 1988), the implication is justified. Additionally, it has been stated that foremost among the internal factors controlling plant development are phytohormones (van Staden *et al.* 1982).

2.2.1 *Cytokinins - chloroplast ultrastructure*

The development and differentiation of cells and organs depends both on internal and external cues. A multitude of studies regarding cytokinins have repeatedly demonstrated that these phytohormones play a major role in chloroplast development. Initial studies on hormonal control of plastid development examined the effects of cytokinin on organellar ultrastructure and on chlorophyll retention during periods of senescence. Over a quarter century ago, it was demonstrated that application of 6–benzylaminopurine (BAP), a cytokinin, to mature green tobacco leaves effectively extended chloroplasts longevity beyond the normal onset of chlorosis and leaf senescence (Sveshnikova *et al.* 1966). Hormonal treatment of mature leaf tissue stimulated the appearance of fully formed grana, thereby restoring the lamellar membranes to a state typically exhibited only by the chloroplasts of young leaves. In the same study, fully senescent (chlorotic) tobacco leaves were removed from the plant, treated with BAP, and maintained under continuous illumination. Remarkably, within 5 d the accumulation of chlorophyll was noted and by 20 d, stacked thylakoids were observed where no lamellar membrane had previously been present.

Years later, Naito and coworkers (Naito *et al.* 1981) examined the effect of BAP on the ultrastructure of bean leaf chloroplasts during various stages of development. Briefly, it was discovered that tissue response to hormone varied with leaf age (development). Application of BAP to young leaves increased both the size and the number of chloroplasts per cell. Treatment of mature leaves, on the other hand, did not promote organellar growth or plastid replication. However, treated tissue of all ages exhibited marked reductions both in lamellar membrane destruction and in loss of chlorophyll.

2.2.2 *Abscisic acid - chloroplast ultrastructure*

In 1971 Mittelheuser and Van Steveninck studied the effect of exogenous abscisic acid and cytokinin on the ultrastructural state of senescing wheat leaves. Senescence was

induced in 10 d old (green) primary leaf explants by depriving the tissue of light. Immediately prior to the transfer to darkness explants were exposed to ABA, cytokinin or no hormone (control). By 48 h, control tissue exhibited a noticeable reduction in the size and number of chloroplast starch grains in addition to a decline in the chloroplast ribosome population. By 72 h, the appearance of plastoglobuli (lipid microbodies derived from the thylakoid membrane) indicated the beginning of lamellar membrane deterioration.

Explants treated with ABA also displayed these features but the process advanced at an astounding pace. By 48 h chloroplast thylakoid degeneration was essentially complete as the organelles had become a mass of globules that were devoid of ribosomes. The effect of cytokinin on these leaf segments was, on the other hand, in direct opposition to that of abscisic acid. In these explants, chloroplast starch grain metabolism was conspicuously delayed until the sixth day after the transfer of tissue to darkness. Remarkably, in these cytokinin treated explants, neither the chloroplast ribosome population nor the lamellar membranes showed signs of degeneration for a further 5 d of light deprivation.

In summary, the work of Mittelheuser and Van Steveninck not only supports the reported function of cytokinins in chloroplast maintenance (as discussed in the previous section) but, additionally, this work documents antagonistic roles for ABA and cytokinin with respect to their interpretation by that organelle. On one hand abscisic acid promotes a rapid degradation of the chloroplast while, on the other hand, cytokinin preserves the ultrastructure and extends the longevity of the organelle.

2.2.3 Cytokinins - chlorophyll accumulation

Over two decades ago, it was recognized that cytokinins play a direct role in chlorophyll biosynthesis. Preliminary experiments on etiolated tissues revealed that cytokinin treated, dark-grown cucumber cotyledons undergo immediate and enhanced chlorophyll synthesis following with the onset of illumination (Fletcher and McCullagh 1971).

Treatment of these tissues with protein synthesis inhibitors demonstrated that the promotory effect of cytokinins on pigment accumulation was nullified by suppression of cytoplasmic ribosomes (Fletcher and McCullagh 1971). Shortly thereafter, it was confirmed that cytokinins enhance the production of δ–aminolevulenic acid (ALA) - a chlorophyll precursor that is normally limiting in the dark and during the initial stages of illumination (Fletcher *et al.* 1973). Interpretation of these findings was that the sole stimulatory effect of cytokinins on green pigment biosynthesis was a dark induction of the ALA formation system - the initial rate limiting step in chlorophyll production (Fletcher and McCullagh 1971; Fletcher *et al.* 1973).

Further analysis established that the regulation of chlorophyll biosynthesis by cytokinins is more complex than originally assumed. It was later recognized that the action of cytokinin on chlorophyll production and accumulation involves two separable events (Dei 1992; Low and Tsuji 1992). The first event is the previously characterized elimination of pigment-lag by hormonal induction of ALA production. It was demonstrated that a short incubation with cytokinin followed by a hormone-free dark period was sufficient to generate this condition. The subsequent illumination of tissue treated in this manner rapidly induced the appearance of chlorophyll from the pool of cytokinin promoted ALA (Low and Tsuji 1992). The second, delayed, effect of cytokinin on pigment production requires extended dark incubation of the tissue prior to illumination. This treatment results in a marked long-term promotion of chlorophyll accumulation. At the time of discovery this effect remained difficult to explain and was speculatively attributed to a nonselective hormonal inhibition of aging (Low and Tsuji 1992) or to an accelerated steady-state rate of chlorophyll synthesis (Dei 1992).

Perhaps the results of a study conducted by Ford and coworkers (1979) added to the confusion surrounding the long-term effect of cytokinins on pigment production. Etiolated sunflower cotyledons were treated with cytokinin in the dark for 24 h after which

time the tissue was illuminated for 2 h. Analysis of the greening tissue demonstrated two incongruities with respect to the anticipated relationship between ALA and chlorophyll. First, it was established that maximal production of chlorophyll by the tissue required a cytokinin incubation period five times that needed for maximal induction of its rate limiting precursor ALA. Secondly, a ten-fold greater cytokinin concentration was required to optimize chlorophyll production than the concentration necessary to trigger maximal ALA production.

These physiological discrepancies suggest an additional role, other than ALA induction, for cytokinin directed chlorophyll accumulation. This alternate cytokinin-mediated event was discovered in the early 1980s and came under intense investigation shortly thereafter wherein it was demonstrated that the major light-harvesting protein of PSII is regulated by this hormone.

2.2.4 Abscisic acid - chlorophyll accumulation

Just as the functions of abscisic acid and cytokinin are antagonistic with regard to chloroplast ultrastructure, this too is the case for their roles in chlorophyll accumulation. More than two decades ago it was recognized that exogenous ABA effectively hampers greening of young etiolated wheat leaves (Beevers *et al.* 1970). In that study, etiolated leaf segments were incubated with abscisic acid for 6 h after which the explants were continuously irradiated to promote chlorophyll synthesis. Tissues treated with the growth regulator exhibited a marked prolongation of the initial lag phase in chlorophyll synthesis, a result in strict opposition to the effect of cytokinins on this process.

Bengston and coworkers (1977) reexamined the effect of ABA and cytokinin on pigment levels in greening wheat leaves. Their work confirmed that of Beevers *et al.* (1970) in that incubation of the etiolated tissue with ABA served to prolong the lag in chlorophyll production during the subsequent period of illumination. Additionally, the rate of chlorophyll accumulation following illumination was strongly suppressed for 48 h in tissues that had

been exposed to ABA. These workers demonstrated that ABA treatment prohibited the generation of protochlorophyllide - a chlorophyll precursor that is, itself, dependent on the level of endogenous ALA. If, however, exogenous ALA was supplied to the ABA treated tissues, protochlorophyllide regeneration was observed. Consequently these researchers hypothesized that ABA inhibits the accumulation of chlorophyll primarily by disabling the ALA forming system, an event that occurs outside the chloroplast.

Shortly after this, the effect of abscisic acid on chlorophyll accumulation by germinating embryos of *Agrostemma githago* was examined (Schmerder *et al.* 1978). Results with this tissue were identical to those from the studies on etiolated wheat leaves discussed above. Seeds were imbibed in the dark for 24 h and excised embryos were incubated in the dark for a further 48 h in the presence of ABA, cytokinin or no growth regulator (control). After this period, embryos were irradiated for 24 h to promote chlorophyll formation. Tissue incubated with 1 $\mu g\ mL^{-1}$ BAP produced greater than 150% of the chlorophyll manufactured in control tissue over the same period. BAP at a concentration one-thousandth of this was sufficient to elevate chlorophyll levels over that of control tissue. Incubation of the embryos with abscisic acid (1 $\mu g\ mL^{-1}$) prior to illumination, however, prohibited any production of chlorophyll during the subsequent 24 h. Additionally, ABA at a concentration one-thousandth of this reduced the accumulation of chlorophyll by the embryo below that of the control tissue. The antagonistic roles of ABA and cytokinin were further established when *A. githago* embryos were simultaneously exposed to both growth regulators. This treatment appreciably reduced the inhibition of greening normally exhibited by the solitary application of abscisic acid (Schmerder *et al.* 1978).

These three studies (Beevers *et al.* 1970; Bengston *et al.* 1977; Schmerder *et al* 1978) clearly demonstrate that abscisic acid obstructs chlorophyll accumulation in etiolated (heterotrophic) tissues. Further to this, Beck and Richmond (1971) showed that ABA can accelate the rate of chlorophyll loss by green (autotrophic) tissues. In this study, green leaf

segments of three species were removed and treated with abscisic acid, cytokinin, or a combination of the two. Senescence was then induced in these explants by light deprivation. Analysis of chlorophyll levels revealed that ABA greatly enhanced pigment loss whereas cytokinin stabilized chlorophyll under these conditions.

Analogous to the results documented by Schmerder *et al.* (1978) regarding the combined influence of ABA and cytokinin (*Agrostemma* embryos), the study by Beck and Richmond (1971) established that if cytokinin is applied to tissue in combination with abscisic acid, the rapid ABA-directed loss of chlorophyll is abolished. Consequently, Beck and Richmond (1971) concluded that an interplay among changing endogenous levels of plant growth regulators modifies their physiological interpretation and thereby brings about diverse developmental responses. The contradictory roles of abscisic acid and cytokinins in the preservation and production of chlorophyll is akin to their opposing effects on the regulation of chloroplast ultrastructure.

2.2.5 Cytokinins - chlorophyll a/b binding apoproteins

The tobacco cell suspension culture AG_{14} is an undifferentiated tissue that is unable to produce thylakoid membranes and chlorophyll in the absence of cytokinin regardless of whether it is illuminated (Seyer *et al.* 1975). In the light, exogenous cytokinin initiates greening and, as electrophoretic analysis demonstrates, stimulates a substantial accumulation of the major light-harvesting protein of photosystem II (Axelos and Pesud-Lenoel 1980). Although a mechanism for the hormonal effect was not defined, it was concluded that cytokinins control LHC IIb biosynthesis because untreated cells lack this protein.

Later, researchers using antibodies specific for the LHC IIb apoprotein, in combination with the highly sensitive technique of radio-immunoassay, were unable to detect even residual levels of this CAB protein in cytokinin-free light grown tobacco suspension

cultures (Axelos *et al.* 1984). In cytokinin treated cells, however, a strong correlation between the rate of chlorophyll synthesis and the specific accumulation of LHC IIb was revealed. Consequently, it was proposed that cytokinins are required for the stabilization of this protein *in vivo* and that in their absence the protein is turned over rapidly resulting in a block in greening (Axelos *et al.* 1984).

Shortly thereafter, the way in which cytokinins exert control over the LHC IIb polypeptide was clarified (Teyssendrier de la Serve *et al.* 1985). An analysis of *in vitro* translation products from cytokinin treated AG_{14} revealed that the translation activity of the polyadenylated RNA was appreciably stimulated for the production of the major PSII antenna. Additionally, Northern blot analysis of the cytokinin treated tobacco suspension cells demonstrated a ten-fold increase in the CAB mRNA over that of controls. As a result, authors of the study concluded that cytokinins promote the accumulation of the light-harvesting apoprotein(s) by amplifying the steady-state level of the relevant mRNA. Whether this amplification of the message occurred transcriptionally or post-transcriptionally, however, remained a predominant question.

The effect of exogenous cytokinin on the aquatic plant *Lemna gibba* (or duckweed as it is commonly known) is in keeping with the results obtained with the tobacco cell suspension system. That is, cytokinins similarly increase the steady-state level of LHC IIb messages in this plant (Flores and Tobin 1986). In the study, photosynthetic (green) *L. gibba* were transferred to darkness and grown heterotrophically on a sucrose medium. Under these conditions, the message for the major light-harvesting apoprotein is reduced to undetectable levels by the seventh day of darkness (this species, unlike most plants, does not degrade chlorophyll or the CAB apoproteins under these conditions). After the extended dark period and in the complete absence of light, cytokinin was applied to the duckweed cultures. Incubation under these conditions for 24 h was sufficient to initiate a marked increase in hybridizable mRNA for the apoprotein(s). To determine whether the gene(s)

encoding the message was transcribed more actively in the presence of cytokinin, nuclei from the duckweed cultures were isolated and the unprocessed transcripts (hnRNA) were examined. The analysis demonstrated no levatory effect of the hormone on the hnRNA of for the CAB protein indicating that cytokinins do not increase Cab gene transcription. However, since the hormone effectively amplifies the level of CAB mRNA, Flores and Tobin (1986) proposed that cytokinins promote the accumulation of these transcripts by enhancing their cytoplasmic stability (a post-transcriptional event).

The technique of nuclear run-off transcription measures the extent to which a particular gene is being transcribed at the time of nuclear isolation. Essentially, the rate of RNA production from a transcription unit is measured without regard to hnRNA processing or mRNA accumulation. Flores and Tobin (1988) used this procedure to confirm that the primary effect of cytokinin on the mRNA of the major light-harvesting apoprotein of dark grown *L. gibba* is a post-transcriptional stabilization of the cytoplasmic molecule. Although the hormone had a slight stimulatory impact on the rate of CAB transcription, this effect was negligible compared to its post-transcriptional function.

Recalling that green heterotrophic *L. gibba* requires no light for cytokinin-mediated CAB mRNA accumulation (Flores and Tobin 1986; 1988), the findings of Abdelghani *et al.* (1991), who were working on tobacco cell suspension cultures, are conflicting. For instance, in the absence of light, cytokinin treated tobacco cells are unable to accumulate the CAB message to an appreciable extent. However, if illuminated tobacco cell suspensions were treated with cytokinin, these researchers reported an extensive accumulation of light-harvesting apoprotein transcripts (Abdelghani *et al.* 1990).

These apparent incongruities in the way the mRNA for light-harvesting antennae respond to cytokinin may be attributable to plastid development. Because duckweed cultures had been treated with light prior to experimentation, chloroplast development was promoted and maintained even in the absence of light. Conversely, the illuminated tobacco

cells are incapable of the developmental transition from etioplast to chloroplast in the absence of cytokinin. Essentially then, the transcriptional response of two different organelles (chloroplasts vs. etioplasts) has been examined with regard to cytokinin and the state of development has proven to be a key factor in hormonal responsiveness.

A study of excised cotyledons of dark-grown watermelon emphasizes this point. Etiolated cotyledons treated with cytokinin in the absence of illumination were able not only to accumulate LHC IIb mRNA but also the relevant CAB protein (Longo *et al.* 1990). Unlike the etiolated tobacco cells, the etiolated cells of watermelon cotyledon interpret the growth regulator (with regard to CAB mRNA accumulation and translation) in the absence of light. Perhaps this discrepancy, too, can be explained by the distinct state of plastid differentiation between tissues - watermelon cells possess amyoplasts while tobacco suspension cells possess etioplasts. This plastid developmental state appears to permit one tissue to interpret cytokinin differently than another tissue. These species comparisons (tobacco suspension culture, heterotrophic duckweed culture, and dark-grown watermelon cotyledon) clearly demonstrates that the state of tissue development is of paramount importance to its phytohormonal responsiveness.

2.2.6 Abscisic acid - chlorophyll a/b binding apoproteins

Whereas cytokinins promote the accumulation of Cab mRNAs, abscisic acid is known to negatively regulate the accumulation of these transcripts. For instance, the soybean (*Glycine max*) Cab 3 gene is turned off by the growth regulator during embryogenesis in this species (Chang and Walling 1991). Expression of Cab 3, the most abundantly transcribed soybean Cab gene, is tightly correlated to the level of endogenous abscisic acid during seed development. Northern blot analysis indicated that the Cab message is at its peak level during the early stages of soybean embryogenesis when the

level of endogenous ABA is low. As seed development proceeds, endogenous ABA peaks and the Cab message begins to decline.

In the same report (Chang and Walling 1991) the effect of exogenous ABA on soybean cotyledonary Cab 3 mRNA levels at various stages of embryo development was examined. Since the attainment of high endogenous abscisic acid by the soybean embryo coincides with the decline of the light-harvesting message, it was hypothesized that the abundance of this transcript is developmentally coordinated by ABA. To test the premise, embryos were removed from the seed coat and the embryonic axis was separated from the remainder of the embryo. Cotyledons were cultured with a defined photoperiod for 5 d in the presence of exogenous abscisic acid. During soybean seed development, 35 days after flowering (DAF) is the stage at which embryonic Cab mRNA levels are highest and ABA levels lowest. Upon completion of a 5 d incubation of 30 DAF cotyledonary explants with 50 μM ABA, no hybridizable Cab transcripts could be detected in the tissue.

At 55 DAF, soybean seed development is typically characterized by a peak in the endogenous ABA concentration accompanied by the commencement of Cab mRNA decline. Cotyledonary explants at this developmental stage differ from cotyledons at 35 DAF in that a 5 d incubation of 50 DAF explants with 50 μM ABA diminishes, but does not eliminate, the hybridizable Cab transcript.

The contradictory action of ABA and cytokinins with respect to chloroplast ultrastructure, chlorophyll accumulation, and Cab transcript accumulation, three distinct aspects of photosystem development, makes this antagonism a recurrent theme.

2.3 NORMAL SEED DEVELOPMENT

2.3.1 Oil deposition

In anticipation of the heterotrophic germinative events that precede its attainment of photosynthetic competence, the developing canola embryo synthesizes fatty acids as an energy reserve. The majority of these fatty acids do not remain free but, rather, are esterified to glycerol to form triacylglycerols (TAGs). These neutral lipids are the primary energy reserve of the seed and are sequestered within organelles termed oilbodies (Stymne and Stobart 1987). The production of TAGs during rapeseed embryogenesis is temporally regulated with rapid synthesis and accumulation commencing circa 16 to 18 DPA and persisting for a further 20 to 24 d (Perry and Harwood 1993; Tzen *et al.* 1993). Depending on growth conditions of the parent sporophyte, 35 to 45% of the seed weight at maturity will be derived from these energy reserves (Johnson-Flanagan *et al.* 1991; Stymne and Stobart 1987; Weselake *et al.* 1983).

2.3.2 Regulation of oleosin, cruciferin and napin accumulation

The production and accumulation of oleosin proteins is a characteristic embryonic event associated with TAG deposition. The developing canola embryo initiates the extensive synthesis of oleosins, concurrent with TAG deposition, such that at maturity these molecules comprise approximately seven percent of the total seed protein (Tzen *et al.* 1993). The oleosins are a group of low molecular mass alkaline polypeptides that are incorporated solely into the oilbody phospholipid monolayer (Huang 1992). Furthermore, because these integral membrane polypeptides maintain a negative charge at physiological pH so too does the oilbody surface. By virtue of this property, in combination with the steric hinderance imposed on the organelle surface by oleosin residues, coalescence of individual oilbodies is prevented (Huang 1992). Additionally, oleosins mediate oilbody volume thereby

ensuring an organellar surface area that is suitable for rapid and efficient lipase-mediated catabolism of TAG during germination (Huang 1992). Besides their proposed structural functions, it has been suggested that oleosins serve as oilbody docking signals for lipases during periods of TAG catabolism (Huang 1992). Not unlike the production of the oleosin proteins, at a defined point in rapeseed embryogenesis the synthesis and deposition of cruciferin and napin - the major seed storage proteins of this species - is initiated. Furthermore, synthesis of these proteins is unique to the embryogenic stage of the canola life cycle (Crouch and Sussex 1981). In a strategy similar to that of TAG deposition, cruciferin and napin are accumulated by the developing seed in anticipation of germination. The stores of carbon and nitrogen within these polypeptides are mobilized during this heterotrophic period and are utilized for growth by the young sporophyte (Crouch and Sussex 1981).

Although both polypeptides are seed storage proteins, cruciferin and napin are biochemically and antigenically distinct (Murphy *et al.* 1989). Cruciferin is a high molecular mass complex (300 kDa) which is composed of several polypeptides within the range of 20 to 30 kDa. Napin, on the other hand, is a low molecular mass protein consisting of two polypeptides with molecular masses 9 and 4 kDa (Crouch and Sussex 1981). While the reported timing of cruciferin and napin production in rapeseed embryos varies from one laboratory to another (compare Crouch and Sussex 1981 with Murphy *et al.* 1989), those concerned do agree that these proteins do not accumulate in the early stages of seed development.

Transcriptional analysis of these genes has revealed that the level of both cruciferin and napin mRNAs rapidly escalate during embryogenesis (DeLisle and Crouch 1989) and this event is accompanied by a pronounced accumulation of the polypeptides. In the transition from the mid to late stage of embryogenesis, seed development is marked by waning levels of napin mRNA (DeLisle and Crouch 1989) the effect of which is arrested

napin accumulation (Crouch and Sussex 1981). The level of cruciferin mRNA, on the other hand, remains elevated during this period (DeLisle and Crouch 1989) with continued synthesis, albeit at a reduced rate, of the polypeptide products (Crouch and Sussex 1981).

An additional characteristic of these embryonic genes is that they are positively regulated by the phytohormone abscisic acid. Culture of excised zygotic embryos of *B. napus* in the absence of ABA, for instance, results in a drastic decline in the storage protein mRNAs. If ABA is supplied to the excised zygotic embryo, however, napin and cruciferin transcript accumulation approaches that of the normal seed (DeLisle and Crouch 1989) and synthesis of the respective polypeptides is also apparent (Crouch and Sussex 1981).

2.3.3 Seed degreening

Just as TAG, storage protein, and oleosin accumulation are characteristic canola embryonic events, so too is the temporal degradation of embryonic chlorophyll. Johnson-Flanagan and Thiagarajah (1990) demonstrated that as the seed proceeds through the 60 to 40% seed moisture range (a period normally requiring 5 to 6 wk) there is a direct correlation between percent seed moisture[3] and the quantity of embryonic chlorophyll. Chromatographic analysis of chlorophyll catabolite generation by the developing canola seed revealed that, near 50% seed moisture, a genetic event is initiated which results in the degradation of seed chlorophyll. In the same study, electrophoretic analysis revealed that specific chlorophyll-associated photosystem protein components are, similarly, removed from the maturing embryo. As a consequence of these events, by the time the seed matures the embryonic photosystem is altogether degraded and seed chlorophyll is not detectable (Johnson-Flanagan and Thiagarajah 1990).

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[3] It should be noted that these researchers maintain embryo development is better judged by seed moisture content than it is temporally (DPA).

2.4 FROST STRESSED SEED DEVELOPMENT

2.4.1 Hastened embryo development

In stark contrast to normal canola seed development where maturation takes several weeks, embryogenesis is hastened following a frost and seed maturation (desiccation) is completed within a matter of days (Johnson-Flanagan *et al.* 1991; Johnson-Flanagan et. al. 1992). Interestingly, although frost has this profound and rapid impact on the rate of embryo development, the stress does not adversely affect the germination potential of seed at or below a moisture content of 80% (Johnson-Flanagan *et al.* 1991). Conversely, lipid and storage protein synthesis are influenced by frost stress. For instance, not only is TAG fatty acid elongation and desaturation altered by a frost but so too is the prolonged post-frost accumulation of the seed storage proteins (Johnson-Flanagan *et al.* 1991). It should be noted, however, that the *de novo* synthesis of cruciferin (and that of napin depending on the stage of seed development) occurs to an appreciable extent for a number of hours following the stress. This post-stress synthesis permits a significant accumulation of these polypeptides prior to seed desiccation (Johnson-Flanagan *et al.* 1991).

2.4.2 Improper retention of embryonic chlorophyll

Furthermore, the frost-cued shift from predesiccation embryogenesis to accelerated desiccation and seed maturation (Johnson-Flanagan *et al.* 1992) interrupts the typical catabolism of seed chlorophyll and leaves behind a mature canola embryo that has failed to degreen (Johnson-Flanagan *et al.* 1990). Early frost is the primary factor responsible for the generation of green canola seed, and hence green oil, in Canada. Because early frost is not uncommon in the northern regions of the country where this crop is often grown, the potential for green seed production in these areas is genuine.

2.4.3 Green oil

Food oil contaminated with chlorophyll is undesirable because it is displeasing to the consumer (Singh and Chuaqui 1991), difficult to process, and photochemically instable. For example, the presence of chlorophyll in canola oil 1) reduces the rate of oil hydrogenation by acting as a catalyst poison (Abraham and deMan 1986), 2) generates "off tastes" and oil rancidity by supporting photooxidation reactions (Dahlen 1973; Usuki *et al.* 1984a) and 3) reduces product shelf-life (by the previous mechanism) proportional to the extent of pigment contamination (Usuki *et al.* 1984b).

Customarily, oil derived from green seed is purified to reduce the level of contaminant pigment. Bleaching clays are regularly used for this purpose but are expensive and environmentally unsound because the spent material must be discarded in limited landfill space. Furthermore, this method of chlorophyll removal is wasteful and inefficient because the clays retain an appreciable amount of valuable oil that cannot be recovered.

Canola oil regularly contains levels of chlorophyll in excess of the maximum allowable level set by the Canadian Grain Commission. Because this condition is expected periodically, it is the strategy of food oil scientists to develop more efficient and less costly procedures for the removal of pigment from canola oil. At best, recent oil purification schemes have achieved rather modest successes on small scale trials (Diosady 1991, Singh and Chuaqui 1991). The cost of large scale industrial application of these methods, however, is expected to be too high to allow their implementation.

2.6 MICROSPORE-DERIVED EMBRYO CULTURE

2.6.1 Rationale for exploitation of embryo culture

B. napus microspore-derived (MD) embryo culture has been employed herein as a model to study the effect of phytohormones on the changes in embryonic photosystem components that are associated with seed development. MD embryos were chosen over seed (zygotic) embryos for the examination of these seed events for three fundamental reasons. First, it is not uncommon for the *B. napus* MD embryo culture system to furnish literally hundreds to thousands of individual embryos within the space of a conventional petri plate. This characteristic eliminates the need and the maintenance of many seed-bearing donor plants.

Second, unlike seed development, MD embryogenesis occurs independently of the sporophytic mother plant and is not subject to parental plant influences. Embryonic autonomy is particularly important to the work reported herein because responsiveness of this tissue to phytohormones is examined.

Third, it is of the utmost importance to accurately stage embryo development in an examination such as this because responsiveness to phytohormones is often dependent on the developmental stage of the tissue. An accurate evaluation of canola embryogenesis is particularly troublesome because the flowering habit of this species is indeterminant. That is, new flowers are continually initiated and, as a consequence, seed development is asynchronous. If seed embryo development were to be properly monitored, the task of recording 'days post anthesis' must be undertaken for every flower of every silique that is to be analyzed. Furthermore, seed development is not accurately staged in this manner because the rate of development varies among the multiple embryos of a single silique (Johnson-Flanagan and Singh 1993). Consequently, an accurate assessment of canola seed development is not only inconvenient but also difficult to perform accurately. Although

asynchronous embryogeny is not circumvented by the culture of MD embryos, developmental studies are greatly simplified because these embryos are rapidly staged on the basis of morphology (size) by passing the culture through filters with diminishing pore diameters.

Regardless of the potential advantages of the MD system, if the molecular and biochemical properties of this tissue do not accurately mimic the events known to take place throughout typical seed development the usefulness of the culture will be limited. Therefore, it is the purpose of the following section to examine the legitimacy of MD embryo culture as a canola seed developmental model.

2.5.2 Oil biosynthesis - MD embryogenesis

In an examination of MD and zygotic embryos of two cultivars of *Brassica napus*, Pomeroy and coworkers (1991) noted that MD embryos are much larger than their zygotic counterparts at parallel developmental stages. Despite this morphological divergence, MD embryos properly initiate TAG accumulation during development and the total fatty acid content of this tissue compares favorably, for an extended period of embryogenesis, to that of seed embryos. However, beyond the mid-cotyledonary stage MD embryos are incapable of further fatty acid accumulation and in that way differ from the zygotes (Pomeroy *et al.* 1991). In the same study, it was noted that during *B. napus* seed development there is a characteristic temporal variation in the fatty acid profile of the storage triacylglycerols. Consequently, a detailed comparison was made between MD and zygotic embryos with regard to these developmental fluctuations in fatty acid profiles. Lending support to the validity of MD embryogenesis as an accurate seed developmental model, MD embryos from both *B. napus* cultivars precisely imitate developmental fatty acid modifications of the seed. For instance, seed of the high erucic acid (22:1) cultivar Reston initially contains a paucity of this fatty acid, yet there is a pronounced accumulation of 22:1 commencing at the mid-

cotyledonary stage and proceeding through to the very late-cotyledonary stage of embryo development. Microspore-derived embryos of this cultivar accurately mimic this temporal pattern of 22:1 accumulation as they begin and complete erucic acid synthesis at morphological stages identical to that of the seed embryo. Moreover, the developmental changes of other prominent Reston seed fatty acids are also accurately reflected during MD embryogenesis. Likewise, Pomeroy and coworkers (1991) reported that MD embryos of Topas accurately mimic the specific fatty acid changes associated with seed development in that rapeseed cultivar.

The three carbon atoms of the glycerol backbone are stereochemically unique and readily distinguishable by the acylation enzymes that participate in TAG formation. Adding further complexity to TAG biosynthesis, particular glycerol acylation enzymes can have stringent preferences for distinct fatty acid species (Stymne and Stobart 1987). Taylor and coworkers (1991), for instance, reported that during TAG deposition in the Reston cultivar of *B. napus* embryonic acylation enzymes exclusively insert erucic acid at glycerol positions sn 1 and sn 3. Likewise, these researchers report the identical stereospecific incorporational pattern of 22:1 into the glycerol backbone of TAGs from microspore-derived embryos of Reston.

In an ensuing comparison of the MD and zygotic embryos of *B. napus*, Taylor and coworkers (1992) examined the capability of tissue homogenates from both embryo systems to synthesize very long chain fatty acids (VLCFAs) and to incorporate these units into the triacylglycerol storage reserve. On a percent acyl lipid basis, it was reported that MD embryo homogenates elongate oleic acid (18:1 to 20:1 and 22:1) and incorporate the resultant VLCFAs specifically into the TAG pool at a rate comparable to zygotic embryos of an analogous morphological stage. These findings proved that the zygotic pattern of VLCFA biosynthesis and localization during embryogenesis is a property shared by embryos of microspore derivation (Taylor *et al.* 1992).

Diacylglycerol acyltransferase (DGAT - [E.C. 2.3.1.20]) is the enzyme which catalyses the acylation of sn 1,2–diacylglycerol to form TAG. The specific activity of this enzyme was determined throughout development in the seed and MD embryo tissues of *B. napus* (Weselake *et al.* 1993). In this developmental analysis, it was revealed that the temporal appearance and activity of this enzyme is similar in both tissues. Although the trend is not as prominent during MD embryogenesis, DGAT activity increases in the early stages of development, peaks during rapid TAG accumulation and then declines as TAG accumulation plateaus (Weselake *et al.* 1993).

In summary MD embryos accurately mimic the following seed developmental events. 1) The temporal appearance and accumulation of particular fatty acids (Pomeroy *et al.* 1991), 2) the fatty acid preferences and stereospecificity of the seed acylation machinery (Taylor *et al.* 1991), 3) the production of VLCFAs and the fatty acid elongase activity (Taylor *et al.* 1992), and 4) the temporal regulation of DGAT activity (Weselake *et al.* 1993). The strong developmental semblances between seeds and MD embryos has prompted a general conclusion among these researchers - MD embryo culture is an excellent system for the investigation of lipid enzymology and lipid biosynthetic pathways during seed development.

2.5.3 Regulation of embryonic proteins - MD embryogenesis

The synthesis and regulation of seed-specific proteins during MD embryogenesis is of direct interest to the validity of this culture system as a potentially useful seed developmental model. Taylor and coworkers (1990) have shown that torpedo-stage zygotic (21 BPA) and torpedo-stage MD (21 d culture) embryos of *B. napus* cv. Tapas are devoid of detectable quantities of napin and cruciferin mRNA. If, however, either embryo type is cultured with 10 µM ABA for a period of 48 h, they both exhibit a marked accumulation of these seed-specific transcripts (Taylor *et al.* 1990). The promotion of napin and cruciferin transcript accumulation in zygotic and MD embryos of this species by abscisic acid has

since been confirmed by Wilen and coworkers (1991). Similarly, these researchers demonstrated that transcription of the cruciferin and napin genes is induced by the plant growth regulator jasmonic acid (JA) in both zygotic and MD embryo systems. Additionally, like conventional seed development, there is a temporal accumulation of the oleosin proteins during MD embryogenesis, this accumulation is positively regulated by ABA, and subcellular fractionation confirms that these proteins are appropriately located in the oilbody fraction (Holbrook et. al. 1991). The precise emulation of zygotic embryogenesis by embryos of microspore derivation further illustrates their validity as a potential model for the study of seed development.

2.6 FROST INDUCED GREEN SEED - SPECULATIVE MECHANISMS

A number of proposals have been put forth in an attempt to explain the abnormal retention of chlorophyll by frost stressed canola seed. These postulations are discussed, in turn, below.

2.6.1 *Arrested seed development*

Because frost stressed canola seed rapidly desiccates, it has been suggested that this environmental circumstance prevents further seed development by prohibiting the completion of embryonic events that would normally extend into, or initiate beyond, the time of the burden. Since degreening is a process that is not complete until late in seed development, proponents of the above theory attribute green seed production to embryonic immaturity.

Recently, this idea lost credibility when it was revealed that frost stressed seed undergoes an accelerated initiation and successful completion of events normally associated with the late stages of standard embryo development (Johnson-Flanagan *et al.* 1992). In this study it was revealed that transcription of the late embryogenesis abundant (LEA) genes

(a set of seed-specific genes that are normally induced toward the tail end of embryogenesis) is rapidly activated following a sublethal frost. Successful initiation of this otherwise late embryogenic event is in direct opposition to the proposal that frost leaves the seed green by arresting subsequent development.

Moreover, although it appears that the rapid induction of LEA transcripts by frost is temporally improper with respect to the time line of typical seed development, induction of LEA transcription after frost stress properly coordinates to normal embryo development with regard to seed moisture content (Johnson-Flanagan *et al.* 1992). The main conclusions of this study were that 1) canola embryo development is best followed by changes in seed moisture rather than temporally and 2) frost stress does not obstruct canola seed maturation but rather accelerates the successful achievement of that state (Johnson-Flanagan *et al.* 1992).

2.6.2 Germinative cue

Another hypothesis, which is restricted to seed in the 50% moisture range, suggests that the abnormal retention of embryonic pigment occurs because the canola seed misinterprets the frost stress as a germinative cue (Johnson-Flanagan *et al.* 1991). This proposal dictates that the seed remains green, not because it is immature but, because the onset of autotrophic growth is anticipated and chlorophyll will be required for the pending shift to photosynthesis. Seemingly contrary to the hypothesis is that frost stressed seed is not observed to precociously germinate. This discrepancy can be resolved, however, by considering that rapid embryonic desiccation acts as an effective barrier to radicle protrusion (Johnson-Flanagan *et al.* 1991).

Most recently, it has been confirmed that frost stress induces renewed synthesis of embryonic chlorophyll (Johnson-Flanagan *et al.* 1994) but does not stimulate molecular events, like TAG mobilization, typically associated with germination (Johnson-Flanagan *et al.*

1992). During standard canola germination, the glyoxylate cycle is activated thereby allowing the heterotrophic embryo to synthesize complex carbohydrates from stored triacylglycerols. The onset of this heterotrophic phase is marked by the substantial transcription of isocitrate lyase - a key enzyme in the glyoxylate biosynthetic pathway (Comai *et al.* 1989). Johnson-Flanagan *et al.* (1992) revealed that frost stressed canola seed does not initiate production of the mRNA for this enzyme implying that frost does not cue a developmentally abnormal shift from embryogenesis to germination. As a consequence, it is unlikely that the stress provokes the retention of embryonic chlorophyll by providing an improper cue for germination.

2.6.3 Impairment of catabolic enzymes

Aside of greatly accelerating seed desiccation and maturation, then, it is evident that frost stress does not invoke a major shift in seed development. Therefore, it is conceivable that frost may promote green seed by specifically impairing enzymatic reactions involved in the degradation of embryonic chlorophyll. Although there is evidence for multiple, species-specific, chlorophyll degradative pathways in senescing plant tissues (Amir-Shapira *et al.* 1987), the mechanism of the process is so poorly characterized that it has recently been referred to as a biological enigma (Hendry *et al.* 1987). Nevertheless, the programmed catabolism of embryonic chlorophyll is of direct interest to this project.

Although both senescence and canola seed maturation are superficially characterized by chlorophyll degradation, embryonic degreening is a phenomenon that is otherwise quite unrelated to that of senescence. Despite the dissimilarity between these events, thylakoid peroxidase and chlorophyllase, enzymes presumed to catabolize chlorophyll during senescence, have been identified as potential mediators of pigment degradation during normal canola embryogenesis (Johnson-Flanagan and MacLachlan 1990a; 1990b). These researchers reasoned that if frost affects the function or production

of either of these enzymes, the failure of the seed to degreen might be explained by an impairment of pigment catabolism.

In their study of the thylakoid peroxidase (1990a), an enzyme known to catalyze the oxidative destruction of chlorophyll (Hutt 1982), Johnson-Flanagan and McLachlan demonstrated low enzymatic activity early in normal seed development when embryonic chlorophyll levels are high. As seed development progresses, however, it was revealed that the peroxidase activity intensifies and peaks at the stage where embryonic chlorophyll is normally rapidly degraded. Finally, as the seed approaches full maturity, chlorophyll is fully catabolized and the activity of the thylakoid peroxidase is diminished (Johnson-Flanagan and McLachlan 1990a).

Western blot analysis of seed peroxidase during the typical seed maturation process indicates that enzyme quantity increases preceding, and is maximal during, the period of greatest peroxidase activity. Additionally, the level of this enzyme decreases later in seed development when peroxidase activity is reduced (Johnson-Flanagan and McLachlan 1990a). Perhaps relevant to the green seed phenomenon is the finding that sublethally frost stressed canola seed in the 65% moisture range is strongly repressed in thylakoid peroxidase activity for the first 24 h period following frost. This set back is minor, however, because the seed thylakoid peroxidase activity fully recovers after this brief period of catabolic repression (Johnson-Flanagan and McLachlan 1990a).

Not unlike the thylakoid peroxidase, the activity of chlorophyllase, the enzyme which catalyses the removal of phytol from chlorophyll (Hendry *et al.* 1987), is minimal early in normal canola embryogenesis when seed chlorophyll is accumulating (Johnson-Flanagan and McLachlan 1990b). Additionally, chlorophyllase activity rapidly increases later in seed development as embryonic chlorophyll is degraded and diminishes upon seed maturation (Johnson-Flanagan and McLachlan 1990b). In contrast to thylakoid peroxidase, however, the activity of chlorophyllase is not adversely affected by frost stress. Furthermore, at 4 d

following the stress, chlorophyllase activity is nearly three-fold greater than it is in unstressed seed. Western blot analysis indicates that the frost stressed embryo rapidly synthesizes chlorophyllase, thus explaining its elevated post-stress activity (Johnson-Flanagan and McLachlan 1990b).

The induction of green seed by frost, then, cannot simply be attributed to a disruption of enzymatic pigment catabolism. Although peroxidase activity in canola seed is initially repressed by the stress, this activity recovers shortly afterward. Moreover, chlorophyllase synthesis and activity is not adversely affected by frost but is, rather, stimulated.

2.6.4 Current deliberation

If the cumulative knowledge regarding green seed is taken into consideration, it is clear that frost stress does not restrict seed maturation, does not induce precocious germination (the shift to autotrophic development), and does not effectively impair the suspected paths of pigment catabolism. What remains disturbingly unclear is how frost does promote this undesirable event.

The demonstration that frost stressed canola seed has the unusual ability, over specific developmental stages, to renew synthesis of chlorophyll (Johnson-Flanagan *et al.* 1990) and the apoproteins with which they associate (Johnson-Flanagan *et al.* 1994) may provide a clue. It is currently presumed, by these researchers, that the inappropriate accumulation of chlorophyll in the stressed seed arises from a developmentally aberrant induction or stabilization of photosystem chlorophyll-proteins which somehow dismisses chlorophyll degradation from the normal embryonic maturation sequence.

Support of the hypothesis is two-fold. First, *in vivo*, all green photosynthetic pigment is found in strict association with chlorophyll binding proteins (Markwell *et al.* 1979; Thornber 1975) and second, the level of canola embryonic chlorophyll a/b binding proteins is elevated

in the tissues of frost stressed canola seed (Johnson-Flanagan *et al.* 1994). It is not unlikely, therefore, that these photosystem apoproteins encourage or stabilize the improper retention and/or accumulation of pigment in the seed following a frost.

3. MATERIALS AND METHODS

3.1 PLANT MATERIAL

Plant material used in the present study was *Brassica napus* L. cv. Topas. Topas was chosen for its ability to yield large numbers of microspore embryos by the methods outlined in the following section. Donor plants were cultivated 2 per pot in Peat–Lite Cornell Mix at a temperature regime of 20°C/15°C (day/night) until bolting occurred at which time plants were grown at a temperature regime of 10°C/7°C (day/night). Cotyledon, leaf, and seed samples were harvested from donor plants that had been cultivated 2 per pot in the soil mixture described above. Plants were cultivated in environmental growth chambers under a 16 h photoperiod with a minimum irradiance of 400 µmol $m^{-2}s^{-1}$ at a temperature regime of 20°C/15°C (day/night). Illumination was provided by a combination of 40 W incandescent bulbs and 215 W very high output (VHO) cool white fluorescent tubes (2:1 ratio). Plants were fertilized once a week with a complete, instant soluble, 20:20:20 (N:P:K) fertilizer applied at a concentration of 200 mg L^{-1} (200-300 mL per application) using a Cameron bucket syphon injector.

3.2 MICROSPORE-DERIVED EMBRYOS

3.2.1 *MD embryo production*

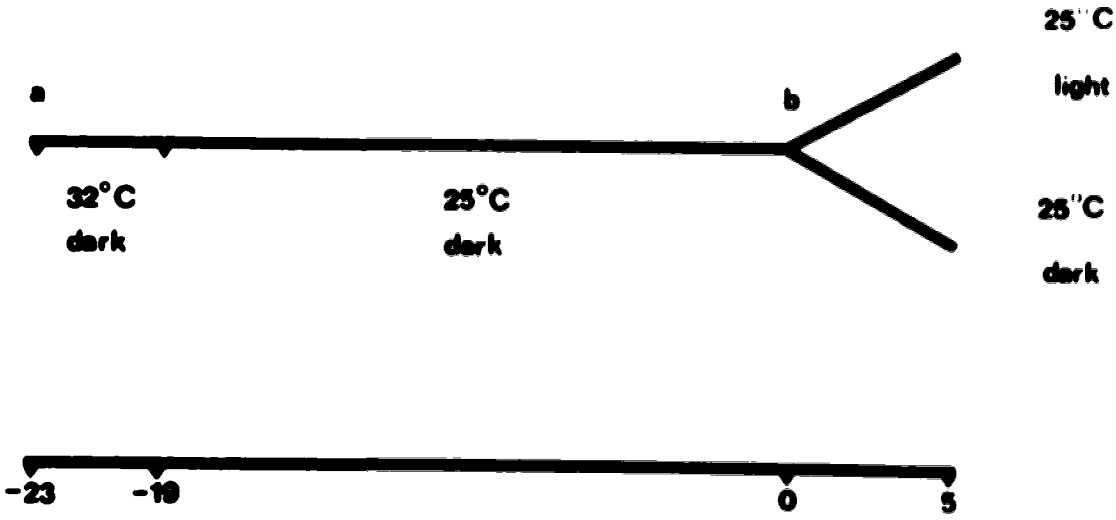
The method of microspore isolation and haploid embryo development described by Orr *et al.* (1990) was employed with one modification - the centrifugation step involving a 32% Percoll cushion was deleted. Microspores were initially cultured in modified Lichter medium (Lichter 1982; Orr *et al.* 1990) containing 13% sucrose (MLN–13%). The cellular suspensions were incubated at 32°C in the dark for 4 d to induce embryogenesis after which

the cultures were transferred to 25°C with rotary agitation (90 rpm). The duration of this period of embryo growth at 25°C varied depending on the experiment (see below).

3.2.2 Photosystem assembly - MD embryo greening

At 23 d post microspore isolation, embryos intended for greening studies were either left under the previous conditions of culture (negative controls) for a further 5 d or, greened by subjecting the culture to 5 d of continuous irradiance at 30 to 35 μmol $m^{-2}s^{-1}$ provided by fluorescent tubes (Sylvania 40 W cool white - GTE, Drummondville, Que). Like their dark grown counterparts, these greening embryos were maintained with constant rotary agitation (90 rpm). An outline of the manipulations is provided in Figure 2.

Figure 2. Culture of *B. napus* MD embryos used in the analysis of greening. Embryos maintained in NLN–13% throughout the 28 d period of development. Conditions of growth are indicated on the uppermost line and those points coordinate with the time-line below; (a), point of microspore isolation (day −23 on the time-line); (b), point at which one-half of the embryos were greened by transfer to continuous illumination of 30 to 35 μmol $m^{-2}s^{-1}$ (day 0). Tissue harvest was initiated on day 0 and continued until day 5.


a
b
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dark
25°C
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light
25°C
dark
-23
-19
0
5
DAYS

3.2.3 *Photosystem disassembly - MD embryo degreening*

If the influence of phytohormones on degreening was studied, greening of the MD embryos was initiated 14 d following microspore isolation. A complete exchange of media, in addition to the selective retention of a culture composed essentially of one morphological stage (early to mid-cotyledonary embryos) was simultaneously accomplished by screening the culture through sterile polyester mesh with a pore diameter of 530 µm (PeCap, Tetko Inc., Elmsford, NY) and rinsing the selected tissue with fresh media. Filtrate embryos were aseptically transferred to fresh NLN–13% and greened over a 5 d period by exposure to continuous illumination of intensity 80 to 100 µmol $m^{-2}s^{-1}$. Illumination was provided by fluorescent tubes with long wavelength supplementation (Sylvania 40 W cool white tubes in a one to one ratio with Sylvania 34 W warm white tubes - GTE, Drummondville, Que). At the end of the greening period cultures were 19 d old.

After the 5 d greening period, developmentally delayed embryos were again removed by passing the culture through the sterile polyester mesh (530 µm pore diameter). Filtrate embryos (mid-cotyledonary stage) were aseptically subcultured into fresh NLN media with a sucrose content of 20% (NLN–20%) containing either 1) no growth regulator, 2) ABA[3] at a concentration of 50 µM or, 3) BAP at a concentration of 5 µM. Both the abscisic acid (±cis–trans isomer) and the BAP utilized in this report were sterilized by filtration, rated 'plant cell culture tested', and obtained through Sigma Chemical Company (St. Louis, MO).

Half of the embryos within each of the three treatments (ABA, BAP or control) were returned to the previous conditions of culture (25°C, 80–100 µmol $m^{-2}s^{-1}$ and rotary agitation) and the other half of the embryos were cultured in the absence of light at 25°C with rotary agitation. The duration of embryo culture under these conditions and all

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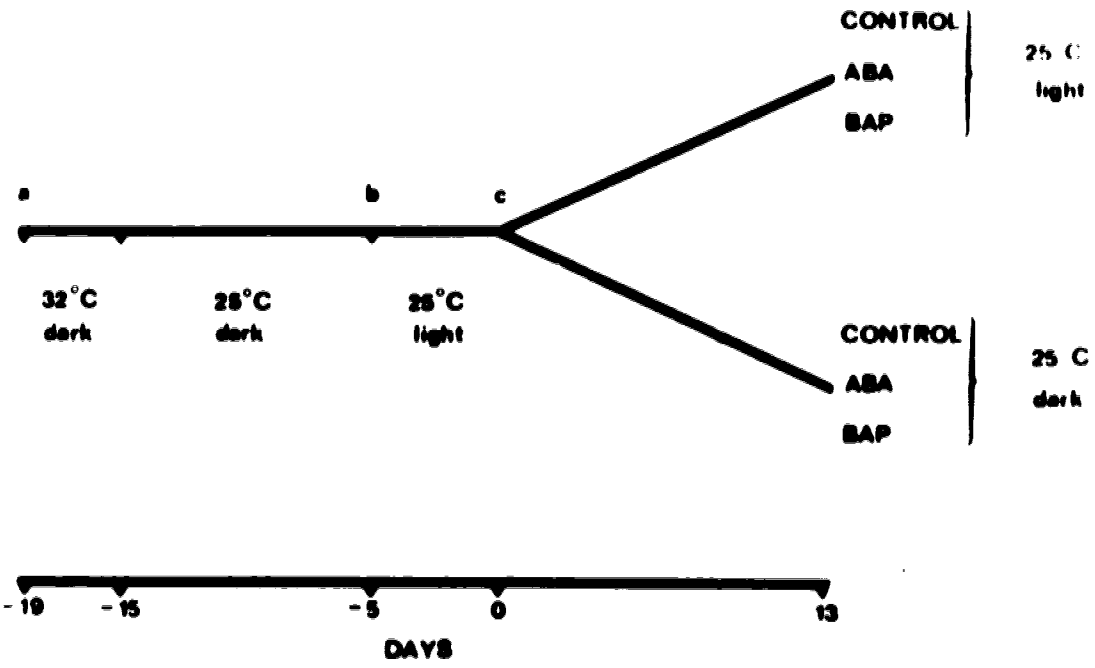
[3] It should be noted that the effect of ABA on the chlorophyll and chlorophyll-catabolite content of greened MD embryos of *B. napus* cv. Topas cultured in NLN-13% has been previously documented (Johnson-Flanagan and Singh 1993).

manipulations to these cultures including microspore isolation, MD embryo greening, phytohormone treatment, and MD embryo degreening are presented in Figure 3.

Figure 3. Culture of *B. napus* MD embryos used in the analysis of degreening. Conditions of growth are indicated on the uppermost line and those points coordinate with the time-line below; (a), point of microspore isolation (day -19 on the time-line); (b), point of media exchange (NLN–13% to fresh NLN–13%) and transfer of all cultures to continuous illumination of 80 to 100 μmol $m^{-2}s^{-1}$ (day -5); (c), point of embryo transfer to NLN–20% (day 0). On day 0, one-half of the cultures were left illuminated while the other half were transferred to darkness. Additionally, on day 0, cultures were either supplemented with ABA (to a final concentration of 50 μM), BAP (to a final concentration of 5 μM), or no growth regulator (controls). Tissue harvest was initiated on day 0 (immediately prior to the addition of growth regulators) and continued for a period no longer than day 13.


CONTROL
ABA
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25°C
dark
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CONTROL
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25 C
dark
-19
-15
-5
0
13
DAYS

3.2.4 *Day 0 - MD embryo greening and degreening*

The timing and number of harvests varied depending on the experiment conducted. Regardless of the type of analysis, MD embryos were harvested throughout a developmental period measured in days beginning at a point termed 'day 0'. Day 0 of the MD embryo greening experiment occurred with the onset of illumination, 23 d after microspore isolation. Tissue was harvested on this day and every day afterward for a further 5d. Both day 0 and the duration of the harvest period are indicated in Figure 2. Dark grown cultures (negative controls) were harvested under green light[4]. Day 0 of the MD embryo degreening experiment occurred following the greening period, 19 d after the isolation of microspores (Figure 3). Dark grown cultures (controls, ABA and BAP) were harvested under green light.

3.3 TISSUE COLLECTION AND STORAGE

MD embryos were harvested over nylon fabric, rinsed with distilled H_2O to remove the media, and immediately immersed in liquid nitrogen. Leaves, cotyledons, and seeds were detached from the donor plant and immediately placed into liquid nitrogen. Frozen tissues intended for Northern blot analysis were stored at –80°C until RNA could be extracted. Frozen tissues intended for SDS–PAGE, Western blot analysis, or chlorophyll quantitation were lyophilized (usually 24-48 h) in the absence of light and the dry masses recorded. These samples were stored at –20°C over a desiccant until particular analyses were executed.

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[4] Light provided by one 34W cool white fluorescent tube (General Electric Canada) was filtered by two 2.5 mm thick green and one 2.5 mm thick yellow PERSPEX transparent acrylic sheets (Imperial Chemical Company, UK).

3.4 COMBINED CHLOROPHYLL EXTRACTION AND PROTEIN PRECIPITATION

3.4.1 Chlorophyll extraction and determination of concentration

All operations pertaining to source tissues collected for the extraction of chlorophyll and simultaneous precipitation of polypeptides by acetone were conducted either under the illumination provided by a dim green light or in the absence of light. Lyophilized tissue was homogenized in the presence of 80% acetone (v/v) in a 5 mL conical glass tissue grinder (Beico Glass, Inc., Vineland, NJ). Grinding was performed within a dry ice/acetone bath. Following homogenization, samples were quantitatively transferred to 15 mL capacity high-speed Corex brand glass centrifuge tubes (Fisher Scientific, Ottawa, Ont.) and left at −20°C for 15 min. Samples were centrifuged at 10,000*g* for 15 min in a high speed refrigerated centrifuge maintained at a temperature of −15°C. The solvent (containing extracted pigments) was gently aspirated from the pellet with a pasteur pipette, transferred to a 14 mL capacity Falcon 2059 polypropylene tube (Fisher Scientific, Ottawa, Ont.), capped, and stored on ice. The pellet was again extracted with 80% acetone (v/v) for 15 min at −20°C and centrifuged as above. The solvent was aspirated and combined with the previous extraction. The final extraction was performed with 100% acetone for 15 min at −20°C and centrifugation was carried out as above. The 100% acetone was aspirated away from the pellet and combined with the two previous extracts. Distilled H_2O was added to the combined pigment extracts to bring the final concentration of acetone to 80% (v/v).

Because the presence of co-extracted storage-lipids was a major obstacle to the accurate determination of chlorophyll concentration by spectrophotometry (light scatter), the pigment extract was subjected to centrifugation (3,000*g* for 10 min at room temperature) to clear the solution. Absorbances of extracts were recorded at 665 nm and 649 nm and chlorophyll concentrations were resolved according to the equations described by Vernon (1960).

3.4.2 *Protein extraction and determination of concentration*

The pellet remaining after pigment extraction was dried of residual acetone with a stream of nitrogen gas in a water bath maintained at 60°C[6]. The acetone-precipitable proteins were extracted from the dried powder by adding an appropriate volume (0.5 to 2.5 mL) of SDS reducing buffer (10% glycerol (v/v), 5% 2–β–mercaptoethanol (v/v), 2% SDS (w/v), 0.05% bromophenol blue (w/v) in 62.5 mM TRIS–HCl, pH 6.8) and incubating the slurry for 15 min at 95°C with occasional vigorous mixing. The solubilized proteins were transferred to a screw cap polypropylene microfuge tube after the insoluble cellular material had been pelleted by room temperature centrifugation at 10,000*g* for 10 min. An aliquot (100 to 200 µL) of the solution was retained for protein determination (described below) and the remainder of the sample was immediately frozen in liquid nitrogen and stored at –20°C until the time of electrophoretic analysis.

Protein was determined according to the method of Bradford (1976) using bovine serum albumin (Sigma Chemical Company, St. Louis, MO) as standard. Proteins were precipitated by adding two volumes of 10% (w/v) trichloroacetic acid (Sigma Chemical Company, St. Louis, MO). The supernatant was discarded and protein pellets were air dried following micro-centrifugation at 10,000*g* for 10 min. Pellets were then dissolved in an appropriate volume (100 to 200 µL) of 0.1 N NaOH and this solution was subjected to the Bradford analysis.

3.4.3 *Protein electrophoresis and Western blot analysis*

SDS polyacrylamide gel electrophoresis was performed on 1.5 mm thick gels with a 4% stacking gel using the discontinuous buffer system described by Laemmli (1970). Separating gels presented in this report are one of two lengths, 16cm or 7cm. The former were either 12% acrylamide gels or 7.5 to 12% linear acrylamide gradient gels run on the

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[6] At this stage, manipulation of the material was no longer conducted under green light.

'Protean xl' cell (Bio–Rad Laboratories, Hercules, CA) at 9 mA/gel (constant current). The latter were 15% acrylamide gels run on the Hoefer 'SE 250 - Mighty Small II' cell (San Francisco, CA) at 180 V (constant voltage). Gels not intended for Western blot analysis were stained overnight in 0.1% (w/v) Coomassie blue R–250 in fixative (40% methanol, 10% glacial acetic acid) and destained by diffusion in fixative (5h).

Gels for Western blot analysis were electrophoretically transferred onto nitrocellulose membrane (BA–S 83 - 0.2 µm pore, Schleicher and Schuell, Keene, NH) in a carbonate buffer of composition: 10 mM $NaHCO_3$, 3 mM Na_2CO_3, pH 9.9 in 20% methanol (Dunn 1986). Electrophoretic transfers were performed either at 25 V (constant voltage) for 15 h or, at 40 V (constant voltage) for 4 h in a Hoefer blotting cell (model TE 50, San Francisco, CA) equipped with a glass cooling coil. The temperature of the carbonate transfer buffer, in both cases, was maintained below 25°C by running cold water through the glass coil.

Upon completion of transfer, blots were incubated overnight with blocker (3%, w/v, Teleostean gelatin - from cold water fish skin - Sigma Chemical Company, St. Louis, MO in PBS (1.37 M NaCl, 27 mM KCl, 81 mM Na_2HPO_4, 15 mM KH_2PO_4, pH 7.4)) and transferred to antiserum (1/300 dilution) in blocking solution for 1h. Blots were washed three times, for 10 min each, in PBS containing 0.05% (w/v) Tween–20 followed by one 10 min wash with PBS and then a 1 h incubation with alkaline phosphatase conjugated to goat anti-rabbit secondary antibody (Sigma Chemical Company, St. Louis, MO) in blocking solution. Following incubation with the conjugated secondary antibody, blots were again washed three times, for 10 min each, in PBS containing 0.05% (w/v) Tween–20 followed by one 10 min wash with PBS and one 10 min wash with 50 mM TRIS–HCl, pH 9.5. Antigenic polypeptides were enzymatically decorated with 0.1% (w/v) disodium naphtol AS–MX phosphate and 0.2% (w/v) fast red TR salt (both from Sigma Chemical Company, St. Louis, MO) in TRIS–HCl, pH 9.5 as described by White and Green (1987a).

The antibodies α–CPIa and α–CPII were the generous gift of Dr. Beverly R. Green (Department of Botany, University of British Columbia, Vancouver, BC). The α–CPIa antibody was raised against purified CPIa, a complex of the reaction centre (CPI) and the CAB antenna (LHCI) of PSI, from barley (White and Green 1987a). This antibody recognizes the four chlorophyll a/b binding proteins of PSI (21–24 kDa on these denaturing gels) and two of the chlorophyll a/b binding proteins, LHC IIa and LHC IIb, of PSII (White and Green 1987a).

The α–CPII antibody was raised against CPII (a form of LHC IIb that does not include the minor 25 kDa polypeptide of that CAB complex) from barley. This antibody recognizes both the major and minor polypeptides (27 kDa and 25 kDa respectively) of LHC IIb but does not react with polypeptides of LHC I (White and Green 1987b).

3.5 NORTHERN BLOT ANALYSIS

3.5.1 RNA extraction and quantitation

A modified method described by Natesan *et al.* (1988) was employed for the extraction of total cellular RNA from the source tissue. A 5 mL conical glass tissue homogenizer (Belco Glass, Inc.) was used to homogenize frozen source tissue in 65°C phenol saturated with 1 M TRIS–Cl, pH 8.0 (3 mL per gram source tissue). The homogenate was cooled rapidly by placing the homogenizer into a dry ice/acetone bath for 2 to 3 s intervals with mixing. Care was taken to avoid freezing the homogenate. Once cold, the sample was placed on ice, followed by additional grinding (30 s) in an equal volume of 0.2 M NaAc pH 5.2 and one–sixth volume of 10% SDS. The homogenate was transferred to sterile 14 mL Falcon 2059 round bottom polypropylene centrifuge tubes (Becton Dickinson & Company, Lincoln Park, NJ) and incubated at 65°C for 5 min with occasional

mixing. Immediately following the incubation, samples were centrifuged at 3,000*g* for 5 min at 4°C, and the aqueous phase was transferred to a clean Falcon polypropylene tube on ice. An equal volume of ice cold chloroform:isoamyl alcohol (24:1, v/v) was added with vigorous mixing followed by centrifugation as described above. The aqueous phase was transferred to a fresh Falcon polypropylene tube and the chloroform:isoamyl alcohol extraction repeated. After centrifugation the aqueous phase was again transferred to a clean Falcon polypropylene tube and total cellular RNA was precipitated overnight at −20°C in the presence of one–sixth volume of DEPC treated 6 M LiCl and two and one half volumes of 95% ethanol. Precipitated RNA was pelleted by centrifugation at 12,000*g* for 20 min at 4°C and washed with cold 70% ethanol to remove salts. The dried samples were dissolved in a suitable volume of DEPC treated sterile water and, according to Maniatis *et al.* (1982), the concentration of RNA was determined by the absorbance at 260 nm.

3.5.2 RNA electrophoresis and photography

A volume of solution corresponding to 150 µg of total cellular RNA was transferred to a sterile 2.0 mL screw cap microfuge tube (Fisher Scientific, Ottawa, Ont.) and lyophilized. The sample was prepared for electrophoresis by dissolving the pellet in formaldehyde RNA sample buffer (53% (v/v) deionized formamide, 11% (v/v) 10X MOPS, 17% (v/v) formaldehyde[6], 7% (w/v) glycerol, and 0.6% (w/v) bromophenol blue in DEPC treated sterile H_2O) and heating at 65°C for 15 min (Fourney *et al.* 1988). Denatured samples were electrophoresed at 100 V (constant voltage) for 2 h at room temperature in a denaturing 1.2% agarose-formaldehyde gel (Fourney *et al.* 1988). RNA was photographed following the electrophoresis on a short wave transilluminator using an orange filter and Polaroid type 665 PN film.

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[6] Volume pertaining to 37% (w/v) formaldehyde.

3.5.3 *Transfer and crosslinking of RNA*

Following photography, gels and Zeta–Probe nylon membranes (Bio–Rad Laboratories, Hercules, CA) were prepared for transfer by soaking in autoclaved 10X SSC (1.5 M NaCl, 175 mM NaCit) for 10 min. RNA was transferred to the membrane over a 24 h period in 10X SSC by capillary action as described by Fourney *et al.* (1988) except that a sponge was not employed to enhance the capillary action. Transferred RNA was fixed to the damp (10X SSC) membrane by a 2 min exposure (RNA-side up) to an overhead short wave ultra-violet light source consisting of two 40 cm, 15 W, UV–C fluorescent tubes (Xymotech, Mt. Royal, Que) with a maximum output at 254 nm. The distance between the membrane and the energy source was 30 cm.

3.5.4 *Probe preparation and hybridization*

A full length (857 bp) *Brassica napus* cDNA clone representing the LHC II Type I Cab gene of PSII (White *et al.* 1992b) was kindly provided by Dr. Jas Singh (Plant Research Centre, Agriculture Canada, Ottawa, Ont) in *Escherichia coli* strain MV 1193 on a recombinant plasmid (pGEM 4Z). The bacterium was grown to saturation on LB media supplemented with ampicillin (100 $\mu g\ mL^{-1}$) and the plasmid isolated according to the alkaline lysis method of Maniatis *et al.* (1982). The plasmid was dissolved in TE pH 8.0 and treated, for 1 h at 37°C, with RNase A from bovine pancreas (Gibco BRL, Burlington, Ont.).

The plasmid was digested with EcoR1 as described by Maniatis *et al.* (1982) and electrophoresed in a 1.2% agarose gel. The 857 bp Cab cDNA fragment was excised from the gel and purified with Gene Clean as described by the supplier (BIO 101, Inc., La Jolla, CA). The purified fragment was labelled to high specific activity with [α-^{32}P]dCTP (Amersham, Oakville, Ont.) via Amersham's Multiprime DNA labelling kit (RPN 1601). Upon completion of the labelling reaction, unincorporated deoxynucleotides were removed from the probe by size exclusion chromatography through centrifugation columns made from the

barrel of a 1 cc plastic hypodermic syringe containing sephadex G–50 resin equilibrated with TE buffer pH 8.0 (Maniatis *et al.* 1982).

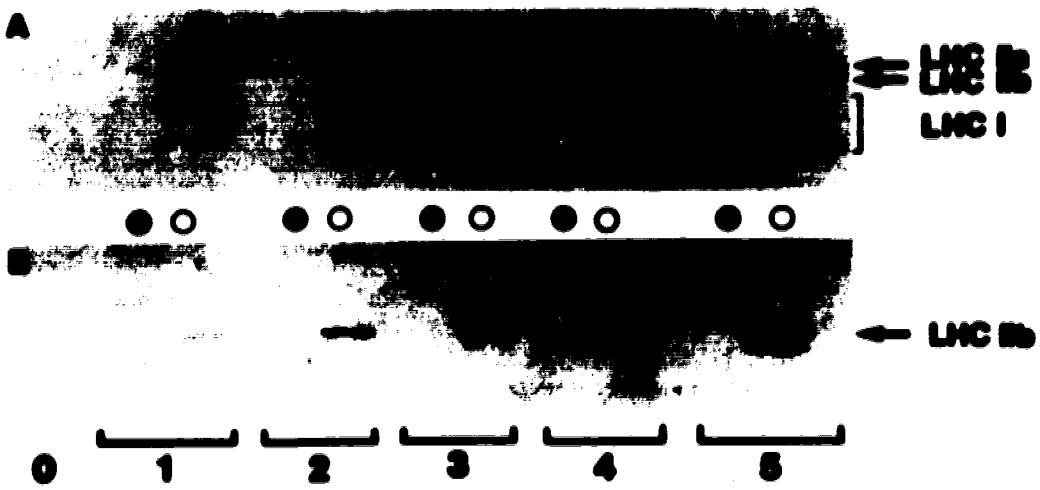
Prehybridization and hybridization procedures were performed in heat sealable plastic bags. Prehybridization was conducted for 1 h in a solution consisting of 50% formamide, 0.25 M $NaHPO_4$ pH 7.2, 0.25 M NaCl, 7% (w/v) SDS, and 1 mM EDTA at 43°C according to the Zeta–Probe manual (Bio–Rad Laboratories, Inc.). Hybridization was conducted over a period of 24 h at 43°C in the presence of the oligonucleotide probe in a solution of identical composition to that of the prehybridization buffer. At the completion of hybridization, the membranes were rinsed briefly in 2X SSC and washed for 15 min at 22°C in 2X SSC/0.1% SDS followed by another 15 min wash at 22°C in 0.5X SSC/0.1% SDS. The final wash was at 65°C in 0.1X SSC/0.1% SDS for 15 min. After washing, the blotted membranes were autoradiographed with Fuji RX medical X–ray film for 2 to 3 h at –80°C in an X–ray cassette equipped with intensifying screens.

4. RESULTS

4.1 MD EMBRYO GREENING - ACCUMULATION OF CAB POLYPEPTIDES

Continual irradiance of 23 d old etiolated MD embryos resulted in a time-dependent accumulation of CAB polypeptides from both photosystems (Figure 4). Accumulation of LHC IIb was examined with two antibodies, α–CPIa (Figure 4A) and α–CPII (Figure 4B), both of which demonstrate identical patterns of accumulation for that PSII CAB protein. The accumulation trends of the light-harvesting antenna of PSI (LHC I) and LHC IIa were consistent with the trend exhibited by LHC IIb (Figure 4A, B). CAB polypeptides were missing from MD embryos cultured in the absence of light (Fig 4A, B).

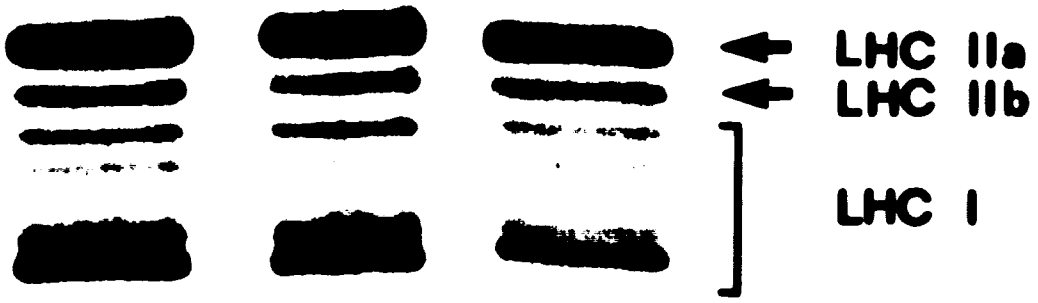
Figure 4. Western blot analysis of the light-dependent accumulation of CAB polypeptides by greening MD embryos of *B. napus*. Twenty-three d old dark grown embryos maintained in NLN–13% were cultured for a further 5 d in the absence of light (●) or, under constant illumination (○) of 30 to 35 μmol $m^{-2}s^{-1}$. Total embryonic protein was separated by SDS–PAGE on 7.5 to 12% linear acrylamide gradients, transferred to nitrocellulose, probed with α–CPIa or α–CPII, and antigenic polypeptides detected with 2° antibody conjugated to alkaline phosphatase. **A**, 60 μg total protein loaded per lane - blot probed with α–CPIa. **B**, 30 μg total protein loaded per lane - blot probed with α–CPII. Days of harvest, and the location and identity of CAB polypeptides are indicated.


A
LHC Ib
LHC Ia
LHC I
LHC IIb
0
1
2
3
4
5

4.2 CAB POLYPEPTIDES - INTRASPECIES TISSUE COMPARISON

Protein extracts from green cotyledons, leaf, and MD embryo were electrophoresed on the basis of equal total chlorophyll. The proportion of CAB polypeptide to chlorophyll was analyzed by Western blotting. Under the conditions of plant growth and tissue culture employed in this study, there was a lower proportion of CAB polypeptides to chlorophyll in the MD embryos than in the cotyledons or leaves (Figure 5 - compare signal intensity of lanes a-c).

Figure 5. A tissue comparison of CAB polypeptides from leaf, cotyledon and MD embryos of *B. napus* - Western blot analysis. Total acetone-precipitable protein was separated by SDS–PAGE on a 12% acrylamide gel, transferred to nitrocellulose, probed with α–CP1a, and antigenic polypeptides detected with 2° antibody conjugated to alkaline phosphatase. CAB polypeptide abundance was analyzed by equal loading on the basis of total chlorophyll (2.5 μg chl per lane). Lanes are as follows, (a), Cotyledon - 5 DAP; (b), leaf; (c), 19 d old greened MD embryos cultured under constant illumination (80-100 μmol $m^{-2}s^{-1}$) for 5 d in NLN–20%.



a b c

4.3 MD EMBRYO DEGREENING

4.3.1 *Western blot analysis*

Western blot analysis of 19 d old greened MD embryos cultured under continuous illumination (control) revealed a gradual synchronous decline in CAB polypeptides over the 13 d period of investigation (Figure 6A). The decline of the photosystem components was most noticeable after day 7. In comparison, 19 d old embryos cultured under continuous illumination in the presence of exogenous abscisic acid underwent an accelerated decline in these polypeptides (Figure 6B). By day 3, the quantity of CAB polypeptides in ABA treated embryos was akin to that of illuminated control embryos that had aged a further 10 d (compare Figure 6A - lane f with Figure 6B - lane a). Similar to the abscisic acid treated tissue, greened MD embryos cultured without plant growth regulator or illumination underwent a rapid loss of antenna polypeptides beginning prior to day 3 (Figure 6C). Unlike the ABA treated embryos, however, these embryos did not fully degrade the CAB polypeptide components over the 13 d period of analysis (compare Figure 6B with Figure 6C). Instead, these embryos begin to accumulate the antenna polypeptides, in the absence of light, on or before day 11 (Figure 6C - lane e).

Treatment of 19 d old greened MD embryos with BAP neither altered the pattern of, nor prevented the gradual depreciation in, CAB polypeptide abundance exhibited by illuminated control embryos (compare Figure 7A with Figure 7B). BAP treated MD embryos displayed a prominent decline in the overall abundance of these photosystem polypeptides after day 7 (Figure 7).

4.3.2 *SDS-PAGE*

Electrophoretic analysis of 19 d old greened MD embryos cultured in the presence or absence of plant growth regulators (ABA or BAP) and with or without illumination revealed conspicuous differences in polypeptide composition (Figures 8 and 9). Embryos

cultured under continuous illumination with abscisic acid (Figure 8B) initiated an extensive time-dependent accumulation of specific polypeptides. Novel polypeptides with apparent molecular masses 32, 29 to 26.5, and 20 kDa were detectable within the first 24 h of ABA treatment. There was a delayed but pronounced accumulation of three more polypeptides beginning on or about day 3. The largest of the three polypeptides had an apparent molecular mass of 24 kDa while the other two resolved directly above and below the 6.5 kDa standard marker (the mass of these molecules cannot be accurately determined on these gels). In contrast, embryos cultured under continuous illumination (Figure 8A), in the absence of light (Figure 8C) or, in the absence of light and the presence of ABA (Figure 8D), did not accumulate these polypeptides to a comparable extent.

Analysis of 19 d old greened MD embryos cultured under continuous illumination by SDS–PAGE revealed two closely migrating but distinct polypeptides with apparent molecular masses in the 17 to 20 kDa range (Figure 9A - polypeptides marked with closed arrowheads). The polypeptides appeared on day 1 and were maintained through day 7. These embryos showed a faint but persistent band that migrated in the vicinity of the 6.5 kDa standard marker (Figure 9A - polypeptide identified with an open arrowhead). This faint band also migrated with a prominent polypeptide in the mature seed (Figure 9A - lane f). Conversely, 19 d old MD embryos cultured under continuous illumination with BAP initiate production of only one of the two polypeptides in the 17 to 20 kDa range (Figure 9B - polypeptide identified with a closed arrowhead) characteristic of illuminated embryos cultured in the absence of this growth regulator. Moreover, the polypeptide that migrates in the vicinity of the 6.5 kDa standard marker in illuminated MD embryos was markedly diminished in illuminated BAP treated embryos throughout the 7 d period of analysis (Figure 9B - position indicated by an open arrowhead).

4.3.3 Chlorophyll analysis

Among 19 d old greened MD embryos cultured in NLN–20% and the dark, the chlorophyll content (based on dry weight) was not affected by ABA or BAP treatments. In other words, in the absence of illumination degreening was not influenced by chemical (Figure 10). However, among illuminated 19 d old MD embryos cultured in NLN–20%, chlorophyll content varied significantly between all treatments (ABA, BAP and control). Embryos incubated in BAP possessed 24% less chlorophyll than illuminated control tissue while embryos incubated in ABA possessed 52% less chlorophyll than illuminated control embryos (Figure 10). With regard to all treatments, illuminated control embryos exhibited the greatest quantity of chlorophyll whereas illuminated embryos cultured in media supplemented with ABA exhibited the least quantity of chlorophyll (Figure 10).

Illuminated control tissue had 33% more chlorophyll than tissue cultured in the dark. However, the converse is true of MD embryos treated with ABA. In the light these embryos displayed significantly less chlorophyll (25%) on a dry weight basis than they did when cultured in the dark (Figure 10). Comparison of BAP treated embryos demonstrated no significant difference in chlorophyll content among illuminated and light deprived tissue (Figure 10).

Finally, the chlorophyll content of MD embryo tissues declined linearly ($p < 0.05$, $r^2 = 0.96$) over the period of analysis (days 1,2,3,5, and 7) and the rate of pigment loss was the same (slope = −80 ng chlorophyll (mg dry wt)$^{-1}$ d^{-1}) regardless of the presence/absence of light or the treatment (ABA, BAP or control).

4.3.4 *Northern blot analysis*

Northern blot analysis of total RNA from 19 d old greened MD embryos cultured under continuous illumination demonstrated that the level of hybridizable Cab transcript[7] moderately increased from day 0 to day 2. After this time, the level of transcript gradually declined through day 5 and then dropped below the point of detection by day 7 (Figure 11A). In comparison, 19 d old green MD embryos incubated with abscisic acid under continuous illumination exhibited a level of hybridizable Cab transcript that was reduced to barely detectable levels within 6 h and did not recover within the period of analysis (Figure 11B).

In contrast, 19 d old greened MD embryos cultured in the absence of light initiated a massive accumulation of Cab transcript prior to day 1 that proceeded through the period of analysis (Figure 11C). If, however, the culture medium was supplemented with abscisic acid and embryos were cultured in the absence of light, the dark-induced increase in hybridizable Cab transcript was eliminated (Figure 11D). Instead, throughout the analysis, the transcript was maintained at a level well below that of illuminated control embryos (compare Figure 11A with Figure 11D).

Treatment of 19 d old greened MD embryos with BAP under continuous illumination (Figure 12B) reduced the level of hybridizable Cab transcript below that of illuminated control embryos (Figure 12A). The depression of Cab transcript by BAP was apparent within 6 h and by day 5 the transcript was no longer detectable (Figure 12B).

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[7] Unless otherwise stated, 'Cab transcript' refers to the LHC IIb type I transcript.

Figure 6. Western blot analysis of the effect of ABA in the light and the effect of darkness on the level of CAB polypeptides from degreening MD embryos of *B. napus*. Nineteen d old greened embryos were cultured over a 13 d period in NLN–20%. Total acetone-precipitable protein was separated by SDS–PAGE on 7.5 to 12% linear acrylamide gradients, transferred to nitrocellulose, probed with α–CP1a, and antigenic polypeptides detected with 2° antibody conjugated to alkaline phosphatase. Samples were loaded on the basis of dry weight (1.0 mg). **A**, Embryos cultured under constant illumination (80-100 μmol $m^{-2}s^{-1}$). **B**, Embryos cultured under constant illumination (80-100 μmol $m^{-2}s^{-1}$) in media supplemented with ABA (50 μM). **C**, Embryos cultured in the absence of light. Lanes are as follows, (a) day 3, (b) day 5, (c) day 7, (d) day 9, (e) day 11 and (f) day 13. The location and identity of CAB polypeptides is indicated.

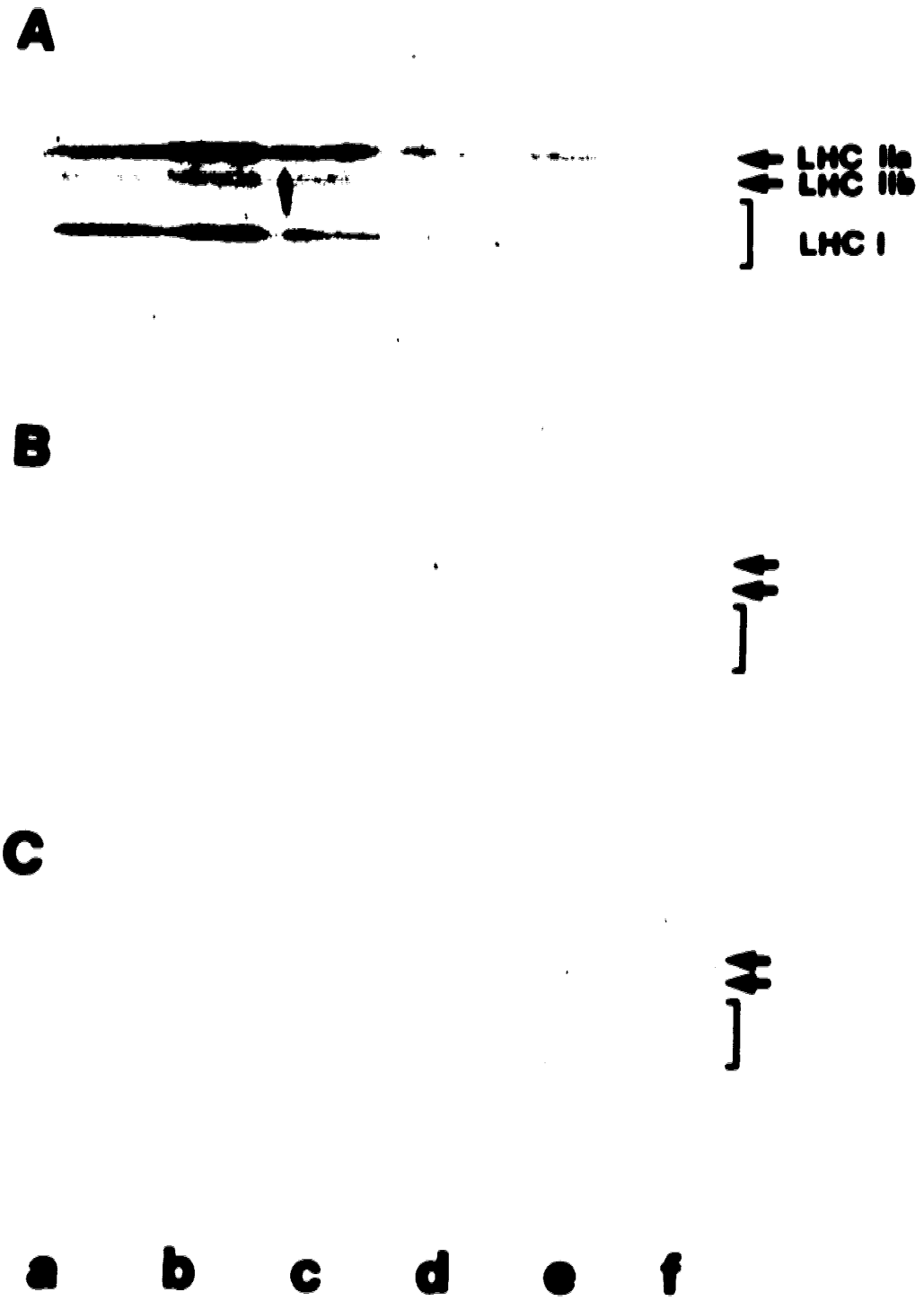
A
LHC IIa
LHC IIb
LHC I
B
C
a
b
c
d
e
f

Figure 7. Western blot analysis of the effect of BAP on the level of CAB polypeptides from MD embryos of *B. napus*. Nineteen d old greened embryos were cultured under constant illumination (80-100 μmol $m^{-2}s^{-1}$) in NLN–20% supplemented with (B) or without (A) 5 μM BAP. Total acetone-precipitable protein was separated by SDS–PAGE on 7.5 to 12% linear acrylamide gradients, transferred to nitrocellulose, probed with α–CP1a, and antigenic polypeptides detected with 2° antibody conjugated to alkaline phosphatase. Samples were loaded on the basis of dry weight (1.0 mg). Lanes are as follows, (a) day 3, (b) day 5, (c) day 7, (d) day 9, (e) day 11 and (f) day 13. The location and identity of CAB polypeptides is indicated.

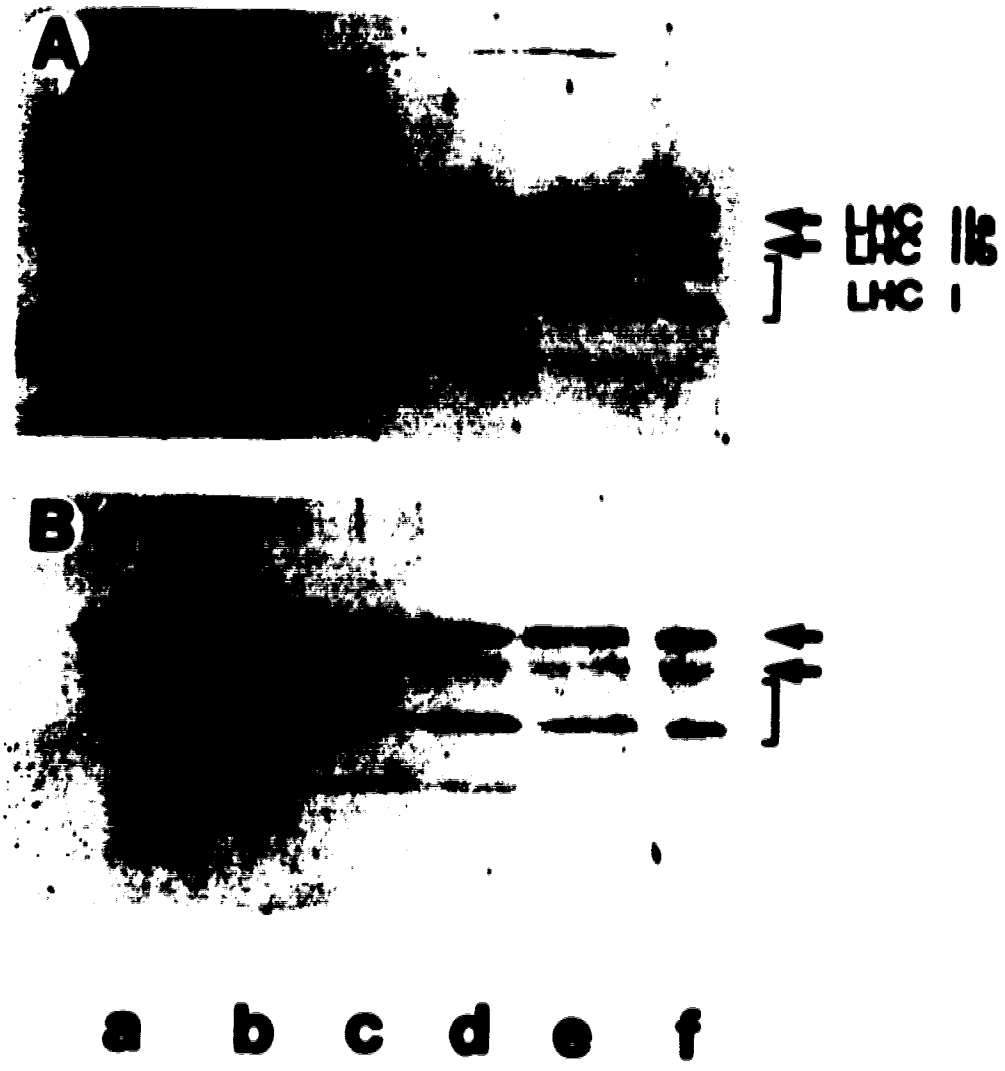
A
LHC IIa
LHC I
B
a b c d e f

Figure 8. Electrophoretic analysis of the effect of ABA on the total proteins from degreening MD embryos of *B. napus*. Acetone precipitable proteins were electrophoresed (SDS–PAGE) on 15% polyacrylamide gels and visualized by staining with Coomassie brilliant blue R–250. Samples were loaded on the basis of constant dry weight (500 μg). **A**, Electrophoresis of protein from 19 d old greened embryos cultured under constant illumination (80-100 μmol $m^{-2}s^{-1}$) in NLN–20%. **B**, Electrophoresis of protein from 19 d old greened embryos cultured under constant illumination (80-100 μmol $m^{-2}s^{-1}$) in NLN–20% supplemented with ABA (50 μM). **C**, Electrophoresis of protein from 19 d old greened embryos cultured in the absence of light in NLN–20%. **D**, Electrophoresis of protein from 19 d old greened embryos cultured in the absence of light in NLN–20% supplemented with ABA (50 μM). Closed arrowheads mark the position of polypeptides mentioned in the text. Open arrowheads (numbered 1-9) mark the position of protein molecular weight standards (Table A-1). Gel lanes are as follows, (a) day 0, (b) day 1, (c) day 2, (d) day 3, (e) day 7 and, (f) mature seed (gels **A** and **C** only).

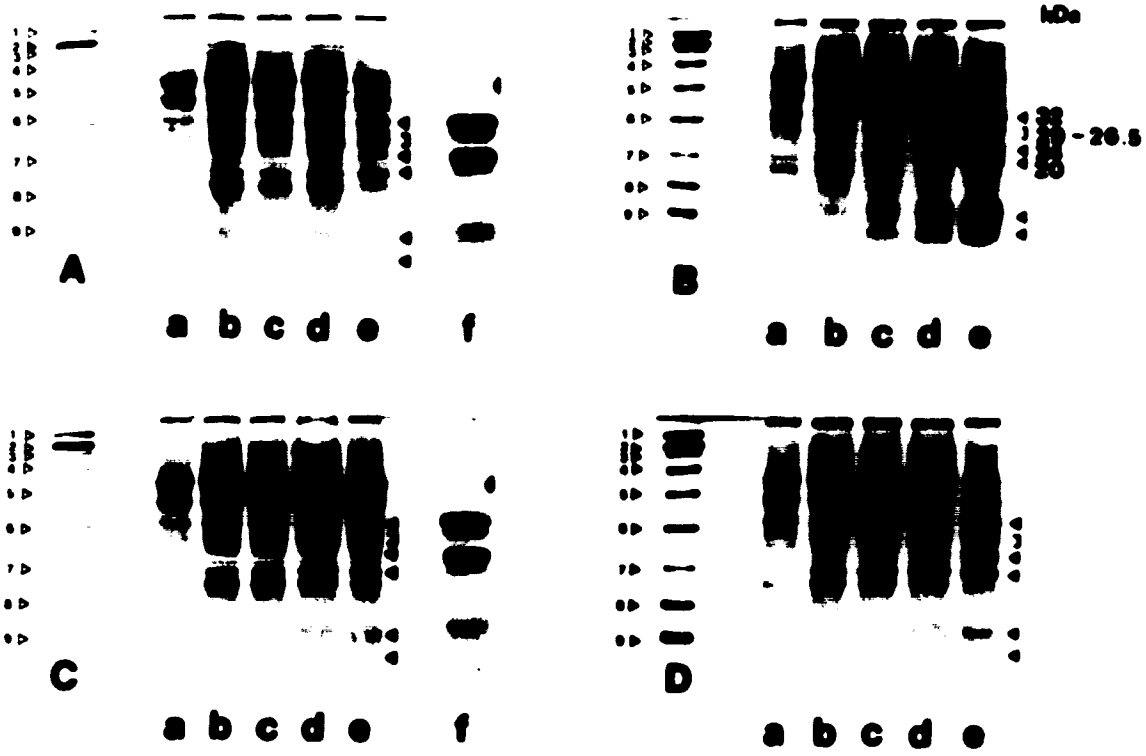
A
a b c d e f
B
kDa
20.5
a b c d e
C
a b c d e f
D
a b c d e

Figure 9. Electrophoretic analysis of the effect of BAP on the total proteins from degreening MD embryos of *B. napus*. Acetone precipitable proteins were electrophoresed (SDS–PAGE) on 15% polyacrylamide gels and visualized by staining with Coomassie blue. Samples were loaded on the basis of constant dry weight (500 µg). **A**, Electrophoresis of protein from 19 d old greened embryos cultured under constant illumination (80-100 µmol $m^{-2}s^{-1}$) in NLN–20%. **B**, Electrophoresis of protein from 19 d old greened embryos cultured under constant illumination (80-100 µmol $m^{-2}s^{-1}$) in NLN–20% supplemented with BAP (5µM). Large arrowheads mark the position of polypeptides mentioned in the text. Smaller arrowheads (numbered 1-9) mark the position of protein molecular weight standards (Table A-1). Gel lanes are as follows, (a) day 0, (b) day 1, (c) day 2, (d) day 3, (e) day 7 and, (f) mature seed (gel **A** only).


A
a b c d e f

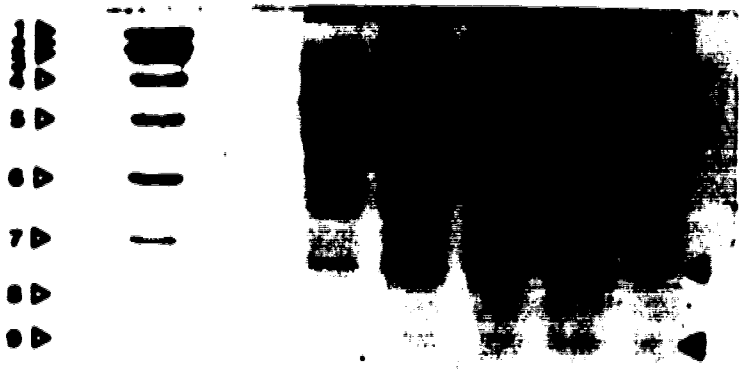
B
a b c d e

Figure 18. The effect of ABA or BAP on the total chlorophyll (a+b) content of degreening MD embryos of *B. napus*. Nineteen d old embryos were cultured in NLN–20% (control), NLN–20% supplemented with ABA (50 μM) or, NLN–20% supplemented with BAP (5 μM), in light (80-100 μmol $m^{-2}s^{-1}$) or in the dark. Embryonic tissue was collected 1, 2, 3, 5 and 7 d after day 0 and the chlorophyll content of each collection analyzed individually. The experiment was repeated three times. The phytohormone by time interaction was not significant and thus the mean chlorophyll values (averaged over the 7 d developmental period) are presented. Because the phytohormone by light interaction was significant ($p < 0.05$), the phytohormone effects were analyzed separately within the context of light and dark. Additionally, the differences between light and dark effects were analyzed separately by phytohormone treatments. Within the light or dark, means with different letters are significantly different at the 0.05 level according to Duncan's Multiple Range Test. An asterisk (*) above a bar representing a light treatment indicates that this mean is significantly different than same mean in the dark ($p = 0.05$).

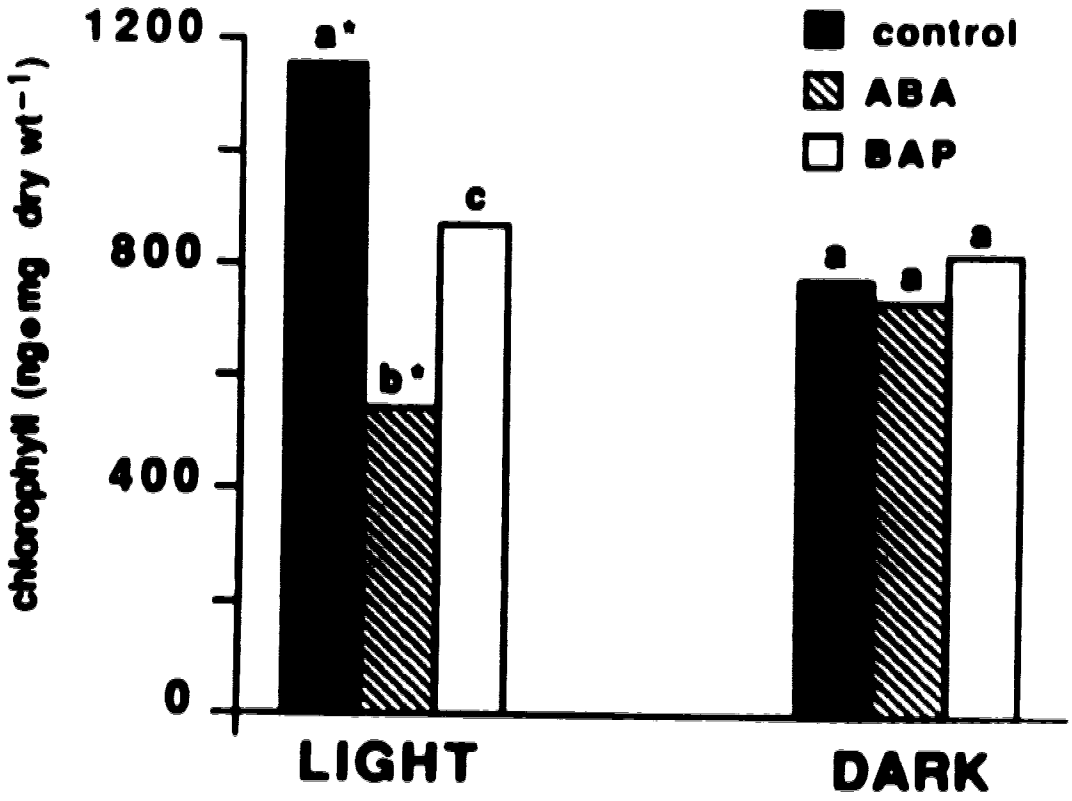
1200
800
400
0
chlorophyll (ng•mg dry wt⁻¹)
a*
b*
c
a
a
a
control
ABA
BAP
LIGHT
DARK

Figure 11. Northern blot analysis of the effect of ABA on LHC IIb Type I mRNA from degreening MD embryos of *B. napus*. Each lane of the RNA resolving gel was loaded with 150 µg of total RNA. The blot was probed with the LHC IIb Type I cDNA from *B. napus*. **A**, RNA from 19 d old greened embryos cultured under constant illumination (80-100 µmol $m^{-2}s^{-1}$) in NLN–20%. **B**, RNA from 19 d old greened embryos cultured under constant illumination (80-100 µmol $m^{-2}s^{-1}$) in NLN–20% supplemented with ABA (50 µM). **C**, RNA from 19 d old greened embryos cultured in the dark in NLN–20%. **D**, RNA from 19 d old greened embryos cultured in the dark in NLN–20% supplemented with ABA (50 µM). Arrowheads mark the position of the 857 bp mRNA. Gel lanes are as follows, (0) day 0 (gel A only), (a) 6 h, (b) day 1, (c) day 2, (d) day 3, (e) day 5 and, (f) day 7.

A

O a b c d e f

'B

a b c d e f

C

a b c d e f

D

a b c d e f

Figure 12. Northern blot analysis of the effect of BAP on LHC IIb Type I mRNA from degreening MD embryos of *B. napus*. Each lane of the RNA resolving gel was loaded with 150 µg of total RNA. The blot was probed with the LHC IIb Type I cDNA from *B. napus*. **A**, RNA from 19 d old greened embryos cultured under constant illumination (80-100 µmol $m^{-2}s^{-1}$) in NLN–20%. **B**, RNA from 19 d old greened embryos cultured under constant illumination (80-100 µmol $m^{-2}s^{-1}$) in NLN–20% supplemented with BAP (5 µM). Arrowheads mark the position of the 857 bp mRNA. Gel lanes are as follows, (0) day 0 (gel **A** only), (6) 6 h, (1) day 1, (2) day 2, (3) day 3 and, (5) day 5.

A

B

0 a b c d e

a b c d e

5. DISCUSSION

It is known that etiolated MD embryos of *B. napus* cv. Topas are capable of chlorophyll synthesis when continuously illuminated (Johnson-Flanagan and Singh 1993). However, it was uncertain whether greening MD embryos were capable of the production and accumulation of the chlorophyll a/b binding proteins characteristically found in other photosynthetic plant tissues. In the present study this question was specifically addressed and it is clear that illuminated MD embryos do accumulate CAB polypeptides in response to illumination (Figure 4A, B). This occurrence was not unexpected because it is known that immature (green) seed embryos of this species possess CAB polypeptides (Johnson-Flanagan *et al.* 1994) and because all chlorophyll is associated with chlorophyll-binding polypeptides *in vivo* (Markwell *et al.* 1979). Nevertheless, it was important to verify the presence of CAB polypeptides in green MD embryos in an effort to demonstrate the usefulness of these embryos as a potential developmental model for canola seed degreening.

An intraspecies comparison of the CAB polypeptide complement of green MD embryos, cotyledons and leaves demonstrated that these tissues possess CAB polypeptides that are electrophoretically indistinguishable (Figure 5). However, MD embryos maintain a lower CAB polypeptide to chlorophyll ratio than do the leaf and cotyledon (Figure 5). This effect is likely attributable to the conditions of embryo culture employed in this study. The proportion of pigment to light-harvesting protein is not fixed and plants manipulate this ratio depending on the growth conditions (Bustow *et al.* 1988; Lichtenthaler *et al.* 1982). Photosynthetic tissues subjected to low light (shortage), for instance, compensate for reduced photosynthetic capacity by producing larger antenna (CAB polypeptide) assemblies. Conversely, photosynthetic tissues subjected to high light adapt by eliminating unnecessary pigment antennae (CAB polypeptides) and increasing the number of photosynthetic reaction

centers. Both processes are designed to maximize the photosynthetic efficiency of the tissue (Lichtenthaler *et al.* 1982). The fact that green MD embryos used in this study exhibit a CAB polypeptide to chlorophyll ratio that is lower than that of the leaves or cotyledons should not, therefore, be viewed as an inappropriate assembly of the light-harvesting apparatus. Instead, this effect is more likely an embryonic response to sufficient or excessive illumination which provokes a reduction in the light-harvesting assemblies (CAB polypeptides).

Once it was established that greening MD embryos developed typical light-harvesting components, our objective was to use the greened embryos as a tool to study the influence of ABA and BAP on the process of seed degreening. From a survey of the literature, it was clear that these phytohormones should have specific predictable effects on this process. We hypothesized that the light-harvesting components of MD embryos cultured with abscisic acid would be negatively influenced while, on the other hand, these components would be positively influenced by BAP. Essentially, abscisic acid had the expected impact whereas the result of BAP treatment was unanticipated.

Exogenous abscisic acid directed the loss of hybridizable Cab mRNA (Figure 11B), chlorophyll a/b binding polypeptides (Figure 6B), and chlorophyll (Figure 10) in illuminated MD embryos of *B. napus*. The transcriptional effect has been documented for soybean embryos (Chang and Walling 1991) and for tomato leaves (Bartholomew *et al.* 1991). The negative effect of this phytohormone on the level of chlorophyll in greening plant tissues has been documented by numerous investigators (Beck and Richmond 1971; Beevers *et al.* 1970; Bengston *et al.* 1977). To my knowledge, however, the effect of ABA on the level of CAB polypeptides in green tissue has not been previously investigated. Considering the conclusions drawn by Bartholomew *et al.* (1991) and the results of steady-state transcript analysis presented in this study (Figure 11B), it is likely that this ABA-directed degreening in

MD embryos is, at least in part, attributable to a restriction of Cab transcription (this hypothesis cannot be verified without conducting nuclear run-on analysis).

CAB polypeptides are subject to turnover (Bennett 1981; Cuming and Bennett 1981). If this turnover occurs to an appreciable extent in MD embryos, the ABA-directed disappearance of these antenna polypeptides could be singularly attributable to the steady-state level of Cab mRNA. MD embryos cultured in the presence of ABA and light have little or no detectable transcript (Figure 11B) - a deficiency that negates *de novo* production of these polypeptides. If the proteins are turned over in illuminated MD embryos treated with ABA they could not be replaced. As a consequence, these photosystem components would be eliminated from the embryo (Figure 6B). It is conceivable that ABA may effect the degreening process in another manner. For instance, the phytohormone could deactivate Cab transcription and simultaneously activate a proteolytic enzyme that specifically degrades these antenna proteins.

MD embryos cultured in the dark, like illuminated MD embryos cultured with ABA, showed a decline in the steady-state level of the CAB polypeptides (compare Figure 6C with Figure 6A). However, light deprivation did not completely eliminate, and permitted a delayed accumulation of, these proteins (Figure 6C - lane e,f). This is an effect not exhibited by MD embryos cultured with abscisic acid under continuous illumination (Figure 6B). Again, if CAB polypeptide turnover occurs to an appreciable extent in MD embryos, the level of antenna proteins in embryos cultured in the absence of light (Figure 6C) could be attributable to the steady-state level of Cab mRNA. These embryos have elevated levels of the LHC IIb transcript (Figure 11C) which might be translated and thereby maintain a basal level of these polypeptides in the absence of light (Figure 6C). Although not resolved from these experiments, it is suspected that transcription of the MD embryonic LHC IIa and the PS I Cab genes also occurs in the dark. This would explain the maintenance of a basal level of these polypeptides in the absence of light (Figure 6C) under conditions of protein turnover.

The effect of light on the appearance of Cab mRNA in etiolated seedlings has been studied since the late 1970s and it is well established that the appearance of Cab mRNA is generally induced by light (Apel 1979; Apel and Kloppstech 1978; Cuming and Bennett 1981). Although it is known that light is not essential for the expression of chlorophyll a/b binding protein genes, it is understood that light is required for maximal Cab mRNA accumulation (Bustow 1988; Mathis and Burkey 1987). Certainly, then, an interesting outcome of the present analysis is the excessive accumulation of Cab mRNA by green MD embryos that are deprived of light (Figure 11C). Remarkably, the level of this photosystem associated transcript is much higher in embryos cultured in the dark than it is in illuminated embryos (compare Figure 11A with 11C).

Previously greened dark-grown MD embryos possess photosynthetic components prior to light deprivation. These green tissues are biochemically and developmentally different from the etiolated tissues studied in the past (Apel 1979; Apel and Kloppstech 1978; Cuming and Bennett 1981). Perhaps, then, it should not be surprising that greened MD embryos accumulate Cab mRNA when deprived of light.

In a recent investigation of greening etiolated pea, for example, the tissue differentiation was a paramount factor in determining the expression characteristics of various members of the Type I LHCII gene group (White *et al.* 1992a). Not only is there a differential expression of Cab genes in the buds and leaves of this species, but specific Type I LHCII genes are transcribed either "early" or "late" (White *et al.* 1992a). Although this study examined greening tissue rather than light deprived green tissue, it established that the expression of Cab genes is a complex developmental and organ-specific matter.

Despite the biochemical and developmental differences between green and etiolated tissues, Tobin (1981) revealed that light deprivation has the anticipated negative effect on the level of translatable Cab mRNA in green *L. gibba*. This is contradictory to the effect of light deprivation on the steady-state level of Cab mRNA in greened dark-grown MD embryos

of *B. napus*. It is conceivable that the conflicting reaction of these green tissues to light deprivation is attributable to the state of tissue differentiation (embryonic versus vegetative).

Why abscisic acid does not fully eliminate the Cab transcript in greened MD embryos cultured in the dark (compare Figure 11D with 11B) remains difficult to explain. Perhaps ABA cannot adequately repress Cab transcription without light. Illuminated photosynthetic tissues translocate protons across the thylakoid membrane and into the lumen of that organelle in response to photon reception by reaction center chlorophyll. This process essentially depletes the chloroplast stroma of protons thereby elevating the pH of that compartment (Anderson 1992). Since ABA is a weak acid (pK_a 4.7), it is protonated to a greater extent in more acidic environments. In this uncharged state, ABA is able to traverse hydrophobic lipid membranes (Zeevart and Creelman 1988). If protonated ABA traverses a membrane separating an acidic compartment (such as the cytoplasm) from a more basic compartment (such as the illuminated chloroplast stroma), the molecule will be ionized to a greater extent in the latter. In this charged state, ABA is no longer free to traverse the membrane and, as a consequence, accumulates within the more alkaline compartment (Zeevart and Creelman 1988).

The transition from light to dark affects chloroplast pH and provokes a subcellular redistribution of the phytohormone (Zeevart and Creelman 1988). Light deprivation eliminates proton translocation into the chloroplast lumen and spoils the thylakoid pH gradient. Therefore, the stromal compartment of MD embryos cultured in the dark should be less basic than it is in illuminated MD embryos. If that is correct, it is conceivable that ABA incompletely reduces the level of Cab transcript in MD embryos cultured in the dark because the phytohormone is not concentrated in a functional subcellular location.

Bearing this in mind, the transitory decline in Cab mRNA exhibited by greened MD embryos cultured with ABA in darkness (Figure 11D - lane a) may be rationalized. Because these embryos were cultured under continuous illumination prior to their transfer to the dark

(Figure 3), presumably the light-dependent thylakoid pH gradient would have existed briefly during the initial period of light deprivation. If this were the case, it is possible that some ABA accumulated in the alkaline chloroplast stroma and directed Cab mRNA repression for the earliest period of ABA treatment. The incomplete repression and renewed transcription of the gene (Figure 11D - lane b) might also be explained in this manner. Because the alkalinity of the chloroplast stroma would deteriorate in the absence of light, stromal ABA would become protonated to a greater degree and, as a consequence, would tend to diffuse from the stroma to a subcellular location where it might less effectively repress the Cab transcript.

This model assumes that the functional location of ABA (with respect to the embryonic degreening process) is the chloroplast stroma. Furthermore, the model suggests that the phytohormone, in a light-dependent manner, regulates the repression of nuclear genes (Cab transcripts) from a position outside the nucleus. It is important to note, therefore, that a signal of chloroplast origin (destroyed under conditions of photooxidative stress) specifically optimizes the expression of Cab genes in a number of plant species (Taylor 1989). As stromal ABA could act on this chloroplast factor, it is not unreasonable to speculate that a light-mediated subcellular localization of ABA may promote the repression of Cab mRNA accumulation in MD embryos from a location outside the nucleus.

Further to this, MD embryos treated with abscisic acid display markedly different patterns of polypeptide accumulation depending on whether the tissue is cultured in light or dark. For instance, embryos cultured with ABA in the presence of light extensively accumulate novel polypeptides with apparent molecular masses of 32, 29 to 27 kDa, 24 and 20 kDa. Two additional, low molecular mass, polypeptides also appear during this treatment (Figure 8B). None of these polypeptides significantly accumulated in MD embryos cultured without ABA or in MD embryos cultured with ABA in the absence of illumination (Figure 8A, C, D). This result serves to underline the importance of light in the physiological

interpretation of abscisic acid. Again, this differential response to ABA (light/dark) might be interpreted as a light-mediated distribution of the phytohormone (Zeevart and Creelman 1988) into a subcellular compartment where these phenotypic modifications can be properly initiated.

Treatment of greened MD embryos with BAP under continuous illumination reduced the level of Cab mRNA below that of illuminated control embryos (Figure 12A, B) and failed to increase the level of CAB polypeptides (Figure 7A, B). This result was unanticipated since the promotory effect of cytokinins on Cab mRNA accumulation has been well documented (Axelos and Pesud-Lenoel 1990; Flores and Tobin 1986, 1988; Longo *et al.* 1990). The interpretation of exogenous cytokinin by plants, however, is not necessarily congruous. Caers and Vendrig (1986) established that the effect of BAP can vary depending on the state of cellular differentiation. In their study of maize seedlings, it was found that BAP encouraged thylakoid development in mesophyll cells but discouraged this process in bundle sheath cells. Cohen *et al.* (1988) showed that BAP treatment could either inhibit or promote pigment synthesis in etiolated cucumber seedlings depending on the length of hormonal incubation. Most recently it has been reported that the expression of a reporter gene driven by the promoter sequence of Cab 3 (a Type I LHC II gene) in illuminated *Arabidopsis thaliana* treated with cytokinin is 58% of that expression in control transformants (Chory *et al.* 1993). In light of these reports, the negative effect of BAP on the level of Cab mRNA and its lack of influence on CAB polypeptides in greened MD embryos becomes less difficult to accept.

Cytokinins are known to alter the steady-state levels of polypeptides in tobacco cells (Abdelghani *et al.* 1991). Since BAP had little or no influence on the antenna polypeptides (Figure 7B), the electrophoretic polypeptide patterns of illuminated (Figure 9A) and illuminated BAP treated (Figure 9B) MD embryos were examined to determine whether BAP was perceived by the embryo. MD embryos exposed to this growth regulator failed to

accumulate a polypeptide in the 17 to 20 kDa range and one in the 6.5 kDa range - molecules typical of greened control embryos. Although the polypeptides remain unidentified, their failure to accumulate in BAP treated embryos verifies cytokinin perception by that tissue.

Analysis of the chlorophyll content of degreening MD embryos revealed that neither BAP or ABA significantly alter the level of this pigment in the absence of light (Figure 10). On the other hand, the effect of abscisic acid is strongly negative under conditions of continuous illumination (Figure 10). Whether this is an ABA induced catabolism of chlorophyll or a side-effect of CAB polypeptide elimination from the tissue (Figure 6B) is not known. BAP treatment fails to enhance the level of chlorophyll in illuminated MD embryos but, rather, causes a significant reduction in the level of this photosynthetic pigment (Figure 10).

Johnson-Flanagan and Singh (1993) have previously reported ABA-mediated chlorophyll catabolism in greened MD embryos of *B. napus*. In that study, illuminated MD embryos fully degreened within 15 d if cultured with ABA (50 μM) in NLN medium containing 13% sucrose. In the present study, the strong negative effect of ABA on the chlorophyll content of illuminated MD embryos of *B. napus* reported by Johnson-Flanagan and Singh (1993) was confirmed with MD embryos cultured in NLN medium containing 20% sucrose (Figure 10).

MD embryos examined in this study degreened at the same linear rate (~80 ng chlorophyll (mg dry wt)$^{-1}$ d^{-1}, r^2 = 0.98) over the 7d period of analysis regardless of the type of treatment (ABA, BAP or control - light/dark). The chlorophyll content of MD embryos cultured in the light and MD embryos cultured in the light with ABA were significantly different (Figure 10). Because the rate of degreening in these tissues was identical (from day 1 - 7), it appears that ABA initiated a change in embryo development prior to the first chlorophyll measurement (day 1). This change in development may have negatively

affected the rate of chlorophyll synthesis or positively affected the rate of chlorophyll degradation. After this point the growth regulator did not affect the rate degreening since the chemical by time interaction was not significant (Figure 10).

The fact that the MD embryos degreened at the same rate regardless of treatment might be attributable to the condition of embryo culture following greening. After the 5 d period of greening, MD embryos were transferred from NLN medium containing 13% sucrose to NLN medium containing 20% sucrose (Figure 3). Transfer of this tissue to NLN of a higher sucrose concentration imposes an osmotic stress on the greened embryos. It is known that the culture of excised zygotic (Finkelstein and Crouch 1986) and microspore-derived (Wilen *et al.* 1990) embryos of *B. napus* on high osmotic media promotes events, such as napin and cruciferin gene expression and embryo desiccation, that are characteristic of normal seed development and maturation. Since degreening is a typical seed developmental event in this species (Johnson-Flanagan and Thiagarajah 1990), it is not surprising that culture of greened MD embryos on high osmoticum induces this maturation event.

The induction of chlorophyll degradation in illuminated control embryos by high osmoticum is a result in apparent contradiction to that reported by Johnson-Flanagan and Singh (1993) where it was revealed that illuminated MD embryos cultured in NLN medium containing either 13 or 20% sucrose failed to degreen appreciably over a 15 d developmental period. It is possible that these conflicting results are attributable to differing embryonic ages. In this experiment, MD embryos were subjected to a 5 d greening period 14 d after microspore isolation. At 19 d post microspore isolation the greened tissue was transferred to NLN–20% and degreening was examined. In the study of Johnson-Flanagan and Singh (1993), MD embryos were not greened until 23 d after microspore isolation after which time they were 28 d old. The 28 d old greened MD embryos were then transferred to NLN–13% or NLN–20% and chlorophyll content was quantitatively examined over a 15 d

period (Johnson-Flanagan and Singh 1993). MD embryos used in this experiment, therefore, are 9 d younger than those examined by Johnson-Flanagan and Singh (1993) - a difference which may explain the degreening incongruities between the two studies. Perhaps the age-dependent induction of degreening by high osmoticum in MD embryos is an indication that the younger embryos used in this study mimic seed maturation events (like degreening) more appropriately than do older embryos.

Like desiccation, abscisic acid regulates development and maturation of microspore-derived (Wilen *et al.* 1990; Johnson-Flanagan *et al.* 1992) and zygotic (Finkelstein *et al.* 1985) embryos of *B. napus*. This growth regulator induces the appearance of seed-specific napin and cruciferin transcripts (Wilen *et al.* 1990), reduces the level of chlorophyll in green MD embryos (Johnson-Flanagan and Singh 1993; present study) and has a negative effect on the light-harvesting apparatus of MD embryos (present study).

The application of ABA to MD embryos of *B. napus* induces napin and cruciferin transcript accumulation more rapidly than does MD embryo culture on high osmoticum (Wilen *et al.* 1990). Moreover, osmotic stress induces the accumulation of endogenous ABA in MD embryos (Wilen *et al.* 1990). Therefore, these researchers proposed that endogenous ABA mediates the changes in gene expression exhibited by the desiccating (maturing) *B. napus* embryo. Seed degreening is a genetic event that occurs at a point in canola development near the developmental switch from predesiccation embryogenesis to that of desiccation (Johnson-Flanagan *et al.* 1990; Johnson-Flanagan *et al.* 1992). The finding that ABA mediates the process of degreening and light-harvesting disassembly in MD embryos of *B. napus* is consistent with the hypothesis of Wilen *et al.* (1990) that ABA mediates seed events associated with desiccation (maturation).

Following sublethal freezing, the canola seed rapidly desiccates, the level of endogenous seed ABA rises appreciably, and the seed fails to degreen (Johnson-Flanagan *et al.* 1992). If the findings of this thesis are correct - that ABA mediates embryonic

degreening - it would appear that frost-stress uncouples the degreening process from endogenous seed ABA. It should be noted, however, that this frost-induced impairment of ABA perception by the seed embryo is specific to the catabolism of the photosystem components and that other ABA responsive events such as the accumulation of isocitrate lyase and LEA transcripts (Johnson-Flanagan *et al.* 1992) and the accumulation of seed storage proteins (Johnson-Flanagan *et al.* 1991) are largely unaffected by the stress.

6. SUMMARY AND CONCLUSION

Clearly, abscisic acid efficiently provokes an overall depreciation of light-harvesting capacity in MD embryos of *B. napus* in the presence of light. BAP treatment does not have a positive influence on chlorophyll, chlorophyll a/b binding polypeptides or the Cab mRNA during MD embryogenesis.

Under conditions of normal canola seed development, endogenous abscisic acid might regulate the programmed degradation of the photosystem antenna components. The failure of frost-stressed seed to properly degreen may be attributable to an impairment of ABA recognition by the zygote with respect to the process of light-harvesting disassembly and chlorophyll degradation. This could then lead to the stabilization and renewed synthesis of CAB polypeptides and chlorophyll that has been documented by Johnson-Flanagan *et al.* (1994).

At least under the conditions of culture employed in the present study, it is evident that cytokinins do not enhance light-harvesting capabilities during MD embryogenesis. If MD embryos are a reliable seed developmental model, this indicates that cytokinins do not play a role in the aberrant stabilization of green pigment in the frost-stressed seed. However, the influence of cytokinins in this seed process cannot be ruled out without an analysis of the endogenous level of seed cytokinins preceding and following frost stress.

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8. APPENDICES

8.1 APPENDIX A PROTEIN MOLECULAR WEIGHT STANDARDS

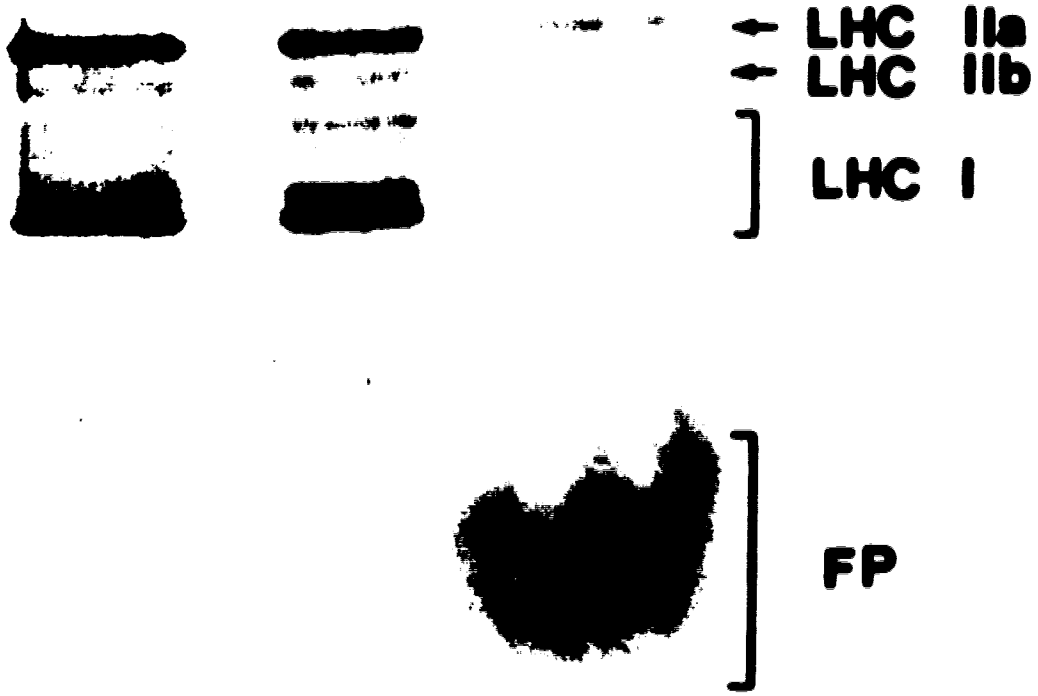
Table A-1 Identity and molecular weight of broad-range electrophoretic protein standards (Bio–Rad Laboratories, Hercules, CA).

Standard	Identity	kDa
1	Myosin (rabbit)	200.0
2	β–galactosidase (*E. coli*)	116.0
3	Phosphorylase b (rabbit)	97.4
4	Serum albumin (bovine)	66.2
5	Ovalbumin (hen)	45.0
6	Carbonic anhydrase (bovine)	31.0
7	Trypsin inhibitor (soybean)	21.5
8	Lysozyme (hen)	14.5
9	Aprotinin (bovine)	6.5

8.2 APPENDIX B CAB POLYPEPTIDE PRECIPITATION BY ACETONE

The objective of the combined chlorophyll extraction and protein precipitation by acetone was to exploit a single tissue sample for two evaluations - chlorophyll determination and Western blot analysis. However, since neither the proficiency of CAB polypeptide precipitation by acetone, the effect of solvent treatment on CAB electrophoretic mobility, nor the effect of acetone treatment on CAB antigenicity were known, Western blot analysis was conducted to reveal these details. In the analysis, a comparison of CAB leaf proteins from lyophilized tissue and from an acetone powder of the same tissue was made (Figure B-1). The blot was probed with α–CPIa so that CAB polypeptides from PSI and PSII would be represented. The analysis demonstrated that a negligible quantity of these polypeptides were present in the solvent following precipitation (compare lanes b and c) and, thus, acetone is an effective agent for the precipitation CAB polypeptides. Acetone precipitation also enhanced the definition of individual CAB polypeptides on these gels (compare lane a with lane b), a favorable effect that is probably attributable to polypeptide delipidation by solvent. Furthermore, it is apparent that the antigenicity of CAB polypeptides was not impaired by solvent precipitation (all polypeptides detectable by α–CPIa were represented in the acetone powder - lane b).

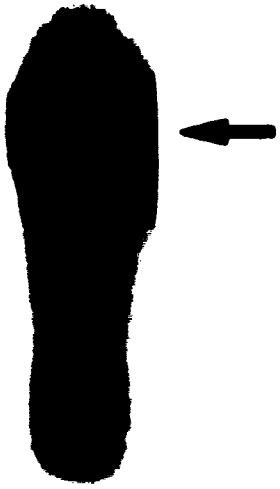
Figure B-1. Western blot analysis of the effectiveness of CAB protein precipitation by acetone. *B. napus* leaf proteins were separated by SDS-PAGE on a 7.5 to 12% linear acrylamide gradient, transferred to nitrocellulose, probed with α–CP1a, and antigenic polypeptides detected with 2° antibody conjugated to alkaline phosphatase. Lane (a), 10 μg total leaf proteins; lane (b), 10 μg total acetone-precipitable leaf proteins; lane (c), the acetone soluble component of the extract in (b). Prior to electrophoresis of (c), the organic solvent was evaporated under reduced pressure and the remaining components dissolved in boiling SDS–PAGE sample buffer. A fraction of this sample was loaded corresponding to the fraction of total sample loaded in (b). FP denotes free pigment (chlorophylls). Location and identity of the CAB polypeptides is indicated.


LHC IIa
LHC IIb
LHC I
FP
a
b
c

8.3 APPENDIX C TISSUE SPECIFICITY OF THE LHC IIb TYPE I cDNA PROBE FROM *B. napus*

Northern blot analysis of total RNA from mature seed and leaf demonstrated that mature seed exhibits no hybridizable Cab transcript while the leaf exhibits an enormous quantity of this photosystem message (Figure C–1).

Figure C-1. Northern blot analysis of LHC IIb Type I mRNA from mature seed and leaf of *B. napus*. Both lanes of the RNA resolving gel were loaded with 150 µg of total RNA. The blot was probed with the LHC IIb Type I cDNA from *B. napus*. Arrowheads mark the position of the 857 bp mRNA.



seed leaf

8.4 APPENDIX D AGAROSE-FORMALDEHYDE RNA RESOLVING GEL

Because the RNA sample buffer contained ethidium bromide, the quality of RNA following electrophoretic separation was examined by visualizing the 28 S and 18 S ribosomal RNA bands with a transilluminator (Fig D-1). Inspection of these ribosomal RNA species prior to capillary transfer allows the researcher to estimate whether samples are equally loaded and whether RNA degradation occurred during isolation or electrophoresis.

Figure D-1. Total RNA from MD embryo, mature seed, and leaf of *B. napus* resolved on a 1.2% agarose-formaldehyde gel. Each lane loaded with 150 µg of total RNA. Gel lanes are as follows. **A**, (a) to (g) RNA from MD embryos cultured under constant illumination (80-100 µmol $m^{-2}s^{-1}$) in NLN–20% (day 0, 6 h, days 1, 2, 3, 5 and 7 respectively), (h) to (m) RNA from MD embryos cultured in NLN–20% in the absence of light (6 h, days 1, 2, 3, 5 and 7 respectively), (n) RNA from mature seed, and (o) RNA from leaf. **B**, (a) to (f) RNA from MD embryos cultured under constant illumination (80-100 µmol $m^{-2}s^{-1}$) in NLN–20% supplemented with ABA to a final concentration of 50 µM (6h, days 1, 2, 3, 5 and 7 respectively), (g) to (l) RNA from MD embryos cultured in the absence of light in NLN–20% supplemented with ABA to a final concentration of 50 µM (6h, days 1, 2, 3, 5 and 7 respectively), (m) to (q) RNA from MD embryos cultured under constant illumination (80-100 µmol $m^{-2}s^{-1}$) in NLN–20% supplemented with BAP to a final concentration of 5 µM (6 h, days 1, 2, 3 and 5). Arrowheads indicate the position of the 25S and 18S ribosomal RNA bands (visualized by ethidium bromide staining).


28 S
18 S
a b c d e f g h i j k l m n o


28 S
18 S
a b c d e f g h i j k l m n o p q