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In Vivo Photomodification of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Holoenzyme by Ultraviolet-B Radiation[1]

Formation of a 66-Kilodalton Variant of the Large Subunit

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Increased levels of solar ultraviolet (290–320 nm) (UV-B) radiation could have profound effects on plant proteins because the aromatic amino acids in proteins absorb strongly in this spectral region. We have investigated the effects of UV-B radiation on plant proteins and have observed a novel 66-kD protein. This product was formed in vivo when *Brassica napus* L. plants grown for 21 d in 65 μmol m^{-2} s^{-1} photosynthetically active radiation were subsequently exposed to 65 μmol m^{-2} s^{-1} photosynthetically active radiation plus UV-B radiation (1.5 μmol m^{-2} s^{-1}). The protein appeared after 4 h of UV-B irradiation and accumulated during the next 16 h in UV-B. The 66-kD protein cross-reacted with an antiserum against the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) holoenzyme. Analysis of soluble leaf proteins revealed that the 66-kD product had a number of isoforms corresponding closely to those of the large subunit of Rubisco (LSU). Partial proteolytic digests of the LSU and the 66-kD protein resulted in an equivalent pattern of protein fragments, leading to the conclusion that the 66-kD protein was a photomodified form of the LSU. A similar high molecular mass variant of Rubisco was observed in soluble protein extracts from leaves of tomato (*Lycopersicon esculentum*), tobacco (*Nicotiana tabacum*), and pea (*Pisum sativum* L.) plants treated in vivo with UV-B, suggesting that it might be a common product, at least among C_3 plants. It is interesting that the 66-kD product appears to be generated after incorporation of the LSU into holoenzyme complexes. This conclusion was drawn from two lines of evidence. First, the LSU variant co-purified with holoenzyme complexes isolated by nondenaturing polyacrylamide gel electrophoresis. Second, a UV-B-specific 66-kD protein did not accumulate in a tobacco mutant that synthesizes the Rubisco subunits but does not assemble them into normal holoenzyme complexes.

Decreased levels of ozone in the stratosphere from organochlorine contamination are resulting in higher levels of UV-B radiation reaching the surface of the earth (Frederick, 1990; Frederick et al., 1991; Kerr and McElroy, 1993). Within the UV-B spectral region, the relative enhancement in irradiance resulting from ozone depletion is disproportionately greater at shorter wavelengths (Caldwell et al., 1989). Biological organisms can be damaged by UV-B when essential molecules are photomodified, and the frequency of photochemical damage, especially to proteins and nucleic acids, generally increases as the wavelength within the UV-B spectral region decreases (Caldwell, 1971; Tevini and Teramura, 1989; Quaite et al., 1992).

Plants are especially vulnerable to increased UV-B radiation, since they cannot avoid exposure to sunlight. Numerous studies have shown that plants are responsive to UV-B irradiation (for reviews, see Caldwell et al., 1989; Tevini and Teramura, 1989; Ballaré et al., 1991; Ensminger, 1993; Greenberg et al., 1995). Plants grown under little or no UV-B radiation differ physiologically, biochemically, and developmentally from those exposed to moderate or high levels of UV-B (Caldwell et al., 1989; Tevini and Teramura, 1989; Barnes et al., 1990; Ziska et al., 1992). At moderate levels of UV-B radiation, plants may acclimate to this form of stress, whereas at higher UV-B fluence rates damage occurs. For instance, increased concentrations of UV-absorbing flavonoids in epidermal cells occur in some plants as they acclimate to UV-B radiation. This has been demonstrated to minimize UV-B-induced damage by screening molecular targets, such as the photosynthetic apparatus (Flint et al., 1985; Murali and Teramura, 1986; Barnes et al., 1990; Bornman and Vogelmann, 1991; Tevini et al., 1991; Cen and Bornman, 1993; Wilson and Greenberg, 1993).

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Abbreviations: 2D, two dimensional; LSU, large subunit of Rubisco; ND, nondenaturing; SSU, small subunit of Rubisco; UV-A, 320–400 nm; UV-B, 290–320 nm; $UV\text{-}B_{BE}$, biologically effective UV-B; UV-C, 200–290 nm.

Several mechanisms exist by which UV-B can trigger acclimation and damage responses in plants. In all cases, the initial step involves photochemical modification of a biomolecule(s), either activating a photoreceptor(s) or damaging a sensitive target(s). Molecules that are potentially sensitive to UV-B in situ include DNA, proteins, auxin, photosynthetic pigments, and phenylpropanoids (Ray and Curry, 1958; Imbrie and Murphy, 1984; Quaite et al., 1992; Braun and Tevini, 1993). Proteins may be particularly sensitive targets, since aromatic amino acids absorb UV-B radiation up to 305 nm. Moreover, proteins can undergo a variety of modifications when exposed to UV-B, including photodegradation of Trp, modification of sulfhydryl residues, increased aqueous solubility of membrane proteins, and fragmentation of peptide chains (Fujimori, 1981; Grossweiner, 1984; Caldwell, 1993; Staxén et al., 1993). These modifications can lead to inactivation of enzymes and disruption of structural proteins. Plant membrane proteins sensitive to UV-B include the D1 PSII reaction center protein and plasma membrane ATPases (Imbrie and Murphy, 1984; Greenberg et al., 1989; Zhang et al., 1994). The highly abundant chloroplast stromal protein, Rubisco (EC 4.1.1.39), has also been reported to be inhibited by UV-B radiation in pea (*Pisum sativum* L.), rice, and soybean (Vu et al., 1984; Strid et al., 1990; Jordan et al., 1992; He et al., 1993). Inactivation of this enzyme by UV-B could be due to modification of the peptide chain, degradation of the protein, and/or diminished transcription of the gene (Vu et al., 1984; Jordan et al., 1992; Caldwell, 1993). However, to our knowledge specific effects on Rubisco at the molecular level have not been described.

We examined the in vivo effects of UV-B on soluble proteins using Rubisco as a model. Rubisco is a 550-kD enzyme complex consisting of four catalytic dimers of LSUs and eight SSUs (Andrews and Lorimer, 1987). In this study, we report that the LSU (54 kD) is converted in vivo to a 66-kD variant by UV-B radiation. This high-molecular-mass variant was observed in four C_3 dicot species exposed to UV-B. The 66-kD UV-B-induced product appears to be formed while LSU is in holoenzyme complexes.

MATERIALS AND METHODS

Plant Growth and Lighting Conditions

Seeds of *Brassica napus* L. cv Topas (canola), *Pisum sativum* L. cv Trapper, *Lycopersicon esculentum* cv Subarctic Maxi, and *Nicotiana tabacum* cv Xanthi were sown in Pro-Mix potting media (Premier Brands, Rivière-du-Loup, Quebec, Canada) and placed in growth chambers at 22°C. Plants were grown under PAR (400–700 nm) with a 16-h light:8-h dark photoperiod for 21 d and then placed in PAR plus either UV-A (control conditions) or UV-A and UV-B (UV-B treatment conditions) (Fig. 1). The XV1 and *Su/+ N. tabacum* mutants were grown on tissue culture media under continuous PAR (Avni et al., 1989) and then exposed to PAR plus either UV-A (control conditions) or UV-A and UV-B (UV-B treatment conditions). PAR (65 μmol m^{-2} s^{-1} at the leaf surface) was generated with cool-white fluorescent lamps filtered through clear polyester film (0.08 mm) to screen out UV-B and UV-C radiation. During the UV-B treatment period, PAR (65 μmol m^{-2} s^{-1}) plus UV-B (1.5 μmol m^{-2} s^{-1}) and UV-A (1.7 μmol m^{-2} s^{-1}) were generated with fluorescent lamps (F48T12-CW for PAR and FS40 for UV-B and UV-A; National Biological Co., Twinsburg, OH) filtered through cellulose diacetate (0.08 mm) to screen out UV-C (>290 nm) (Fig. 1). To generate PAR (65 μmol m^{-2} s^{-1}) plus UV-A (1.7 μmol m^{-2} s^{-1}) for the control treatments, the irradiation from FS40 lamps was filtered through polyester film (0.08 mm Mylar D) to remove most of the UV-B. (Fig. 1).

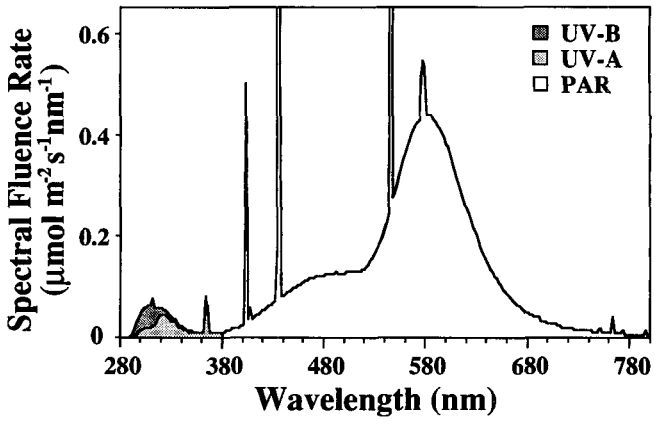

Figure 1. Spectral output of light sources used for growth and treatment of the plants. Prior to experimental treatments, all plants were germinated and grown under PAR from cool-white fluorescent lamps. Note that the cool-white fluorescent lamps emit a small amount of UV-A radiation from 380 to 400 nm. For all experiments, the spectral distribution of the light source used for UV-B treatment of plants contained PAR plus the UV-A emitted by FS-40 lamps plus the UV-B emitted by FS-40 lamps. The light source used for the control plants during the experimental treatment period contained PAR plus the UV-A that remains when the FS-40 lamp is filtered through polyester film. See "Materials and Methods" for a description of the light sources.

The spectral photon distributions and fluence rates of all light sources were measured at the level of the leaves using a calibrated spectroradiometer (Optronics, Inc., Orlando, FL, or Oriel, Inc., Stratford, CT) or a radiometer (Photodyne, Camarillo, CA) and interference filters (10-nm half-power bandwidth) to isolate individual bandwidths. The fluence rate of PAR was measured using a calibrated quantum sensor (Li-Cor, Lincoln, NE). The biologically effective irradiance of UV-B used was equivalent to 260 mW m^{-2} $UV\text{-}B_{BE}$ for treated plants (25–50% of the levels currently found in the southern United States and approximately equal to the levels currently found in southern Canada) and 75 mW m^{-2} $UV\text{-}B_{BE}$ for control plants using Caldwell's general plant damage action spectra normalized at 300 nm (Caldwell, 1971; Caldwell et al., 1980). Also, the visible: UV-B ratio on a photon basis was approximately 100:2, which is similar to the ratio in the environment (100:1) (Greenberg et al., 1995).

Protein Isolation and Analysis

Leaf discs (0.4 cm^2) were taken from the oldest fully expanded leaves of individual plants at various times during the treatment period, immediately frozen in liquid N_2, and then stored at −70°C for subsequent protein analysis. Frozen leaf discs were homogenized in protein extraction buffer (50 mM Tris-HCl, pH 8.0; 0.5 mM EDTA; 2 mM DTT, 2 mM PMSF; 10 μM leupeptin; 1 mM *p*-aminobenzamidine; 1% polyvinylpolypyrrolidone; 0.02% sodium azide) at 4°C. After centrifugation (14,000*g*, 30 min) the concentration of soluble proteins in the supernatant was determined according to the method of Ghosh et al. (1988).

Proteins were prepared for SDS-PAGE by diluting protein extracts with one-half volume of SDS sample buffer (30% [w/v] glycerol; 9% [w/v] SDS; 120 mM Tris-HCl [pH 6.6]; 15% [w/v] 2-mercaptoethanol) and heating at 90°C for 2 min. Proteins were separated by SDS-PAGE (12% [w/v] acrylamide) in the buffer system of Laemmli (1970). Separated proteins were visualized using either Coomassie blue or silver staining (Bio-Rad).

For protein detection with antisera, proteins were electroblotted from SDS-polyacrylamide gels onto a 0.2-μm nitrocellulose membrane (Bio-Rad) using 16 mM Gly; 25 mM Tris-HCl, pH 8.7; 0.02% (w/v) SDS; and 20% methanol as the transfer buffer. Electrophoretic transfer was carried out for 2 h at 300 mA constant current. The efficiency of protein transfer was examined by silver staining the gels after transfer. Protein blots were blocked with 1% nonfat milk powder in Tris-buffered saline (25 mM Tris-HCl, pH 7.6; 140 mM NaCl) and incubated with a 1:3000 dilution of a rabbit anti-Rubisco (holoenzyme) antiserum. The antiserum was raised against Rubisco holoenzyme from *Euglena gracilis* and has been shown to cross-react with higher plant Rubisco (Avni et al., 1989). Antibody binding was visualized using a goat anti-rabbit IgG secondary antibody conjugated to alkaline phosphatase according to manufacturer's protocol (Bio-Rad). The molecular masses of detected protein bands were estimated with prestained standards.

Protein samples for 2D gel electrophoresis (O'Farrell, 1975) were isolated by extraction from plant tissue as above. Protein aliquots (50 μg in approximately 25 μL) were prepared for the first dimension (IEF gel electrophoresis) by addition to an equal volume of a buffer containing 9.5 M urea, 10% Suc, 8% 3-([cholamidopropyl])dimethylammonio)-1-propanesulfonic acid, 5% ampholines (pH 3–10), 15 mM DTT and loaded onto 4% polyacrylamide tube gels with a pH 5 to 10 gradient. The IEF was carried out at 20°C for 20 h at 800 V. After IEF, proteins were separated in the second dimension by equilibrating each tube gel in SDS sample buffer, placing them on 1.5-mm-thick 10 to 15% SDS gels with 5% stacking gels, and electrophoresing for 4 h at 100 V constant voltage. Proteins were visualized by silver staining or electroblotted onto nitrocellulose for subsequent immunodetection with anti-Rubisco immune serum.

For ND PAGE, proteins were extracted from plant tissue as described above with the following modifications. The extraction buffer for tissue homogenization contained 50 mM Tricine (pH 8.0), 150 mM NaCl, 0.04% sodium azide, 2 mM PMSF, 10 μM leupeptin, 50 μM aprotinin, and 100 μM iodoacetamide. Samples were homogenized and centrifuged (13,000*g*, 10 min) after which glycerol was added to a final concentration of 10% (w/v). Electrophoresis of samples (15 μg of protein) was carried out at 350 V for 2 h in 5.5% acrylamide gels (Fieldes and Gerhardt, 1994). Proteins were detected by staining with Coomassie blue or by immunodetection as described above except acetic acid was not used in the staining solutions. The Rubisco-containing band was excised from stained ND gels and subsequently incubated in SDS sample buffer for 20 min to denature the proteins. The gel slices were placed in the sample wells of an SDS-polyacrylamide gel, and the proteins in the gel slices were separated by electrophoresis.

Partial Proteolytic Digests

Partial proteolysis was performed by co-electrophoresis of individual proteins with papain (Sigma) as described by Cleveland et al. (1977) and modified for chloroplast proteins by Marder et al. (1986). Soluble protein samples from UV-B-treated plants were separated using SDS-PAGE; one-half of the gel was stained with Coomassie blue and the other half was transferred to nitrocellulose for immunodetection using anti-Rubisco antibodies as described above. Protein bands to be isolated for proteolytic analysis were located on the stained gel using the immunoblot as a reference. Each gel slice was immersed in equilibration buffer (0.5 M Tris-HCl, pH 6.6; 0.8% [w/v] SDS; 10% glycerol; 1% 2-mercaptoethanol; 1 mM EDTA) for 15 min and then placed in individual wells of an SDS gradient gel (15–20% [w/v] acrylamide; 10 cm × 10 cm × 1.5 mm). The gel was placed in an electrophoresis unit, covered with upper reservoir buffer, and then covered with equilibration buffer (10 μL). An overlay buffer (10 μL) containing various concentrations of papain (0–25 ng mL^{-1}) was added to each well. The protein mixture was electrophoresed for 1.5 h at 100 V. Electrophoresis was stopped for 30 min to allow proteolysis to occur, after which electrophoresis was continued for 3 h at 100 V to separate the resultant peptide fragments. Proteins were transferred to nitrocellulose and immunodetected with the anti-Rubisco (holoenzyme) antiserum.

RESULTS

Initial Identification of a UV-B-Induced Product of Rubisco

When *B. napus* plants grown in PAR for 21 d were exposed to PAR plus UV-B (Fig. 1), trace amounts of a novel soluble protein of 66 kD were detected in Coomassie blue-stained SDS-polyacrylamide gels (Fig. 2A). The protein was observed after 8 h of exposure to UV-B and seemed to accumulate as a function of exposure time to UV-B radiation. A variety of antisera, including anti-Hsp 70, anti-ubiquitin, and anti-Rubisco, was used in an effort to make a preliminary identification of the 66-kD protein. In immunoblots of soluble leaf proteins from UV-B-treated plants probed with the Rubisco antiserum, a protein band

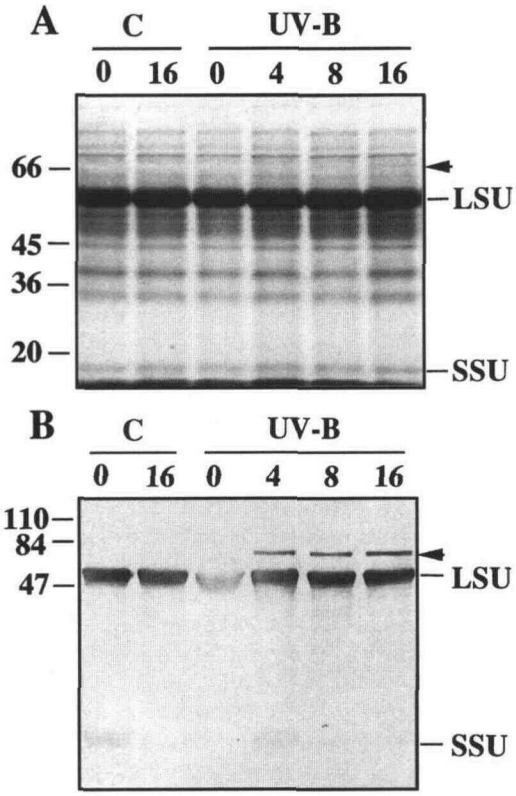

Figure 2. Effect of UV-B on soluble leaf proteins from *B. napus*. Plants were grown for 21 d under PAR and then shifted to control conditions (C; UV-A plus PAR) for 0 or 16 h or to UV-B conditions (UV-B plus UV-A plus PAR) for 0, 4, 8, or 16 h. A, SDS-PAGE (12% [w/v] acrylamide) of protein samples (15 μg/lane) detected by Coomassie blue staining. B, Immunoblot of protein samples (2 μg/lane) probed with a Rubisco holoenzyme antiserum. Positions of LSU, SSU, a protein of approximately 66 kD that accumulates during the treatment (arrow), and the molecular mass standards (in kD) are indicated.

of approximately 66 kD was detected in addition to the LSU (54 kD) and the SSU (14 kD) (Fig. 2B). This 66-kD product observed immunologically accumulated during the 16-h UV-B treatment period. The temporal accumulation of the photoproduct represents a response to dose, as the total UV-B fluence that the plants had been exposed to increased over time. The 66-kD protein appeared to co-migrate with the Coomassie blue-stained 66-kD protein and did not cross-react with the other antisera that were tested.

2D PAGE of Soluble Leaf Proteins from *B. napus*

The LSU and SSU can each be resolved by 2D PAGE into several isoforms based on pI (Robbins and Vaughn, 1983). This property might be useful to determine whether a given protein is related to Rubisco. The major isoforms of LSU (about eight spots in 2D PAGE) were observed in silver-stained 2D polyacrylamide gels of proteins from control samples (Fig. 3A). These isoforms were also detected by the anti-Rubisco immune serum in a protein blot of a 2D gel (Fig. 3C). The soluble proteins from the UV-B-treated plants contained a number of additional protein spots at about 66 kD, each having a pI corresponding closely to one of the isoforms of the LSU. These 66-kD products were detected both by silver staining and by immunological analyses of the gels (Fig. 3, B and D), indicating that the 66-kD product is related to the LSU.

Partial Proteolytic Analysis of the 66-kD UV-B-Induced Protein

To further confirm that the 66-kD protein was related to LSU, partial proteolytic fragmentation patterns of LSU and of the higher molecular mass product were compared (Fig. 4). After the LSU was digested with papain, seven proteolytic fragments were detected with the anti-Rubisco immune serum. This digestion pattern (Fig. 4) is characteristic of LSU as previously reported by Bottomley and Whitfield (1979). Digestion of the 66-kD protein with papain gave rise to five protein fragments that were detected with the Rubisco antiserum (Fig. 4). All of these fragments matched fragments derived from the LSU, including one fragment that co-migrated with intact LSU (Fig. 4). It is possible that fewer fragments were detected from the 66-kD product because of the lower concentration of this protein relative to LSU applied to the papain digestion gel. Thus, we conclude that the 66-kD UV-B-induced protein is a high-molecular-mass variant of LSU.

Prevalence of the High-Molecular-Mass UV-B-Induced Rubisco Product

To determine whether UV-B-induced accumulation of the 66-kD protein is observed in other C_3 plants, seedlings of pea, tomato, and tobacco were exposed to the same UV-B conditions used for *B. napus*. The 66-kD variant of LSU was readily detected in each of these plant species following exposure to UV-B (Fig. 5). However, the product was absent or in trace amounts in the associated control plants.

Co-Purification of the 66-kD Protein with Rubisco Holoenzyme Complexes

We wanted to determine whether the 66-kD UV-B-induced protein is generated while the LSU is bound to holoenzyme complexes and whether it remains there after being produced. To address the latter question, ND PAGE followed by SDS-PAGE were used to determine whether the high-molecular-mass variant co-purified with holoenzyme complexes. ND PAGE revealed that UV-B-treated plants contained protein bands that co-migrated with putative holoenzyme complexes from the control plants (Fig. 6). These complexes from control and UV-B-treated *B. napus* were identified as intact holoenzymes because they migrated in ND PAGE in a manner similar to holoenzyme complexes reported for tobacco (Avni et al., 1989), and subsequent SDS-PAGE of these *B. napus* proteins revealed the presence of the LSU and SSU (Fig. 6).

When the holoenzyme complexes from the UV-B-treated plants were analyzed by SDS-PAGE, the 66-kD LSU variant was present as a strong band along with the LSU and SSU.

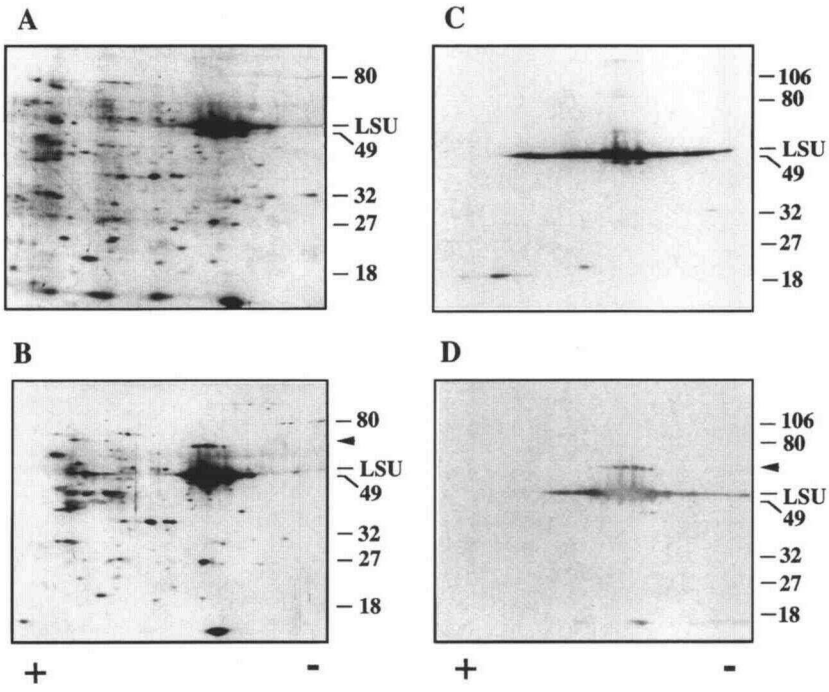

Figure 3. 2D PAGE of soluble leaf proteins from control and UV-B-treated *B. napus*. Protein samples (20 μg) were resolved in the horizontal dimension by IEF in a 4% polyacrylamide gel with a pH 5 to 10 gradient. The vertical dimension was SDS-PAGE (10–15% acrylamide gradient). A and B, Silver-stained gels of soluble leaf proteins from control and UV-B-treated *B. napus*, respectively. C and D, Immunoblots of soluble leaf protein samples from control and UV-B treatments, respectively, probed with the anti-Rubisco holoenzyme immune serum. Positions of molecular mass markers (in kD) and LSU are shown. Arrows indicate the position of the 66-kD protein that accumulates during UV-B treatment.

The holoenzyme complexes from the control plants contained predominantly the LSU and SSU. This indicates that the 66-kD protein either can be incorporated into or is generated within holoenzyme complexes. We note that there was a minor amount of the 66-kD protein in samples from the control plants (Fig. 6). We assume, however, that it arose during the ND PAGE/SDS-PAGE analysis, since it was not present in control samples subjected to only SDS-PAGE (Fig. 2).

UV-B-Induced Degradation of LSU in a Rubisco Assembly Mutant of Tobacco

The XV1 mutant of tobacco has a single amino acid change in LSU that precludes assembly of the LSU and SSU into normal holoenzyme complexes (Avni et al., 1989). We therefore used this mutant to determine whether prior assembly of the Rubisco holoenzyme is required for generation of the 66-kD LSU variant. When *N. tabacum* cv Xanthi (wild type) was exposed to UV-B, the high-molecular-mass product was observed, whereas there was no apparent degradation of LSU or extraneous peptides cross-reacting with the antiserum (Fig. 7). Conversely, a significant fraction of the LSU was lost when the XV1 plants were treated with UV-B (Fig. 7, cf. lanes C and UV for the XV1 plants). Since the XV1 mutant accumulates LSU to only a few percent of the wild-type level (Avni et al., 1989), an excess of XV1 protein was loaded on the gel to allow comparisons to be made to the wild-type plants. This may explain why high-molecular-mass polypeptides and degradation products were detected in extracts from this mutant (Fig. 7). The high-molecular-mass forms from the XV1 plants did not appear to correspond exactly to the 66-kD UV-B-induced protein observed on immunoblots of extracts from the wild-type plants. Furthermore, the levels of

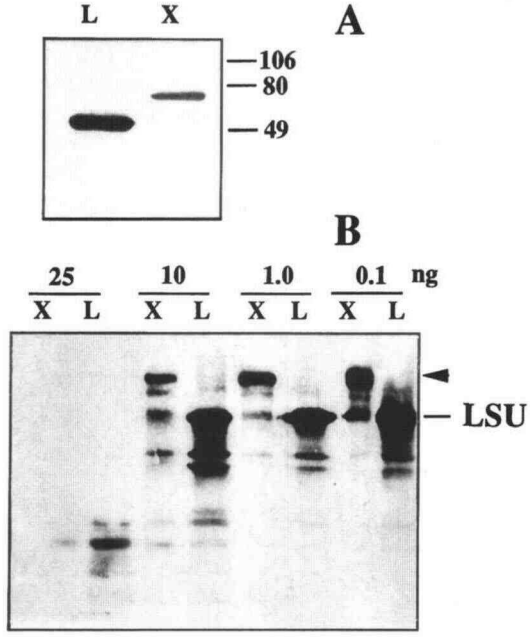

Figure 4. Partial proteolytic analysis of the LSU and the 66-kD UV-B-induced protein. Gel slices containing either LSU or the 66-kD protein were placed into wells of an SDS-polyacrylamide gel (10–15% [w/v] acrylamide) and co-electrophoresed with papain as described in "Materials and Methods." Following SDS-PAGE, peptide fragments were transferred to nitrocellulose filters and detected immunologically with the anti-Rubisco holoenzyme immune serum. A, LSU (L) and the 66-kD protein (X) from excised gel slices separated by SDS-PAGE in the absence of papain. B, LSU (L) and the 66-kD protein (X) from excised gel slices digested with the indicated amounts of papain (0–25 ng mL^{-1}). Positions of LSU and the 66-kD protein (arrow) are indicated.

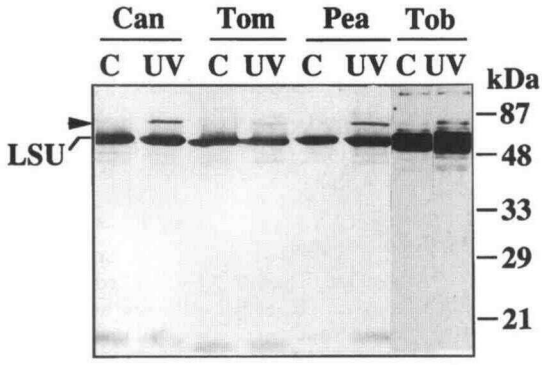

Figure 5. Immunological analysis of soluble leaf proteins from four C_3 plant species. *B. napus* (Can), tomato (Tom), pea (Pea), and tobacco (Tob) plants were irradiated with control (C) or UV-B (UV) lighting for 6 h. Samples of soluble leaf proteins (2 μg/lane) were separated by SDS-PAGE (12% [w/v] acrylamide), transferred to nitrocellulose, and probed with the anti-Rubisco holoenzyme immune serum. Positions of LSU, the 66-kD protein (arrow), and molecular mass markers (in kD) are shown.

the proteins in the 60- to 70-kD region from the XV1 plants did not increase in response to UV-B, so we assume they arose via a non-UV-B-dependent mechanism. It is interesting that in the XV1 mutant a higher molecular mass band at about 110 kD is generated in response to UV-B radiation.

The XV1 mutant has a low level of Chl (approximately 30% of the wild type; Avni et al., 1989). It is known that plants with diminished levels of Chl are susceptible to enhanced UV-B-driven protein degradation (Greenberg et al., 1989). Since a large fraction of the LSU was apparently degraded when seedlings of the XV1 were treated with UV-B (Fig. 7), one might assume that plants with low Chl levels would exhibit accelerated degradation of LSU without visible production of the high-molecular-mass variant. This possibility was examined using a yellow-green (aurea) mutant of tobacco (heterozygous for a semidominant mutation in the nuclear-encoded photosynthetic *Su* gene, designated *Su*/+), which has a normal complement of assembled Rubisco holoenzyme but only about 25% of the wild-type amount of Chl (Kawata and Cheung, 1990). When the *Su*/+ plants were exposed to UV-B, the 66-kD protein was observed at a level commensurate with that of the UV-B-treated wild-type plants, and there was no apparent degradation of the LSU compared to the control *Su*/+ plants (Fig. 7). Therefore, only the XV1 Rubisco assembly mutant failed to accumulate the 66-kD LSU variant in response to UV-B. This is consistent with the recent finding that the 66-kD protein was an SSU-LSU adduct (M.I. Wilson, K.E. Gerhardt, and B.M. Greenberg, unpublished observations), explaining the requirement for holoenzyme assembly to form the photoproduct.

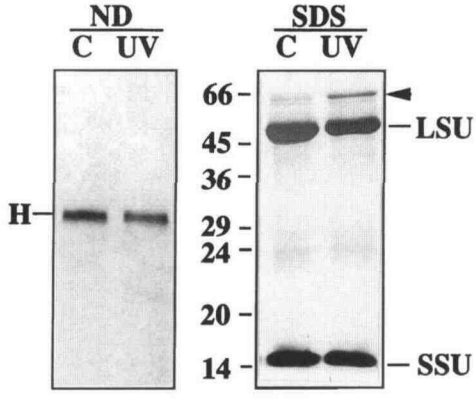

Figure 6. Presence of the 66-kD UV-B-induced LSU variant in holoenzyme complexes. *B. napus*, grown for 21 d in PAR, were shifted to control (C) or UV-B treatment (UV) conditions for 6 h. Soluble proteins from these plants were separated by ND PAGE (ND) and detected by Coomassie blue staining. The only protein detected in the region shown was the Rubisco holoenzyme (H). The holoenzyme bands were excised from the ND PAGE gel and analyzed by SDS-PAGE (SDS). Silver staining of the SDS-polyacrylamide gel revealed bands at 66, 55, and 14 kD. The 66-kD band is indicated with an arrow. The positions of LSU, SSU, and molecular mass markers (in kD) are also shown.

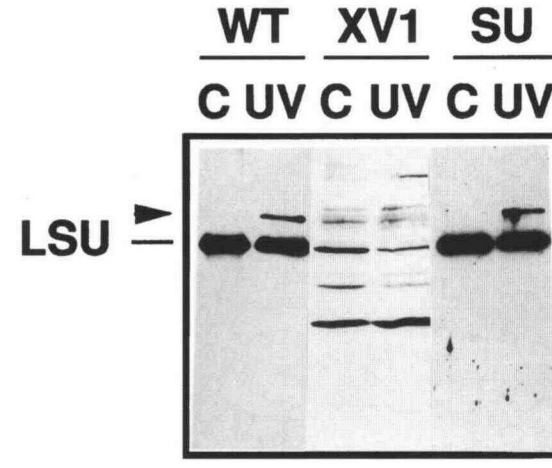

Figure 7. Immunological analysis of soluble leaf proteins from wild-type and mutant tobacco. Wild-type (WT) tobacco and the XV1 and *Su*/+ (SU) mutants of tobacco were exposed to control (C) or UV-B (UV) treatment conditions for 6 h. Proteins were extracted from leaf tissue, separated by SDS-PAGE, and electroblotted onto nitrocellulose filters. The proteins were detected with an antiserum to Rubisco holoenzyme. Positions of the LSU and 66-kD UV-B-induced product are indicated.

DISCUSSION

A number of plant proteins have been shown to be sensitive to UV-B, one of which is Rubisco (Imbrie and Murphy, 1984; Bornman, 1989; Greenberg et al., 1989; Staxén et al., 1993; Zhang et al., 1994). In this study, we found that UV-B irradiation of *B. napus* plants resulted in the accumulation of a novel 66-kD protein. Initial immunodetection with a Rubisco antiserum suggested that the 66-kD protein was related to Rubisco. Positive identification of the 66-kD polypeptide as a photomodified form of the LSU was subsequently demonstrated using immunodetection of 2D polyacrylamide gels and of partial proteolytic digests. The 66-kD UV-B-induced variant of the LSU may be a ubiquitous product in C_3 plants, since it was readily detected in *B. napus*, pea, tomato, and tobacco

plants following exposure to UV-B radiation. A protein of similar molecular weight was found in corn grown under high fluence rate PAR plus UV-B, although the source of this protein was not investigated (Santos et al., 1993).

The 66-kD LSU photoproduct accumulated steadily during the 16-h UV-B exposure period (Fig. 2), and a similar amount remained after a subsequent 6 h of dark incubation (data not shown). This observation implies that prolonged exposure of plants to UV-B radiation could result in the accumulation of substantial amounts of the LSU variant. Indeed, the data in Figure 2 represent a dose response with the increasing amount of photoproduct corresponding to the increasing amount of total UV-B fluence applied to the plants at each time. Note that on an integrated UV-B_{BE} dose basis the 4-h exposure corresponds to the daily dose experienced in the arctic, and the 16-h exposure corresponds to the daily dose experienced at the equator under current stratospheric ozone levels. Studies during longer periods and at varying UV-B fluence rates will now be required to determine how much 66-kD protein can accumulate. Also, it will be interesting to determine whether formation of the 66-kD protein is related to the decreases in total Rubisco protein and enzyme activity that have been observed following extended growth of plants under UV-B (Vu et al., 1984; Jordan et al., 1992; He et al., 1993). Even if the formation of the 66-kD product is the beginning of a pathway that ultimately leads to degradation or inactivation of Rubisco under elevated levels of UV-B radiation, the exceptionally large amounts of this enzyme in chloroplasts may render photomodification of LSU an inconsequential factor in terms of total photosynthetic activity. This argument, however, will not hold for other, less abundant proteins, which are likely to be sensitive to UV-B in a similar manner. Thus, their levels could decrease significantly with resultant negative impacts on plant productivity and/or viability.

Exposure of proteins to UV-B radiation can result in their photooxidation, and Rubisco, in general, seems to be a target for oxidative damage in plants. A protein of about 66 kD that cross-reacted with anti-Rubisco antibodies was observed in chloroplasts isolated from hybrid poplar leaves treated with ozone, indicating that oxidative modifications can lead to formation of high-molecular-weight products of Rubisco (Landry and Pell, 1993). This may explain in part why trace amounts of the 66-kD LSU variant were generated in protein samples from control *B. napus* plants during ND PAGE/SDS-PAGE (Fig. 6), because we did not attempt to protect Rubisco from air oxidation during this analysis. Other high-molecular-mass variants (110 kD) have also been reported to occur in response to cold acclimation (Huner and MacDowall, 1979) and oxidative stress during senescence (Mehta et al., 1992). In both cases, these variants were dimers of LSU likely resulting from intermolecular cross-linking via oxidation reactions. However, the presence of the 66-kD protein and the absence of the 110-kD protein under UV-B indicates that photooxidation of proteins can result in specific products.

Photooxidation of proteins induced by UV-B radiation has been studied by monitoring changes in enzyme activity, protein profiles, or Trp fluorescence (Grossweiner et al., 1976; Fujimori, 1981; Grossweiner, 1984; Imbrie and Murphy, 1984; Pigault and Gerard, 1984, 1989; Greenberg et al., 1989; Kochevar, 1990; Caldwell, 1993). Although other chromophores in proteins can trigger oxidative damage following absorbance of a UV-B photon, in Rubisco there are no UV-B-absorbing cofactors and Trp is the only amino acid that absorbs in the range of UV-B radiation used in this study (<292 nm) (Grossweiner et al., 1976; Pigault and Gerard, 1984, 1989; Kochevar, 1990; Kim and Sancar, 1993). After Trp absorbs a UV photon and reaches an excited state, it can undergo intersystem crossing to form a triplet state, and subsequently the absorbed energy can be passed to O_2, forming singlet oxygen ($^1O_2$). The $^1O_2$ can then oxidize the Trp to an oxindole (Grossweiner et al., 1976; Creed, 1984; Imbrie and Murphy, 1984). One of these photoproducts, *N*-formylkynurenine, can act as a photosensitizer, which, in the presence of UV-A, could react with a nearby amino residue to form a covalent bond (Walrant and Santus, 1974; Grossweiner et al., 1976; Fujimori, 1981). Trp can also be photooxidized to the Trp^+ radical cation (Grossweiner, 1984). Radical cation formation can result in the direct intramolecular transfer of electrons from Trp to a nearby amino acid residue (Pigault and Gerard, 1989; Kochevar, 1990). These reactions may result in covalent cross-linking between residues and/or direct damage to residues in close proximity to the Trp. It is interesting that Caldwell (1993) reported a detailed study of the changes in Trp fluorescence of partially purified Rubisco from cucumber and noted that Trp residues are photooxidized in the presence of UV-B radiation.

If one assumes that the 66-kD product derives from photooxidation of Trp, this could lead to inter- or intramolecular cross-linking. Given the high concentrations of Rubisco in chloroplasts (Lawlor, 1993), intermolecular cross-linking between Rubisco holoenzyme complexes is a possibility. However, aggregates of the holoenzyme were not observed by native gel electrophoresis (Fig. 6). Furthermore, the 66-kD product co-purifies with holoenzyme complexes of a typical size of 550 kD (Fig. 6), making cross-linking between holoenzyme complexes an unlikely explanation for formation of the LSU variant. It should also be noted that the size of the 66-kD LSU variant is inconsistent with incorrect processing of the LSU precursor, because pre-LSU is only 1 or 2 kD larger than the mature LSU (Langridge, 1981; Shinozaki and Sugiura, 1982).

The positions of Trp residues in LSU are highly conserved among plants, and crystallographic analysis of spinach Rubisco reveals that Trps are present in the active site and at interfaces between the LSU and SSU within the holoenzyme (Knight et al., 1990). Accordingly, cross-links between LSU and SSU within the confines of a holoenzyme complex are possible. Thus, it is significant that the molecular mass of the UV-B-induced Rubisco product is consistent with LSU-SSU cross-linking. Indeed, preliminary evidence using an antibody specific to the SSU indicates that the 66-kD product comprises the SSU and the LSU (M.I. Wilson, K.E. Gerhardt, and B.M. Greenberg, unpublished observations).

It is particularly noteworthy that the 66-kD UV-B-induced product did not accumulate in the XV1 Rubisco mutant, especially in view of the report by Avni et al. (1989) that LSU is actively translated in XV1 plants. It then follows that the 66-kD variant is not generated during or immediately after translation of the LSU. Instead, when the XV1 plants are exposed to UV-B a specific 110-kD protein is formed. This may be a dimer of two LSUs (Mehta et al., 1992). Furthermore, since the 66-kD UV-B product was found to co-purify with holoenzyme complexes from wild-type plants (Fig. 6), we may conclude that the LSU is incorporated into a holoenzyme before the UV-B-specific 66-kD protein can be generated. Moreover, once the product is formed it has the capacity to remain associated with a holoenzyme complex.

Since the 66-kD LSU photoproduct is generated at the low UV-B fluence rates used here (25–50% of the levels currently found in the southern United States) and it remains associated with the holoenzyme, its appearance may represent an early event of UV-B damage to Rubisco. Thus, production of the LSU variant might be a sensitive and valuable indicator of UV-B stress in plants. In future studies it will be interesting to assess whether holoenzyme complexes containing the UV-B product lose enzymatic activity. We are currently studying the formation of the 66-kD product in vitro using purified Rubisco holoenzyme, because this would facilitate more detailed dose, structural, and mechanistic analyses of this form of UV-B lesion.

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