ABSTRACT

Title of Dissertation: THE EFFECTS OF SOLAR ULTRAVIOLET-B ON THE PROTEOME AND ANTIOXIDANT DEFENSE SYSTEMS IN SOYBEAN LEAVES

Chenping Xu, Doctor of Philosophy, 2007

Directed By: Associate Professor Joe H. Sullivan
Department of Plant Science and Landscape Architecture

Stratospheric ozone depletion has caused an increase in the amount of ultraviolet-B (UV-B) radiation reaching the earth's surface. Some investigations have demonstrated that UV-B has effects on protein accumulation and active oxygen species (AOS) metabolism in plants. Because of the unrealistically high UV-B level and low levels of ultraviolet-A (UV-A) and photosythetically active radiation (PAR) in indoor studies it has been questioned whether results from these studies can be extrapolated to field responses. In the present study two isolines of the soybean cultivar Clark with different flavonoid contents were grown in the field with or without natural levels of UV-B. The leaf proteome and AOS metabolism were examined.

Ambient solar UV-B radiation changed AOS metabolism by decreasing superoxide dismutase activity and increasing the activities of ascorbate peroxidase, catalase and glutathione reductase relative to UV-B exclusion treatments. This resulted

in decreased ascorbic acid and increased dehydroascorbate content. Proteomic analysis showed that the accumulations of 67 protein spots were significantly affected by solar UV-B. Proteins related to photosystems in photosynthesis were increased in abundance while enzymes involved in the primary carbon and nitrogen metabolism were decreased. This could lead to overreduction of the photosynthetic electron transport chain and enhance the formation of superoxide radicals and singlet oxygen. The magenta line, which has reduced flavonoid levels, had greater proteomic and oxidative responses than the standard line, suggesting that flavonoids act as screening compounds and antioxidants in protecting plants from UV-B radiation. These line-specific differences occurred even under UV-B exclusion, which may be due to high UV-A, PAR or temperature. More detailed studies are needed to elucidate the effects of other environmental factors on the soybean leaf proteome and AOS metabolism under field conditions.

THE EFFECTS OF SOLAR ULTRAVIOLET-B ON THE PROTEOME AND ANTIOXIDANT DEFENSE SYSTEMS IN SOYBEAN LEAVES

By

Chenping Xu

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2007

Advisory Committee:

Associate Professor Joe H. Sullivan, Chair
Associate Professor Irwin N. Forseth
Professor William Kenworthy
Dr. Savithiry Natarajan
Professor Bruno Quebedeaux

ACKNOWLEDGEMENTS

First of all, I am deeply grateful to Dr. Joe H. Sullivan, for his advice and unreserved support whenever I needed throughout this research. Also I want to thank Dr. Savithiry Natarajan for her encouragement and guidance. Dr. Natarajan gave me this great opportunity to work on proteomics.

I am indebted to Dr. Thomas J. Caperna and Dr.Wesley M. Garrett, for opening their labs and assisting in doing analyses for this research; Dr. W. Kenworthy, for providing soybean seeds; Dr. J. Slovin, for critical review of my thesis; Dr. H. Bae, for help with data presentation; and my fellow graduate students, Linda Pope and Darren DeStefano, for assistance with UV exclusion shelters.

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2D-PAGE	two-dimensional polyacrylamide gel electrophoresis.
6-4 product	pyrimidine (6-4) pyrimidinone photoproduct.
ACN	acetonitrile.
ANOVA	analysis of variance.
AOS	active oxygen species.
APX	ascorbate peroxidase.
AsA	ascorbic acid.
ATP	adenosine triphosphate.
BLAST	basic local alignment search tool.
BSA	bovine serum albumin.
CBB	coomassie brilliant blue.
CAT	catalase.
CHAPS	3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulfonate.
CPD	cyclobutane pyrimidine dimmer.
Cyt c	cytochrome c.
KDa	kilodalton.
DHA	dehydroascorbate.
DHAR	dehydroascorbate reductase.
DNA	deoxyribonucleic acid.
DTT	dithiothreitol.
EDTA	ethylenediaminetetraacetic acid.
EST	expressed sequence tag.

GADPH	glyceraldehyde-3-phosphate dehydrogenase.
GCV	glycine cleavage system.
GR	glutathione reductase.
GSH	reduced glutathione.
GSSG	glutathione disulphide.
HEPES	N-(2-hydroxyethyl)piperazine-2'-(2-ethanesulfonic acid).
HSP/HSC	heat shock protein / heat shock cognate.
IEF	isoelectric focusing.
IPG	immobilized pH gradient.
LC-MS/MS	liquid chromatography tandem mass spectrometry.
MALDI-TOF	matrix-assisted laser desorption/ionization-time of flight.
MDHA	monodehydroascorbate.
MDHAR	monodehydroascorbate reductase.
min	minutes.
MOD	modulated.
Mr	relative molecular weight.
mRNA	messenger ribonucleic acid.
MS	mass spectrometry.
MS/MS	tandem mass spectrometric.
$NADP^+$	nicotinamide adenine dinucleotide phosphate, oxidized form.
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form.
NADH	nicotinamide adenine dinucleotide, reduced form.
NCBI	National Center for Biotechnology Information.

nm	nanometer.
OEE	oxygen-evolving enhancer.
PAR	photosythetically active radiation.
pI	isoelectric point.
PMF	peptide mass fingerprinting.
POD	guaiacol peroxidase.
PS	photosystem.
PTM	post-translational modifications.
PVP	polyvinylpyrrolidone.
QTL	quantitative trait loci.
RNA	ribonucleic acid.
Rubisco	ribulose bisphosphate carboxylase/oxygenase.
SDS	sodium dodecyl sulfate.
SHMT	glycine (serine) hydroxymethyltransferase.
SOD	superoxide dismutase.
SQW	square-wave.
TBA	thiobarbituric acid.
TBARS	thiobarbituric acid reacting substances.
TCA	trichloroacetic acid.
TFA	trifluoroacetic acid.
Tris	tris(hydroxymethyl)aminomethan.
UV	ultraviolet.
UV-A	ultraviolet-A.

UV-B	ultraviolet-B.
UV-C	ultraviolet-C.

CHAPTER 1 LITERATURE REVIEW

1.1. ULTRAVIOLET-B RADIATION

Plants use sunlight for photosynthesis and, as a consequence, are also exposed to solar ultraviolet (UV) radiation. UV radiation is generally divided into three classes: UV-C (≤ 280 nm), UV-B (280-320 nm), and UV-A (320-400 nm). UV-C is highly energetic and extremely damaging to biological systems. However, it is strongly absorbed by ozone and oxygen in the stratosphere, and is removed from sunlight reaching the earth's surface (Caldwell et al., 1989). UV-B radiation is also harmful and only partly absorbed by ozone (Madronich et al., 1998). UV-A is less damaging than is UV-B and since it is not attenuated by ozone, the level of UV-A reaching the earth's surface is independent of ozone concentration (Madronich et al., 1998; Caldwell et al., 1989).

The UV-B portion of sunlight has received much attention in recent years, because radiation from this spectral region increases due to the stratospheric ozone depletion, which results from increases of chlorofluorocarbons in the atmosphere (Gleason et al., 1993; McFarland and Kaye, 1992; Blumthaler and Amback, 1990; Caldwell et al., 1989). Since 1980, biologically effective erythemal levels of UV-B have increased 4–7% at mid-latitudes, and 130 and 22% during springtime in the Antarctic and Arctic, respectively (Madronich et al., 1998). Ozone-depleting chemicals are likely to reach their peak over the past few years due to the Montreal Protocol, and ozone levels have been projected to return to pre-1980 levels by the middle of this century (Schrope, 2000). However, many factors, including rising concentrations of greenhouse gases, could delay this return (Newman et al., 2001; Montzka et al., 1999, Shindell et al., 1998). Although UV-B

radiation has important regulatory and photomorphogenic roles (Ballare et al., 1995), excessive UV-B radiation is clearly harmful (Yannarelli et al., 2006a; Bray and West, 2005; Ruhland et al., 2005).

1.1.1. Experimental protocols

Many early studies examining UV-B effects on terrestrial plants were conducted indoors using growth chambers or greenhouses, in which plants were exposed to unnaturally high UV-B from lamps against a background of low UV-A and photosynthetically active radiation (PAR, 400–700 nm). By the 1990s it was widely accepted that UV-B effects on plants were typically exaggerated under this condition because of unnaturally high ratios of UV-B/UV-A and UV-B/PAR, and extrapolation to field responses was questioned (Krizek, 2004; Caldwell and Flint, 1997, 1994; Caldwell et al., 1994). Therefore, outdoor studies, that use visible background irradiance provided by sunlight, are necessary to realistically evaluate the biological effects of solar UV-B radiation. The two most widely used approaches in outdoor studies are the attenuation approach and the enhancement approach.

The attenuation approach uses sheet filters that either absorb or transmit most of UV-B, typically providing a sub-ambient and near-ambient UV-B treatment, respectively. Advantages of this approach are its low cost, simplicity, and lack of the need for electricity, thus they are very portable. A disadvantage is that this method typically only tests the effects of ambient solar UV-B exposure, rather than above-ambient UV-B levels that result from ozone depletion. In order for the results to be used to predict the consequences of ozone depletion, one must assume that responses are linear over a wide

range of UV-B levels. This assumption has not been well tested, although responses are likely to be nonlinear (Ballare et al., 1996). These nonlinear responses make the extrapolation of findings from attenuation studies to ozone depletion scenarios problematic.

The enhancement studies supplement solar UV-B with fluorescent lamps to mimic future UV-B enhancements due to ozone depletion. Most of the enhanced UV-B studies in the field have used square-wave (SQW) delivery systems that provide supplemental UV-B radiation at fixed dose-rates for a certain number of hours centered around solar noon. These systems provide an addition based on the modeled consequences of ozone depletion under clear sky conditions and do not take into account cloud cover. These exposure systems, if not carefully monitored, can lead to greater UV-B supplementation with respect to ozone depletion than intended, and to substantially higher ratios of UV-B to UV-A, and PAR than those that would occur due to ozone depletion (Fiscus and Booker, 1995; Sullivan et al., 1994). These ratios are particularly important in determining how sensitive plants respond to changes in UV-B (Krizek, 2004; Caldwell et al., 1994). The problem can be addressed to some extent by using a step-wise delivery system, in which UV-B output from lamps increases through the morning and then declines after solar noon. In combination with switching lamps off during completely overcast days, this step-wise approach can provide UV-B enhancements similar to those provided by more costly modulated systems (Musil et al., 2002; Sullivan et al., 1994). However, spectral models, estimating the required UV-B supplements for specified geographic locations, times of the year and ozone thickness, may over estimate the supplementary UV-B for a given ozone depletion scenario.

Modulated (MOD) field radiation systems have also been developed and employed by a few laboratories in which UV-B supplements are supplied as proportional increments to constantly monitored background UV-B conditions (Diaz et al., 2006; Bassman et al., 2002; McLeod, 1997; Sullivan et al., 1994; Caldwell et al., 1983). These delivery systems compensate not only for changes in solar radiation due to solar angle, cloud cover, and other atmospheric conditions, but also for factors such as filter photodegradation, lamp aging and temperature, by maintaining proportional supplements to background UV-B. Unfortunately, modulated systems are technically more complex and expensive than SQW systems and have not been widely used.

Whereas the outdoor enhancement approach can examine responses to above-ambient UV-B levels, the UV spectral output from these lamps does not match the spectral enhancement found in sunlight with ozone depletion. In addition to UV-B, fluorescent lamps also produce some UV-A radiation, which is little modified by ozone, so issues of spectral balance must be considered. Although the supplemental UV-A radiation from lamps forms only a small percentage of the solar UV-A, it may have significant effects on plant growth and thus supplemental studies need to include proper controls for UV-A (Heijari et al., 2006; Newsham et al., 1996; Middleton and Teramura, 1993).

1.1.2. UV-B effects on plants

1.1.2.1. Photosynthesis

Many indoor studies have shown that UV-B can impair all of the three main processes of photosynthesis: the photophosphorylation reaction, the CO_2 fixation reactions and stomatal control of CO_2 supply, with photosystem (PS) II appearing to be particularly sensitive (Allen et al., 1998; Teramura and Sullivan, 1994). These studies utilized high UV-B level and low levels of UV-A and PAR, and are useful to identify the potential mechanisms by which UV-B can affect photosynthesis. However, it may be misleading to use such data to predict plant responses to enhanced UV-B due to ozone depletion in field conditions where fewer reports of UV-B damage to photosynthesis exist.

Some studies utilizing filters to compare near ambient and reduced UV-B radiation indicate that current ambient UV-B inhibit photosynthesis (Albert et al., 2005; Ruhland et al., 2005; Xiong et al., 2002; Kolb et al., 2001; Xiong and Day, 2001; Krause et al., 1999; Lingakumar et al., 1999). The impairments in photosynthesis in the upper mesophyll has been associated with enzymatic, rather than PS II, limitations (Xiong and Day, 2001). Other studies indicate that the inhibition of biomass accumulation in response to UV-B primarily is due to reductions in leaf area but not damage to photosynthesis (Xiong and Day, 2001; Ballare et al., 1996; Sullivan, 1994; Teramura and Sullivan, 1994). Xiong and Day (2001) found that photosynthetic function in the upper mesophyll was impaired by ambient UV-B, but this impairment was apparently compensated for by thicker leaves, such that gas-exchange rates per unit leaf area were

not compromised. Also, Bredahl et al. (2004) found that ambient UV-B decreased the maximal photochemical efficiency, while net assimilation was not affected. However, since the response to UV-B is generally nonlinear, exclusion studies may not be reliable estimates of the impact of enhanced UV-B on photosynthesis due to ozone depletion.

The majority of field experiments examining the effect of enhanced UV-B on photosynthesis used a SQW UV-B system (Day and Neale, 2002). Although inhibition of photosynthesis by enhanced UV-B radiation has been observed in many field SQW experiments (Yao and Liu, 2007; Feng et al., 2003; Keiller et al., 2003; Keiller and Holmes, 2001), there are still studies demonstrating no inhibition in photosynthesis (Xu and Qiu, 2007; Shi et al., 2004; Sullivan et al., 2003a; Ziska et al., 1993). However, Keiller and Holmes (2001) found that UV-B enhancements over five years led to inhibition of carbon assimilation in five tree species, in the absence of any apparent PS II damage. Many MOD UV-B experiments indicate that enhanced UV-B have no effects on photosynthesis (Bassman and Robberecht, 2006; Heijari et al., 2006; Bassman et al., 2002; Warren et al., 2002a; Allen et al., 1999; Stephen et al., 1999; Mepsted et al., 1996; Caldwell et al., 1994; Barnes et al., 1988; Beyschlag et al., 1988; Flint et al., 1985). Caldwell and Flint (1994) concluded that reports of morphological changes (especially reduced leaf area) and reduced growth, in response to elevated UV-B, were more common than those that found reduced photosynthesis. Generally photosynthetic gas-exchange rates are usually unaffected by enhanced UV-B based on leaf area (Shi et al., 2004; Searles et al., 2001; Allen et al., 1998; Mepsted et al., 1996; Fiscus and Booker, 1995).

Using meta-analysis, Caldwell et al. (2003) and Searles et al. (2001) found that enhanced UV-B in the field could affect shoot mass, plant height and leaf area, while contents of chlorophyll and carotenoid, net photosynthesis, and PS II activity were not significantly affected. Also, changes to the irradiative environment associated with stratospheric O_3 depletion did not affect the chlorophylls and photochemical yield in two Antarctic bryophytes (Newsham et al., 2002).

1.1.2.2. Secondary metabolism

Foliar concentrations of UV-B absorbing compounds commonly increase in response to UV-B exposure. In the meta-analysis above, Searles et al. (2001) found that increases in foliar concentrations of UV-B-absorbing compounds were the most consistent response to UV-B supplements. Concentrations of these pigments were also significantly positively associated with O_3-dependent irradiance parameters in two Antarctic bryophytes (Newsham et al., 2002). Also, UV-B can alter the foliar flavonoid composition (Martz et al., 2007; Reifenrath and Müller, 2007; Keski-Saari et al., 2005; Turtola et al., 2005; Warren et al., 2002b; Tegelberg et al., 2001), but studies on qualitative changes in flavonoids have not been very common. Several enzymes involved in UV-B absorbing compound synthesis, such as phenylalanine ammonia lyase, chalcone synthase, chalcone isomerase, and flavonoid 3′ hydroxylase, are stimulated by UV-B (Fujibe et al., 2004; Ryan et al., 2002).

Increases in UV-B absorbing compounds appeared to protect DNA in *Arabidopsis* (Fujibe et al., 2004) and maize (Stapleton and Walbot, 1994), and toreduce the sensitivity of PSII to UV-B in grape (Kolb et al., 2001), *Arabidopsis* (Fujibe et al., 2004; Rao and

Ormrod, 1995), and rye (Tevini et al., 1991). Soybean cultivars with higher constitutive concentrations of these compounds experienced less DNA damage and biomass reduction upon UV-B exposure (Mazza et al., 2000; D'Surney et al., 1993). Many studies have looked at UV-B sensitivity in various mutants to test if UV-B absorbing compounds can provide protection against UV-B radiation. Mutants with genetic blocks in phenolic synthesis have been shown to exhibit increased sensitivity to UV-B in comparison with the wild type of that species (Booij-James et al., 2000; Reuber et al., 1996; Landry et al., 1995; Rao and Ormrod, 1995; Lois and Buchanan, 1994; Stapleton and Walbot, 1994; Li et al., 1993; Middleton and Teramura, 1993). Also other mutants with elevated accumulation of UV-B absorbing compounds display a remarkable tolerance to UV-B (Bieza and Lois, 2001; Jin et al., 2000). Hence, it is well established that UV-B absorbing compounds do afford the plant protection against UV-B radiation.

Higher concentrations of UV-B absorbing compounds are usually inferred to reduce epidermal transmittance and provide selective sunscreen protection to targets in the mesophyll, because these compounds absorb effectively in the UV-B region, show little absorption in the visible region and are located predominantly in the vacuoles of epidermal cells (Markstädter et al., 2001; Schmelzer et al., 1988; Robberecht and Caldwell, 1983). A considerable body of evidence has accumulated implicating UV-B absorbing compounds as plant sunscreens. Strong negative correlations between concentrations of soluble UV-B absorbing compounds and epidermal UV-B transmittance have been documented in both field and indoors experiments (Bieza and Lois, 2001; Bilger et al., 2001; Kolb et al., 2001; Markstädter et al., 2001; Barnes et al., 2000; Burchard et al., 2000; Mazza et al., 2000; Olsson et al., 1999). However, the

relationship between concentrations and epidermal transmittance is complicated because of optical heterogeneity and variable distribution of these compounds (Kolb and Pfundel, 2005; Sullivan et al., 2003a; Day and Neale, 2002; Day et al., 1993).

In higher plants UV-B absorbing compounds include a large number of phenylpropanoids, with flavonoids and hydroxycinnamic acids likely to be most important in terms of UV-B sunscreen (Cockell and Knowland, 1999). There are disparate conclusions ranging from the hydroxycinnamic acid sinapoyl esters being the predominant UV-B protectant (Ruhland et al., 2005; Booij-James et al., 2000; Landry et al., 1995), to flavonoids being the predominant UV-B protectant (Hofmann et al., 2003; Markstädter et al., 2001; Ryan et al., 1998), to both the flavonoids and sinapoyl esters providing protection (Bieza and Lois, 2001; Li et al., 1993). The importance of these compounds likely is relational to their relative concentrations, leaf developmental stage, and localization within the leaf (Kliebenstein, 2004; Burchard et al., 2000; Day et al., 1996). In *Arabidopsis* the hydroxycinnamic acids are the most abundant phenylpropanoid and provide the most protection (Booij-James et al., 2000; Landry et al., 1995), while in maize and barley, flavonoids are the most abundant and provide the most effective protection (Reuber et al., 1996; Stapleton and Walbot, 1994). Burchard et al. (2000) found that in early stages of development the epidermal hydroxycinnamates are the dominant UV-B protective compounds in the rye primary leaves, and this function is increasingly replaced by the epidermal flavonoids during leaf maturation and acclimation.

Although the sunscreen role of phenylpropanoids against UV-B has been commonly reported, this does not rule out other protective functions of these compounds.

Anthocyanins and flavonoids act not only as UV filters, but also as active oxygen scavengers (Peng et al., 2003; Gould et al., 2002). Several reports indicate a shift from B-ring mono-hydroxylated flavonoids (kaempferol) towards their ortho-dihydroxylated equivalents (quercetin) under UV-B in growth chambers (Hofmann et al., 2003, 2000; Markham et al., 1998a), greenhouses (Olsson et al., 1999) and field conditions (Reifenrath and Müller, 2007; Ryan et al., 2002, 1998). These two flavonols differ only in the degree of hydroxylation on the B-ring of the flavonoid skeleton, with quercetin being dihydroxylated and kaempferol monohydroxylated. The dihydroxylated compounds are no more efficient as UV-B absorbers than their monohydroxylated equivalents. However, quercetin may confer better UV-B protection (Keski-Saari et al., 2005; Hofmann et al., 2003, 2000; Ryan et al., 1998), because ortho-dihydroxylated flavonoids are more effective antioxidants than their monohydroxylated equivalents (Montesinos et al., 1995), and also are better able to dissipate absorbed UV energy (Markham et al., 1998b; Smith and Markham, 1998). Interestingly, quercetin accumulation has been observed as a response to other stresses, such as heavy metal pollution (Loponen et al., 1998), nitrogen deficiency (Bongue and Phillips, 1995) and paraquat application (Steger-Hartmann et al., 1994). This underscores a prominent role for ortho-dihydroxylated flavonol accumulation in the response of plants to environmental stress. Also UV-B induced flavonoid can affect auxin polar transport and catabolism which has been linked to UV-B tolerance (Jansen, 2002; Jansen et al., 2001; Jacobs and Rubery, 1988).

Additionally, UV-B-induced accumulation of foliar phenylpropanoids and related phenolics act as anti-herbivore compounds (Caputo et al., 2006; Rousseaux et al., 2004;

Warren et al., 2002a) and affect decomposition (Pancotto et al., 2005; Sullivan, 2005). Therefore, UV-B radiation effects at the subcellular level could be translated into significant effects at higher trophic levels within ecosystems (Caputo et al., 2006; Sullivan, 2005; Warren et al., 2002a).

1.1.2.3. DNA damage

DNA is a potentially sensitive target molecule for UV-B, because it absorbs UV-B efficiently and undergoes phototransformations that lead to the formation of the cyclobutane pyrimidine dimer (CPD) (Dany et al., 2001; Mitchell et al., 1990), and the pyrimidine (6-4) pyrimidinone photoproduct (6-4 product). Many studies indicate that UV-B radiation can induce both CPD and 6-4 product dimers in higher plants (Bray and West, 2005). In indoor experiments, artificial UV-B caused DNA dimers in duckweed (Jiang et al., 2007), rice (Hidema et al., 2005; Hada et al., 2003; Hidema and Kumagai, 1998), *Arabidopsis* (Fujibe et al., 2004; Tanaka et al., 2002; Dany et al., 2001), alfalfa (Quaite et al., 1992), *Spinacia oleracea* (Hada et al., 1998), wheat (Taylor et al., 1996), cucumber (Takeuchi et al., 1996a), *Pisum sativum* (Kalbin et al., 2001), *G. magellanica* (Giordano et al., 2004), *Phaseolus vulgaris* (Kucere et al., 2003), and maize (Stapleton and Walbot, 1994). In field studies, supplemental UV-B also was shown to increase the CPD level in rice (Hidema et al., 1999) and soybean (D'Surney et al., 1993). Even ambient solar UV-B caused DNA damage in *G. magellanica* (Giordano et al., 2003; Ballare et al., 2001; Rousseaux et al., 1999), barley (Sullivan et al., 2003b; Mazza et al., 1999), maize (Stapleton et al., 1997), soybean (Mazza et al., 2000) and *Datura ferox* (Ballare et al., 1996).

The biological effects of pyrimidine dimers have been extensively studied in microbes and mammals. CPDs have been reported to inhibit the progress of DNA polymerases. Mammalian RNA polymerase II has been reported to stall at both CPDs and 6–4 products (Mitchell et al., 1989; Protic-Sabljic and Kraemer, 1986). If unrepaired, a single CPD is potentially sufficient to completely eliminate expression of a transcriptional unit. There is evidence that the stalled RNA polymerase II remains bound to the site of the obstruction (Donahue et al., 1994). Thus persisting lesions may actually reduce the overall concentration of free RNA polymerase, in addition to eliminating transcription of the gene in which they are located. Every pyrimidine dimer acts as a block to transcription and replication. Therefore, these DNA lesions, if unrepaired, may interfere with DNA transcription and replication, and can lead to misreading of the genetic code and cause mutations, growth-inhibition, and potential death (Giordano et al., 2004; Jiang et al., 1997).

Although it is clear that UV-B causes DNA damage, there is no evidence to support that this results in any damage at the physiological or whole plant level. Therefore, plants have highly evolved DNA repair mechanisms. DNA repair systems in higher plants broadly include two categories: photoreactivation (photorepair) and excision repair (dark repair) (Bray and West, 2005). In photorepair, the photolyase enzyme binds to a dimer to form a complex that is stable in the absence of light. When a photon in the wavelength range 300-600 nm is absorbed, the dimer is reversed to monomer pyrimidines and the enzyme is released (Sancar, 2003, 1994). In dark repair, dimers are replaced by *de novo* synthesis in which the undamaged complementary strand is employed as a template. Photorepair is the major pathway in plants for repairing UV-

induced DNA damage (Britt, 1999, 1996). In many plants, there are at least two distinct photolyases: one specific for CPD and the other specific for 6-4 product (Waterworth et al., 2002; Nakajima et al., 1998; Takeuchi et al., 1996a; Taylor et al., 1996; Chen et al., 1994).

UV-B and UV-A radiation can increase photolyase activity (Giordano et al., 2003; Waterworth et al., 2002; Pang and Hays, 1991). Therefore, the ability to perform photorepair may be a crucial factor for the ability of plants to withstand UV-B radiation. In rice, cultivars more resistant to UV-B were found to exhibit higher photolyase activities than less resistant cultivars (Hidema and Kumagai, 2006, 1998; Hidema et al., 2005; Teranishi et al., 2004; Hidema et al., 2000). *Arabidopsis* mutants which are defective in photolyase show higher sensitivity to UV-B (Britt and Fiscus, 2003; Jiang et al., 1997; Landry et al., 1997; Britt et al., 1993), whereas mutants with increased photoreactivition of CPD and 6-4 product are resistant to UV-B radiation (Tanaka et al., 2002). Hidema et al. (2005) carried out quantitative trait loci (QTL) analysis to test the linkage between CPD photolyase and UV-B sensitivity, and found that UV-B sensitivity is a quantitative inherited trait, and the tight linkage of the CPD photolyase locus in a QTL explains a major portion of the genetic variation for this trait. Ueda et al. (2005) reported that *qUVR-10*, which showed the largest allelic difference among the QTLs associated with UV-B resistance, encoded CPD photolyase. These findings suggest that CPD photolyase activity is a crucial factor in the determination of UV-B sensitivity.

1.2. AOS METABOLISM

The term active oxygen species (AOS) is generic, embracing not only free radicals such as superoxide (O_2^-) and hydroxyl radicals ($^{\cdot}OH$), but also hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). While it is generally assumed that the hydroxyl radical and singlet oxygen are so reactive that their production must be minimized, superoxide and peroxide are synthesized at very high rates even under optimal conditions. A regulated balance between AOS production and destruction is required if metabolic efficiency and function are to be maintained in both optimal and stress conditions.

There is a wide array of sources of AOS in plants. The electron transport chains of the chloroplast and mitochondria are two important sources of AOS. In the chloroplast, environmental stress may limit CO_2 fixation and reduce the $NADP^+$ regeneration by the Calvin cycle. In this case, the photosynthetic electron transport chain is overreduced, which leads to the formation of superoxide radicals and singlet oxygen (Asada, 1999; Foyer et al., 1994ab). The dismutation of superoxide radicals generates H_2O_2 spontaneously but the velocity of this reaction is greatly increased by superoxide dismutase (SOD) (Bowler et al., 1992). In the mitochondria the electron transport chain may also become overreduced under stress, generating O_2^-. In the peroxisomes, H_2O_2 is formed as a byproduct of both the photorespiratory pathway and β-oxidation of fatty acids. Also in the peroxisomes the oxidation of xanthine to uric acid by xanthine oxidase generates O_2^-, whereas uric acid is oxidized to allantoin, forming H_2O_2. In the cytoplasm and the endoplasmic reticulum, during the detoxification reactions catalysed by the cytochromes, electron leakage to oxygen and the decomposition of the intermediate oxygenate of cytochrome P_{450} can form O_2^-. AOS can also be generated at the plasma

membrane level by NADPH oxidase or extracellularly in the apoplast catalysed by enzymes such as cell wall peroxidase, oxalate oxidase and amine oxidase (Blokhina et al., 2003; Vranova et al., 2002).

AOS can result in damage to a variety of metabolic processes. For example, several Calvin-cycle enzymes within the chloroplast are extremely sensitive to H_2O_2 (Kaiser, 1979). Also superoxide and H_2O_2 can react in the Harber-Weiss reaction to generate the $^{\cdot}OH$ radical, which is the most potent oxidant known. The hydroxyl radicals can rapidly attack all macromoleculars leading to irreparable metabolic dysfunction and cell death (Scandalios, 1993). Therefore to keep this damage to a minimum, plants possess enzymatic and nonenzymatic antioxidantive defense systems. Among the latter are reduced glutathione (GSH), ascorbate, α-tocopherol, carotenoids, flavonoids, mannitol, hydroquinones (Larson, 1988; Ames, 1983). The enzymatic antioxidants include enzymes such as SOD, catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR) and the enzymes involved in the synthesis and regeneration of the low molecular mass antioxidants.

In the chloroplast, efficient destruction of O_2^- and H_2O_2 requires the action of several antioxidant enzymes. Superoxide is rapidly converted to H_2O_2 by the action of SOD, or reduced by ascorbate (Noctor and Foyer, 1998). Dismutation of O_2^- simply converts one destructive AOS to another. H_2O_2 is a strong oxidant that rapidly oxidizes thiol groups. Catalase can convert H_2O_2 to water and oxygen, but it is found predominantly in the peroxisomes, where it functions chiefly to remove the H_2O_2 formed during photorespiration and β-oxidation of fatty acids. However it has low substrate

affinity and is susceptible to photoinactivation and degradation. Even so, it is essential for the destruction of photorespiratory H_2O_2 in C_3 plants. In C_4 plants, where photorespiration is greatly reduced, CAT may not be important. An alternative mode of H_2O_2 destruction is via peroxidase which is found throughout the cell (Jimenez et al., 1997; Yamaguchi et al., 1995), and has a much higher affinity for H_2O_2 than CAT. In plant cells, the most important reducing substrate for H_2O_2 reduction is ascorbate (Mehlorn et al., 1996; Nakano and Asada, 1987). Ascorbate peroxidase uses two molecules of ascorbate to reduce H_2O_2 to water, with the generation of two molecules of monodehydroascorbate (MDHA); MDHA can be reduced to ascorbate, catalyzed by MDHAR, and ascorbate can also be nonenzymatically regenerated from MDHA. Dehydroascorbate (DHA) is always produced during the rapid disproportionation of the MDHA radical, DHA is reduced to ascorbate by the action of DHAR using GSH as the reducing substrate and generating glutathione disulphide (GSSG), which is reduced to GSH by GR. The removal of H_2O_2 through this series of reactions is known as the ascorbate-glutathione cycle (Noctor and Foyer, 1998). It is generally assumed that this cycle is mainly responsible for the scavenging of AOS, especially in the chloroplast, although Polle (2001) and Morell et al. (1997) suppose the coupling between AsA- and GSH-related redox systems is weak.

Oxidative stress is induced by a wide range of stress factors including oxygen deprivation (Blokhina et al., 2003), low temperature (Suzuki and Mittler, 2006), toxic metals (Rodriguez-Serrano et al., 2006; Romero-Puertas et al., 2002), high salinity (Badawi et al., 2004), herbicides (Ekmekci and Terzioglu, 2005; Lascano et al., 2003), drought (Badawi et al., 2004; Tambussi et al., 2000), wounding (Grantz et al., 1995), SO_2

fumigation (Kubo et al.,1995), and ozone exposure (Baier et al., 2005). Also increasing evidence suggests that AOS are involved in the damage caused by UV-B radiation. UV-B radiation can increase AOS levels (Yao and Liu, 2007; Jovanovic et al., 2006; Kalbina and Strid, 2006; Wang et al., 2006; Prasad et al., 2005; Hideg et al., 2003) and lipid peroxidation (Yao and Liu, 2007; Jovanovic et al., 2006; Yannarelli et al., 2006a; Prasad et al., 2005; Yang et al., 2005) in plants. There is also evidence indicating that UV-B radiation has an impact on antioxidants (Agrawal and Rathore, 2007; Yao and Liu, 2007; Jovanovic et al., 2006; Ren et al., 2006; Yannarelli et al., 2006a; Prasad et al., 2005; Giordano et al., 2004; Galatro et al., 2001). However, most of these experiments were conducted indoors using growth chambers or greenhouses in which plants were exposed to unrealistically high UV-B radiation compared to low levels of UV-A and PAR. Very few studies on the impacts of solar UV-B radiation on the AOS metabolism have been conducted under natural conditions (Agrawal and Rathore, 2007; Mazza et al., 1999, Taulavuori et al., 1998). So it is important to make an extensive investigation of various antioxidants after realistic UV-B exposure and to assess their contributions to defense systems under natural conditions.

1.3. PROTEOMIC ANALYSIS

The term proteome is used to describe the complete set of proteins expressed by the entire genome in the life of a tissue, cell or subcellular compartment (Abbott, 1999). With the accumulation of vast amounts of DNA sequences in the database, researchers are realizing that merely having complete sequences of genomes is not sufficient to elucidate biological function. The information on amino acid sequences of known

proteins in the database does not match the wealth of information on nucleotide sequences being generated through plant genome projects. The 'microarray chip' technology is a possible solution, but for a substantial number of proteins there may be only a loose correlation between mRNA and protein levels. Post-translational modification, protein-protein interaction, protein localization, and the molecular composition of cellular structures such as organelles can be determined only at the protein level (Pandey and Mann, 2000).

The two key steps in classical proteomics are the separation of proteins and the subsequent protein identification. The best separation method is two-dimensional (2D) gel electrophoresis, in which the proteins are separated according to isoeletric point (pI) using isoelectric focusing (IEF) in the first dimension, then separated according to molecular weight (Mr) using SDS polyacrylamide gel electrophoresis (PAGE) in the second dimension. Once proteins have been separated, they can be identified by mass spectrometry (MS). MS has essentially replaced the classical technique of Edman degradation in protein identification because it is more sensitive, can deal with protein mixtures, and offers much higher throughput. The most commonly used mass spectrometry is matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS and liquid chromatography MS (LC-MS/MS). Although MALDI-TOF MS analysis is easy to automate and allows high throughput analysis, protein identification relies solely on the accurate matching of the peptide mass. Therefore it is very difficult to correctly identify proteins if there is a protein mixture. Also the database must contain enough of the protein sequence to compare with the experimentally derived mass map. The LC-MS/MS method is technically more complex and less scalable than MALDI MS

fingerprinting. Its main advantage is that sequence information derived from several peptides is much more specific for the protein than a list of peptide masses. A short region of local identity which spans two or more consecutive tryptic cleavage sites may enable identification, even when the remainder of the sequence is divergent (Newton et al., 2004; Pandey and Mann, 2000). In addition, the fragmentation data can be used to search nucleotide databases such as db EST as well as the protein databases.

Recently researchers have reported on subcellular proteomes such as chloroplast (Wijk, 2000) and mitochondria (Kruft et al., 2001; Millar et al., 2001), whereas others have focused on single tissues including *Arabidopsis* seeds (Gallardo et al., 2001), maize root (Chang et al., 2000) and leaves (Porubleva et al., 2001), and barrel medic roots (Mathesius et al., 2002, 2001). Also some researchers have worked on a large-scale project to identify protein from multiple tissues of the barrel medic (Watson et al., 2003) and rice (Komatsu et al., 2004). So far, no protein reference map has been reported for soybean leaves to our knowledge. Proteomic analysis also provides a broad view of plant responses to stress at the protein level. In recent years this approach has been successfully applied to study, at the protein expression level, the effects of drought (Pinheiro et al., 2005), nutrient deficiency (Alves et al., 2006), temperature (Yan et al., 2006; Sule et al., 2004), oxidative stress (Wang et al., 2004), herbicide (Castro et al., 2005), wound (Shen et al., 2003), anoxia (Chang et al., 2000), salt (Yan et al., 2005), and heavy metal (Labra et al., 2006). Also, using proteomic technologies Casati et al. (2005) analyzed the proteome of maize leaves after exposure to UV-B radiation. They found that UV-B radiation altered the accumulation of 178 protein spots and phosphorylated

pyruvate phosphate dikinase. However this represents one of the few studies on the proteomic response to solar UV-B.

1.4. SOYBEAN

Soybeans (*Glycine max* [L.] Merr.) are the most important grain legume crop grown in the U.S. Soybean provides an important source of proteins and bioactive isoflavones for human foods and the animal industry, and has been the dominant oilseed produced since the 1960s. Soybean seeds contain 40 to 55% of protein on a dry weight basis and are an important source of edible vegetable oil throughout the world (Smith and Huyser, 1987; Coates et al., 1985).

Due to its economic importance, there has been considerable research on the effects of UV-B on soybean. Soybean genotypes exhibit a wide range in sensitivity to UV-B radiation, due in part to differences in flavonoid contents (Yanqun et al., 2003; Reed et al., 1992). In sensitive cultivars UV-B reduced photosynthesis and growth (Yao et al., 2006; Feng et al., 2003; Middleton and Teramura, 1994, 1993; Teramura and Sullivan, 1990), and increased the amount of UV-B absorbing compounds (Sullivan et al., 2007; Mazza et al., 2000; Middleton and Teramura, 1994, 1993; Teramura and Sullivan, 1990; Murali et al., 1988; Mirecki and Teramura, 1984). Exposure to UV-B also induced oxidative stress (Yannarelli et al., 2006a; Yao et al., 2006; Galatro et al., 2001; Malanga et al., 1999) in some cultivars, but most of these experiments were conducted indoors so it is not clear to what extent UV-B induces greater oxidative stress in the field. There has been very limited research on the effects of UV-B at the protein

level in soybean (Murali et al., 1988) and no comprehensive assessment of the response of soybean proteins to UV-B has been attempted.

The purpose of this study was to investigate the effects of solar UV-B on AOS metabolism and the proteome in the soybean leaf under natural conditions. Secondly, in order to examine the protective role of flavonoids against solar UV-B, two isolines of the soybean cultivar Clark with different flavonoid contents were grown in the field with or without natural levels of UV-B. The results of this research will add to our knowledge on the response of soybean to UV-B by providing proteomic and oxidative responses to solar UV-B under field conditions.

CHAPTER 2 IMPACT OF SOLAR ULTRAVIOLET-B ON ANTIOXIDANT DEFENSE SYSTEM IN SOYBEAN LINES DIFFERING IN FLAVONOID CONTENTS

ABSTRACT

Exposure to ultraviolet-B (UV-B) radiation can lead to oxidative damage in plants. However, UV protection may be enhanced by increased production or activities of antioxidants. Two isolines of soybean Clark cultivar, the standard line with moderate levels of flavonoids and the magenta line with reduced flavonoid levels, were grown in the field with or without natural levels of UV-B. The first trifoliates were harvested after a 4-12 day exposure to the experimental conditions for analysis of active oxygen species (AOS) metabolism. Solar UV-B radiation caused oxidative stress in both lines, and altered the AOS metabolism primarily by decreasing superoxide dismutase activity and increasing the activities of ascorbate peroxidase, catalase, and glutathione reductase. This resulted in decreased ascorbic acid and increased dehydroascorbate. The magenta line had greater oxidative stress than the standard line in spite of its enhanced oxidative defense capacity, even under UV-B exclusion. The enhanced responses in the magenta line, especially under UV-B exclusion, may be due to increased penetration of solar ultraviolet-A and/or oxidative stress caused by other factors.

2.1. INTRODUCTION

Increases in chlorofluorocarbons in the atmosphere have depleted the earth's stratospheric ozone layer, leading to an increase in ultraviolet-B (UV-B: 280-320 nm) radiation at the earth's surface (Madronich et al., 1998; Gleason et al., 1993; Blumthaler and Amback, 1990). Many studies have indicated deleterious effects of UV-B on plants such as reduced growth and photosynthesis (Germ et al., 2005; Ruhland et al., 2005), and damages to DNA (Bray and West, 2005). UV-B radiation can also cause oxidative stress (Yannarelli et al., 2006a; Yang et al., 2005).

However, many plants are resistant to UV-B radiation and possess a number of UV-B protection mechanisms. One of the most important mechanisms is screening out UV-B radiation by accumulation of flavonoids in the leaf epidermis (Markstädter et al., 2001; Schmelzer et al., 1988; Robberecht and Caldwell, 1983). Other mechanisms that have received less attention than epidermal screening mechanisms are enzymatic and nonenzymatic antioxidantive defense systems that may mitigate UV-induced damage due to production of active oxygen species (AOS).

The AOS include not only free radicals such as superoxide (O_2^-) and hydroxyl radicals ($^{\cdot}OH$), but also hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). These AOS can cause oxidative damage to membrane lipids, nucleic acids, and proteins (Foyer et al., 1994b). To keep this damage to a minimum, plants possess enzymatic and nonenzymatic antioxidative defense systems. Among the latter are reduced glutathione (GSH), ascorbic acid (AsA), α-tocopherol, carotenoids, flavonoids, mannitol and hydroquinones (Larson, 1988). The enzymatic antioxidants include enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), guaiacol peroxidase (POD; EC 1.11.1.7),

ascorbate peroxidase (APX; EC 1.11.1.11), glutathione reductase (GR; EC 1.6.4.2), dehydroascorbate reductase (DHAR; EC 1.8.5.1), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) and others. O_2^- is rapidly converted to H_2O_2 by the action of SOD (Noctor and Foyer, 1998). CAT can convert H_2O_2 to water and oxygen, but it is found predominantly in the peroxisomes, and has low substrate affinity. An alternative mode of H_2O_2 destruction is via peroxidase which is found throughout the cell (Jimenez et al., 1997). APX uses two molecules of AsA to reduce H_2O_2 to water, with the generation of two molecules of monodehydroascorbate (MDHA); MDHA can be reduced to AsA, catalyzed by MDHAR, and AsA can also be nonenzymatically regenerated from MDHA. Dehydroascorbate (DHA) is always produced during the rapid disproportionation of the MDHA radical, DHA is reduced to AsA by the action of DHAR using GSH as the reducing substrate and generating glutathione disulphide (GSSG), which is reduced to GSH by GR. The removal of H_2O_2 through this series of reactions is known as the ascorbate-glutathione cycle (Noctor and Foyer, 1998).

Increasing evidence suggests that AOS are involved in the damage caused by UV-B radiation. UV-B radiation can increase AOS levels (Yao and Liu, 2007; Jovanovic et al., 2006; Kalbina and Strid, 2006; Wang et al., 2006; Prasad et al., 2005; Hideg et al., 2003) and lipid peroxidation (Yao and Liu, 2007; Jovanovic et al., 2006; Yannarelli et al., 2006a; Prasad et al., 2005; Yang et al., 2005) in plants. Although it is not known how plants irradiated with UV-B generate AOS, it is thought that NADPH oxidase may be involved in the generation (Rao et al., 1996). There is also evidence indicating that UV-B radiation has an impact on the non-enzymatic antioxidants such as AsA (Prasad et al., 2005; Giordano et al., 2004; Galatro et al., 2001), GSH (Galatro et al., 2001; Kalbin et

al., 1997), and α-tocoperol (Carletti et al., 2003; DeLong and Steffen, 1998). Studies on the effects of UV-B on the enzymatic antioxidants at both the activity level (Agrawal and Rathore, 2007; Yao and Liu, 2007; Jovanovic et al., 2006; Ren et al., 2006; Yannarelli et al., 2006a; Prasad et al., 2005) and the mRNA level (Zinser et al., 2007; Willekens et al., 1994) have yielded inconsistent results. It is not clear whether this is due to differential responses among species or to differences in experimental conditions. Most of these experiments were conducted indoors using growth chambers or greenhouses in which plants were exposed to unrealistically high UV-B radiation and low ultraviolet-A (UV-A: 320-400 nm) and photosythetically active radiation (PAR: 400-700 nm). Responses of plants in controlled conditions may differ from those in the field conditions, because of the different level of UV-A and PAR (Krizek, 2004; Caldwell et al., 2003). Very few studies on the impacts of solar UV-B radiation on AOS metabolism have been conducted under natural environmental conditions (Agrawal and Rathore, 2007; Yao and Liu, 2007; Mazza et al., 1999; Taulavuori et al., 1998). It is important to make an extensive investigation of various antioxidants after UV-B exposure and to assess their contributions to defense systems under natural conditions.

Soybean (*Glycine max* [L.] Merr.) exhibits a wide range in sensitivity to UV-B radiation, due in part to differences in flavonoid contents (Reed et al., 1992). In addition to epidermal screening of potentially damaging UV-B radiation, flavonoids may also contribute to UV protection by scavenging of AOS. However, the role that flavonoids play in protection against AOS damage has been studied only under artificial condition in *Arabidopsis* (Rao et al., 1996; Landry et al., 1995; Rao and Ormrod, 1995). Isolines of soybean that lack flavonoids provide a valuable tool for evaluating the protection role of

flavonoids. The purpose of this UV-B exclusion study was to examine the effects of solar UV-B radiation on AOS metabolism and to investigate whether flavonoids afforded protection against oxidative stress caused by solar UV-B radiation under field conditions.

2.2. MATERIALS AND METHODS

2.2.1. Plant materials and experiment design

Two soybean isolines with different flavonoid contents were used: the Clark standard line which produces moderate levels of flavonol glycoside, and the Clark magenta line that has reduced flavonol glycoside levels (Buzzell et al., 1977). Seeds of the two isolines were planted in pots in the greenhouse at the University of Maryland and allowed to germinate for 3 days. Following this period the plants were moved to the USDA, Beltsville Southfarm where they were separated into two UV-B treatment regimes. Half of the plants were placed inside either of two open-ended exclusion shelters made of polyester (DuPont, Circleville, OH, USA), which absorbs almost all solar radiation below 316 nm. The second half of plants was placed under another two shelters covered by clear Teflon material (DuPont, Circleville, OH, USA), which is virtually transparent to solar UV radiation. The materials are similar in transmission properties in the UV-A and PAR wavelength. The plants beneath the polyester filters received very little UV-B radiation and served as controls for seasonal changes in temperature and PAR, etc. Plants were rotated every day and watered to minimize the occurrence of drought stress which can alter the response to UV-B radiation (Sullivan and Teramura, 1990). Plants were harvested 3 times at 4-day intervals following the

production of the first trifoliate (V1-V3 developmental stage). Leaflet blades of the first trifoliates from 3-5 plants from each replicate treatment were harvested for each sample and five independent samples were harvested for each treatment replicate. The harvested samples were frozen in liquid nitrogen immediately and then stored at -80 ^{o}C prior to analysis.

2.2.2. Determination of enzyme activities

Plant tissues were ground in a mortar with liquid nitrogen, and ice-cold extraction buffer (100 mM KH_2PO_4/K_2HPO_4, pH 7.8; 1% Triton X-100; 5 mM AsA; 1% PVP). After 15 min at 4 ^{o}C, the homogenates were centrifuged at 21,000 × g for 10 min. The supernatants were passed through a Sephadex G-25 column (PD-10) which had been equilibrated with 100 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.8 containing 5 mM AsA. The elutes were collected for enzymes activities. All assays were performed at 25 ^{o}C, had a final volume of 1 mL and were performed in triplicates. Protein concentrations were determined according to Bradford method (1976) with bovine serum albumin (BSA) as a standard.

APX activity was measured by the method of Nakano and Asada (1981). The reaction mixture contained 50 mM KH_2PO_4/K_2HPO_4 (pH 7.0), 0.25 mM AsA, 0.2 mM H_2O_2 and extract. The APX activity was determined by following the decrease in A_{290}, extinction coefficient of 2.8 $mM^{-1}cm^{-1}$ was used to calculate activity. Correction was made for the low, nonenzymatic oxidation of AsA by H_2O_2. MDHAR activity was measured by the method of Hossain et al. (1984) at 340 nm. Reaction mixture contained 50 mM Tris-HCl (pH 7.6), 0.2 mM NADH, 2.5 mM AsA, 1 unit of ascorbate oxidase and

extract. Extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ was used to calculate activity. To determine the MDHAR activity the rate of MDHA-independent NADH oxidation (without AsA and AsA oxidase) was subtracted from the MDHA-dependent oxidation rate (with AsA and AsA oxidase). DHAR activity was measured as described by Nakano and Asada (1981) at 265 nm. The assay contained 50 mM KH_2PO_4/K_2HPO_4 (pH 7.0), 2.5 mM GSH, 0.2 mM DHA, 0.1 mM EDTA and extract. Extinction coefficient of 14 $mM^{-1}cm^{-1}$ was used to calculate activity. The reaction was corrected for nonenzymatic reaction of DHA by GSH. GR activity was measured by the method of Foyer and Halliwell (1976) at 340 nm. The reaction mixture contained 50 mM HEPES-KOH (pH 8.0), 1.0 mM EDTA, 0.2 mM NADPH, 1.0 mM GSSG and extract. Reaction was started by the addition of GSSG. Extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ was used to calculate activity. Correction was made for the small, nonenzymatic oxidation of NADPH by GSSG. CAT activity was determined as H_2O_2 consuption measured as the decrease in absorbance at 240 nm according to the method of Aebi (1983). The assay contained 50 mM KH_2PO_4/K_2HPO_4 (pH 7.0), 10 mM H_2O_2 in phosphate buffer. Extinction coefficient of 39.4 $mM^{-1}cm^{-1}$ was used to calculate activity. SOD activity was measured spectrophotometrically by measuring the inhibition of O^{2-} dependent reduction of cytochrome c at 550 nm, according to the method of Tanaka and Suigahara (1980). The assay contained 50 mM KH_2PO_4/K_2HPO_4 (pH 7.8), 0.1 mM EDTA, 0.1 mM Cyt c, 0.1 mM xanthine, enzyme extract and xanthine oxidase. One unit of SOD was defined as the quantity of enzyme required to inhibit the reduction of Cyt c by 50%. Guaiacol POD activity was measured at 436 nm by the method of Nakano and Asada (1981). The assay

contained 50 mM KH_2PO_4/K_2HPO_4 (pH 5.25), 40 mM guaiacol, 10 mM H_2O_2, and the extract. The activity was calculated using an extinction coefficient of 25.5 $mM^{-1}cm^{-1}$.

2.2.3. Determination of metabolites

Samples were ground with mortar and pestle at 0 ^oC for 10 min in the presence of 5% trichloroacetic acid (TCA). The slurry was centrifuged at 16,000 g for 5 min at 4 ^oC. The supernatant was used to determine the content of thiobarbituric acid reacting substances (TBARS) or neutralized to pH 6 (for ASA) or 7 (for GSH and H_2O_2) with 4 N KOH. All assays were performed in triplicates.

H_2O_2 was determined according to the method of Okuda et al. (1991), 200 uL of the supernatant was applied to 1-mL column of anion exchange resin (AG-1, Bio-Rad), and the column was washed with 800 uL of distilled water, the eluate was used for H_2O_2 assay. The reaction mixture contained 1 mL of the elute, 400 uL of 12.5 mM 3-dimethylaminobenzic acid in 375 mM phosphate buffer (pH 6.5), 80 uL of 10 mM 3-methyl-2-benzothiazoline hydrazone and 20 ul of peroxidase (0.25 unit). After 3 min at 20 ^oC the absorbency was determined at 590 nm and compared with increases elicited by standard samples of hydrogen peroxide. The lipid peroxidation was determined by the TBARS contents according to the method of Dhindsa and Matowe (1981). The supernatant was combined with equal volume of thiobarbituric acid (TBA) reagent (0.5% TBA in 20% TCA), heated at 95 ^oC for 30 min, cooled and centrifuged. The amount of TBARS in the supernatant was determined from the difference between the absorbance at 532 nm and that at 600 nm using extinction coefficient of 155 $mM^{-1}cm^{-1}$. AsA and DHA was determined by a method of Foyer et al. (1983). AsA was measured by the change in

A_{265} following the addition of ascorbate oxidase. Sample was added to 0.1 mM phosphate buffer (pH 5.6) to make a final volume of 990 uL. The A_{265} was measured and 10 uL (4 units) of ascorbate oxidase was added. The AsA content was measured via the absorption decrease using an extinction coefficient of 14 $mM^{-1}cm^{-1}$. DHA was measured by the same method following reduction to AsA in a reaction mixture containing 20 mM dithiothreitol and 50 mM phosphate buffer (pH 7.0). Glutathione was determined according to the procedure of Anderson et al. (1992). Total glutathione was measured in reaction mixture consisting of 400 uL reagent A containing 110 mM Na_2HPO_4, 40 mM NaH_2PO_4, 15 mM EDTA, 0.3 mM 5,5'-dithiobis-(2-nitrobenzoic acid), and 0.04% BSA; 320 uL reagent B containing 1 mM EDTA, 50 mM imidazole, 0.02% BSA and an equivalent of 1.5 units GR activity; 400 uL of 1:50 dilution of the extract in 5% Na_2HPO_4 (pH 7.5) prepared immediately prior to starting the assay; and 80 uL of 9 mM NADPH to start the reaction. Change in absorbance was measured at 412 nm. For GSSG, 1 mL of the 1:50 dilution was incubated for 1 hour at 25 ^oC with 40 uL of 2-vinylpyridine. The standard curve in which the GSH equivalents present was plotted against the rate of change in A_{412}. For each sample, GSH was estimated as the difference between total glutathione and GSSG.

2.2.4. Statistical analysis

The experimental design was a split plot with the main plots being UV-B treatment, replicated twice, with subplots being the soybean lines. The General Linear Model procedure (SAS PC version 6.04, SAS Institute, Inc., Cary, NC) was used for the

different analysis of variance (ANOVA) to test for main effects of UV-B, lines, time and their interactions.

2.3. RESULTS

2.3.1. TBARS and H_2O_2 contents

During the experiment the TBARS contents were increased by solar UV-B radiation ($P = 0.0002$; Table 2.1 and Fig. 2.1A). However, the differences of TBARS contents between the two lines were not significant ($P = 0.0733$), although the magenta line had higher TBARS content than the standard line at 4 days ($P = 0.0001$). The ambient UV-B radiation had no impact on the leaf H_2O_2 content ($P = 0.2177$) but the magenta line had higher H_2O_2 content than the standard line ($P < 0.0001$; Table 2.1 and Fig 2.1B), and the H_2O_2 content decreased during the experiment.

2.3.2. AsA and glutathione contents

AsA and glutathione contents were determined to examine the effect of ambient UV-B on the water-soluble antioxidants. The DHA and total AsA contents were significantly increased by UV-B ($P < 0.0001$ and $P = 0.0069$ respectively; Table 2.1 and Fig. 2.2). However, the AsA content was decreased by UV-B ($P = 0.0002$). Therefore, the ratio of AsA/DHA was decreased after exposure to UV-B radiation ($P < 0.0001$). The magenta line had higher content of DHA and total AsA than the standard line ($P = 0.0208$ and $P = 0.0484$ respectively; Table 2.1 and Fig. 2.2). There were no differences between the two lines in the contents of AsA and the ratio of AsA/DHA.

The GSSG contents were decreased by solar UV-B radiation ($P = 0.0377$; Table 2.1 and Fig. 2.3), while the GSH and total glutathione contents were not affected by UV-B. Therefore, the ratio of GSH/GSSG was enhanced after UV-B exposure ($P = 0.027$). The magenta line has higher content of GSH and total glutathione than the standard line ($P = 0.0369$ and $P = 0.042$ respectively). However, there were no differences between the two lines in the GSSG content and the ratio of GSH/GSSG.

2.3.3. Enzyme activities

Solar UV-B radiation significantly increased the APX activity and decreased the SOD activity (both at $P < 0.0001$; Table 2.1 and Fig. 2.5D, 2.4A). Also there were differences in the activities of these two enzymes between the two lines, the magenta line had higher APX activity and lower SOD activity than the standard line ($P = 0.0088$ and $P < 0.0001$, respectively). The magenta line had higher activities of CAT, MDHAR, DHAR, and GR, than the standard line (all at $P < 0.0001$; Table 2.1 and Fig. 2.4B, 2.5). The activities of CAT and GR were increased by solar UV-B exposure ($P = 0.0067$ and $P = 0.0203$ respectively), while the MDHAR and DHAR activities were not affected by solar UV-B radiation. Also, the activities of GR and DHAR decreased during the experiment. Guaiacol POD activity greatly increased at 12 days but was not affected by the solar UV-B radiation during the experiment, also there was no difference in its activity between the two lines (Table 2.1 and Fig. 2.4C).

2.4. DISCUSSION

Previous studies on the effects of UV-B on lipid peroxidation have shown that under low or medium levels of UV-B, no increased lipid peroxidation was detected (Giordano et al., 2004; Dai et al., 1997). However, high levels of UV-B were shown to increase lipid peroxidation (Jovanovic et al., 2006; Yannarelli et al., 2006a; Prasad et al., 2005; Hideg et al., 2003; Costa et al., 2002; Alexieva et al., 2001). All these experiments were conducted in growth chambers or greenhouses under artificial conditions. In field supplementation studies, UV-B increased lipid peroxidation in buckthorn (Yang et al., 2005) and *Picea aspirate* (Yao and Liu, 2007), but its effect varied among soybean cultivars (Yanqun et al., 2003). In this study, increased TBARS content by solar UV-B indicates the oxidative damage occurred under ambient levels of UV-B radiation.

Hydrogen peroxide is known to diffuse across biological membranes and cause cellular damage. In this experiment solar UV-B did not alter the H_2O_2 content, which might be due to the increased APX activity and decreased SOD activity. Therefore the increased lipid peroxidation may be caused by other AOS. Although the O_2^- content was not determined in this study, the inhibition of SOD activity by UV-B could lead to increases in O_2^- content. However, this does not necessarily mean that UV-B has no effect on H_2O_2 generation. UV-B exposure enhanced NADPH-oxidase in *Arabidopsis* (Rao et al., 1996), and the involvement of NADPH-oxidase in the H_2O_2 generation has been demonstrated in plants (Rao et al., 1996; Moller and Lim, 1986). There is very limited information about UV-B's effect on H_2O_2 content in higher plants under field conditions. H_2O_2 production was increased by high level of UV-B in experiments conducted indoors (Kalbina and Strid, 2006; Wang et al., 2006; Prasad et al., 2005; Hideg

et al., 2003; Alexieva et al., 2001). However, Murphy (1990) could not detect H_2O_2 accumulation in cultured rose cells treated with broad-band radiation including UV-B. In the present study, the decreased H_2O_2 content at 12 days might be due to the greatly increased activity of POD, which uses H_2O_2 as its substrate.

The glutathione pool was slightly affected by solar UV-B exposure in this experiment. Only GSSG content was decreased by UV-B radiation. Increased thiol content by UV-B radiation were reported in many experiments (Costa et al., 2002; Galatro et al., 2001; Dai et al., 1997; Kalbin et al., 1997; Rao and Ormrod, 1995), but all these increases were found under high levels of UV-B. In the field, long-term exposure to enhanced UV-B radiation did not affect thiol content in *Vaccinium myrtillus* (Taulavuori et al., 1998), increased in wheat and bean (Agrawal and Rathore, 2007), and decreased in pines (Laakso et al., 2001). Increases in AsA pool in response to UV-B exposure have also been observed (Galatro et al., 2001; Dai et al., 1997; Takeuchi et al., 1996b; Rao and Ormrod, 1995). However in maize seedlings, UV-B exposure had no effect on the AsA content (Carletti et al., 2003). Under field conditions, long-term exposure to enhanced levels of UV-B did not change the AsA content (Taulavuori et al., 1998). In the present study, the AsA content was decreased while the DHA content was increased by solar UV-B, resulting in a decreased ratio of AsA/DHA. These UV-B effects on AsA are consistent with the results in wheat and bean (Agrawal and Rathore, 2007; Prasad et al., 2005) and this could be explained by the increase of APX activity under UV-B exposure. Higher APX activity consumes more AsA and produces more DHA. AsA is a major primary antioxidant reacting directly with hydroxyl radicals, superoxide and singlet oxygen, and also a powerful secondary antioxidant, reducing the

oxidized form of α-tocopherol. The importance of AsA in scavenging free radicals and protecting against UV-B damage was highlighted in a study using AsA-deficient *Arabidopsis* mutants (Conklin et al., 1996).

Many responses of SOD to UV-B exposure have been reported revealing no uniform responses. For example, in indoor experiments SOD activity was increased by UV-B radiation in pea and wheat (Alexieva et al., 2001), *Arabidopsis* (Rao and Ormrod, 1995), soybean (Prasad et al., 2005), poplar (Ren et al., 2006), and rice (Dai et al., 1997), but was not affected in buckwheat (Jovanovic et al., 2006) and soybean (Malanga et al., 1999), and was decreased in sunflower cotyledon (Costa et al., 2002). Also, SOD expression was not affected by UV-B radiation in *Nicotiana plumbaginifolia* (Willekens et al., 1994), but was decreased in *Psium sativum* (Strid, 1993). In a field study, supplemental UV-B increased SOD activity in wheat and bean (Agrawal and Rathore, 2007) and *Picea asperata* (Yao and Liu, 2007), had no effects in barley (Mazza et al., 1999), and caused different responses among soybean cultivars (Yanqun et al., 2003). While in other experiments the effects of artificial UV-B on SOD activity were found to vary with temperature (Takeuchi et al., 1996b), duration of the treatment (Dai et al., 1997), leaf age and PAR source even under the same level of PAR (Krizek et al., 1993). Takeuchi et al. (1996b) reported that UV-B increased SOD activity at 20 ^{0}C while it decreased SOD activity at 25 ^{0}C in cucumber cotyledons, which is consistent with our results because in the field in July the temperature is very high. Rao et al. (1996) demonstrated that UV-B exposure preferentially induces peroxidase-related enzymes instead of SOD.

In the present study, UV-B radiation increased APX activity. This is consistent with the results of many other experiments conducted in chambers (Yannarelli et al., 2006a; Rao et al., 1996; Takeuchi et al., 1996b; Landry et al., 1995), or even in the field (Mazza et al., 1999), suggesting APX has an important role in the control of endogenous H_2O_2 content. Solar UV-B radiation also increased the activities of CAT and GR, but had no impact on the activities of POD, MDHAR, and DHAR. However, in this study, the total activities of enzymes were determined, and determinations of total activities may not adequately reflect the compartment-specific changes. Each of the antioxidant enzymes comprises a family of isoforms, often with different characteristics. Enzymes might be affected by UV-B radiation without changes in total activity. For example, UV-B exposure could induce different enzyme isoforms such as POD (Yannarelli et al., 2006b; Murali et al., 1988), CAT (Willekens et al., 1994), SOD (Rao et al., 1996) or APX (Yannarelli et al., 2006b).

During the experiment, the activities of GR and DHAR decreased while the POD activity greatly increased at 12 days. Consistent with our results, Dertinger et al. (2003) reported that the GR activity was maximal in the youngest leaves and was reduced during leaf development in tobacco. Also Chen and Gallie (2006) found that both the protein and activity of DHAR were highest in the youngest leaves and declined with leaf age in tobacco. They concluded that DHAR affected the level of foliar AOS and photosynthetic activity during leaf development, and as a consequence influenced the rate of plant growth and leaf development. It is reported that POD activity increased during early growth stages and then declined during senescence in cucumber (Zheng and Yang, 1991), tobacco (Dhindsa et al., 1981), and maize (Prochazkova et al., 2001). PODs are enzymes

that catalyze the H_2O_2-dependent oxidation of a wide variety of substrates, mainly phenolics (Dunford, 1986). PODs are involved in numerous physiological roles in plant tissues, including lignin biosynthesis, indole-3-acetic acid degradation, wound healing and pathogen defense (Kawano, 2003; Bernards et al., 1999; Bestwick et al., 1998; Sato et al., 1993). The great increase in POD activity at 12 days might be due to lignin biosynthesis and/or indole-3-acetic acid degradation.

The magenta line had higher H_2O_2 content than the standard line. Also the magenta line had higher contents of DHA, GSH, and total glutathione, and higher activities of APX, DHAR, MDHAR, GR, and CAT, while the SOD activity was lower in the magenta line than in the standard line. These results suggest that oxidative stress and damage was greater in the magenta than in the standard line, in spite of its enhanced oxidative defense capacity as compared to the standard line. Most importantly, these occurred even under UV-B exclusion. The enhanced responses in the magenta line, especially under UV-B exclusion, may be due to increased penetration of solar ultraviolet-A and/or oxidative stress caused by other stressors.

Although it is less damaging on a photon basis than UV-B, UV-A comprises a much larger portion of the solar spectrum than does UV-B, and UV-A is able to penetrate to greater depths inside leaves than UV-B (Liakoura et al., 2003). In the field, solar UV-A can cause photoinhibition of PSII (Krause et al., 1999) and growth inhibition (Krizek and Chalker-Scott, 2005; Flint and Caldwell, 2003) in higher plants. UV-A has also been shown to induce oxidative stress in experiments conducted in growth chambers (Yao et al., 2006). According to measurements performed under natural sunlight, at least 50% or more of UV damage was caused by UV-A in Antarctic phytoplankton (Cullen et al.,

1992). UV-A can also induce oxidative stress and lipid oxidation in cyanobacteria (He et al., 2002) and alga (White and Jahnke, 2002). Therefore UV-A is an important photobiological component of sunlight and these data suggest that it plays a role in oxidative stress in soybean.

While flavonoids provide UV-B protection by absorbing radiation in the UV-B region, they also are effective UV-A absorbers (Markstädter et al., 2001). Therefore, flavonoid accumulations in the epidermis would limit UV-A penetration into mesophyll cells as well as mitigating UV-B damage. The greater responses, even under UV-B exclusion, in the magenta line are likely due to its inability to synthesize flavonoids and thus mesophyll cells may be exposed to increased fluxes of UV-A through its more transparent epidermis. Also, flavonoids can act as antioxidants (Peng et al., 2003). These in turn could generate greater oxidative stress, which necessitates more efficient antioxidant scavenging capacity, in the magenta line. Other studies indicated that *Arabidopsis* mutants lacking phenolic sunscreens exhibited enhanced responses in AOS metabolism upon UV-B exposure, but there was no difference under UV-B exclusion (Rao et al., 1996; Landry et al., 1995; Rao and Ormrod, 1995). This might be due to the low UV-A level utilized in these experiments. Further studies are needed to understand the impacts of solar UV-B, UV-A, and PAR irradiance on AOS metabolism.

2.5. CONCLUSION

Our results indicate that ambient levels of solar UV-B caused oxidative stress in both lines, and altered the antioxidant defenses mainly by decreasing SOD activity and increasing activities of APX, CAT, and GR. The magenta line that has reduced flavonoid

levels exhibited greater oxidative stress and damage than the standard line in spite of its enhanced oxidative defense capacity. Since this was the case even under UV-B exclusion, the enhanced responses in the magenta line, especially under UV-B exclusion, may be due to increased penetration of solar ultraviolet-A and/or oxidative stress caused by other stressors such as UV-A, PAR, or temperature. This study also suggests the dual role of flavonoids as screening and antioxidant compounds in protecting plants from UV radiation. More detailed studies are needed to elucidate the effect of solar UV-A and PAR radiation on AOS metabolism.

Table 2.1. p value table for the effects of UV-B (U), line (L), time (T) and their interactions on the metabolite contents and enzyme activities of soybean seedlings grown in the field under two levels of UV-B radiation.

Attributes	U	L	T	U*L	U*T	T*L	U*T*L
H_2O_2	0.2177	<.0001	<.0001	0.5918	0.9661	0.0271	0.8965
TBARS	0.0002	0.0733	<.0001	0.5516	0.2331	0.0014	0.9070
AsA	0.0002	0.8909	<.0001	0.5182	0.0985	0.8132	0.0099
DHA	<.0001	0.0208	<.0001	0.3065	0.1962	0.0023	0.2948
AsA/DHA	<.0001	0.4845	<.0001	0.6645	0.0022	0.2008	0.0169
AsA+DHA	0.0069	0.0484	<.0001	0.5719	0.1585	0.0073	0.3312
GSH	0.3752	0.0369	<.0001	0.3934	0.9224	0.0026	0.7323
GSSG	0.0377	0.8289	<.0001	0.8111	0.0986	0.9299	0.5379
GSH/GSSG	0.0270	0.1774	<.0001	0.6138	0.1065	0.0185	0.2781
GSH+GSSG	0.5735	0.0420	<.0001	0.3940	0.9817	0.0038	0.7843
APX	<.0001	0.0088	<.0001	0.0984	0.0757	0.1231	0.3655
SOD	<.0001	<.0001	<.0001	0.0250	0.0491	<.0001	0.9380
CAT	0.0067	<.0001	0.0121	0.3502	0.6862	<.0001	0.8528
GR	0.0203	<.0001	<.0001	0.4823	0.5599	0.0141	0.4752
DHAR	0.2507	<.0001	<.0001	0.6384	0.6059	0.4816	0.7500
MDHAR	0.1124	<.0001	<.0001	0.2124	0.8547	0.1186	0.7571
POD	0.8323	0.5109	<.0001	0.9776	0.8504	0.8953	0.8286

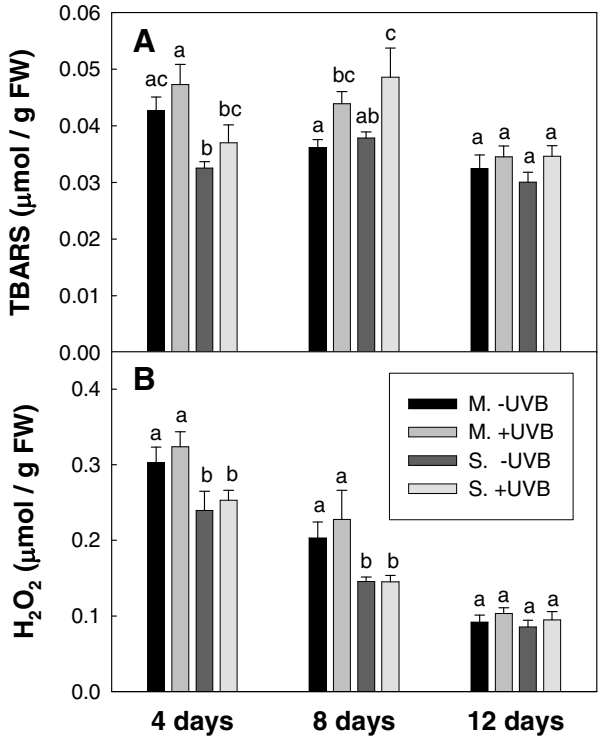

Fig. 2.1. The influence of UV-B radiation on contents of TARBS (A) and H_2O_2 (B) in the Clark standard and magenta lines of soybean following 4, 8, and 12 days exposure. Each bar is the mean ±SE (n=10) for each treatment. Bars with the same letter were not significantly different at $\alpha < 0.05$.

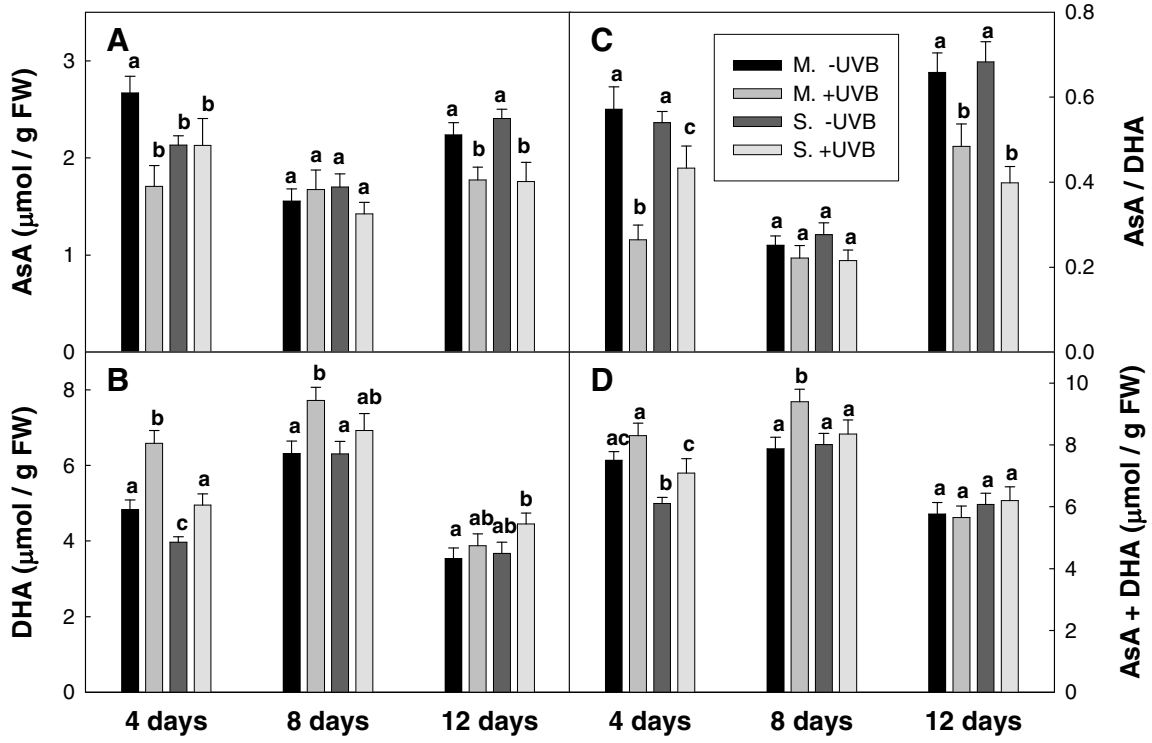

Fig. 2.2. The influence of UV-B radiation on contents of the AsA pool in the Clark standard and magenta lines of soybean following 4, 8, and 12 days exposure. Each bar is the mean ±SE (n=10) for each treatment. Bars with the same letter were not significantly different at $\alpha < 0.05$.

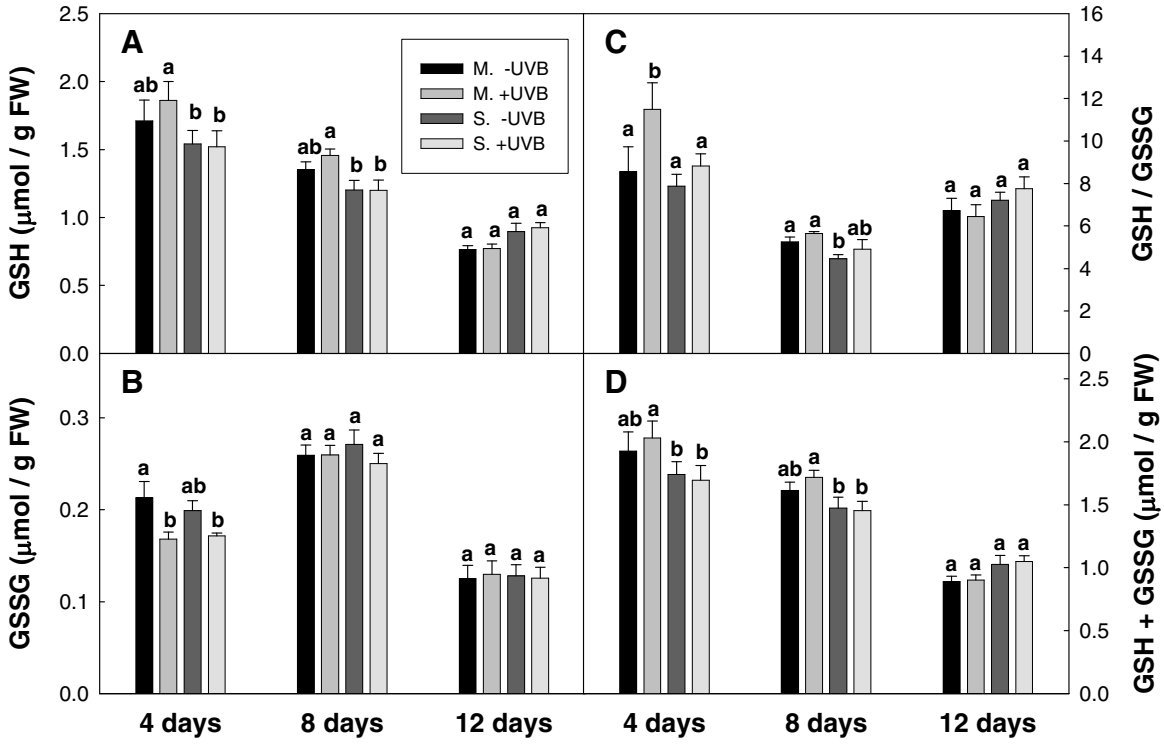

Fig. 2.3. The influence of UV-B radiation on contents of glutathione pool in the Clark standard and magenta lines of soybean following 4, 8, and 12 days exposure. Each bar is the mean ±SE (n=10) for each treatment. Bars with the same letter were not significantly different at $\alpha < 0.05$.

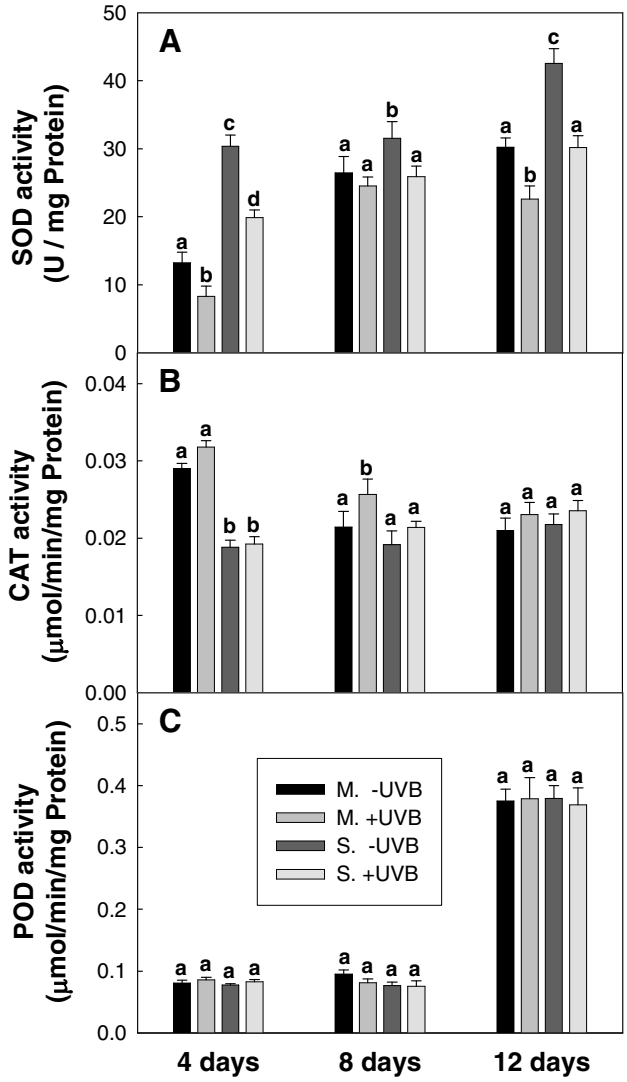

Fig. 2.4. The influence of UV-B radiation on activities of SOD (A), CAT (B), and POD (C) in the Clark standard and magenta lines of soybean following 4, 8, and 12 days exposure. Each bar is the mean ±SE (n=10) for each treatment. Bars with the same letter were not significantly different at $\alpha < 0.05$.

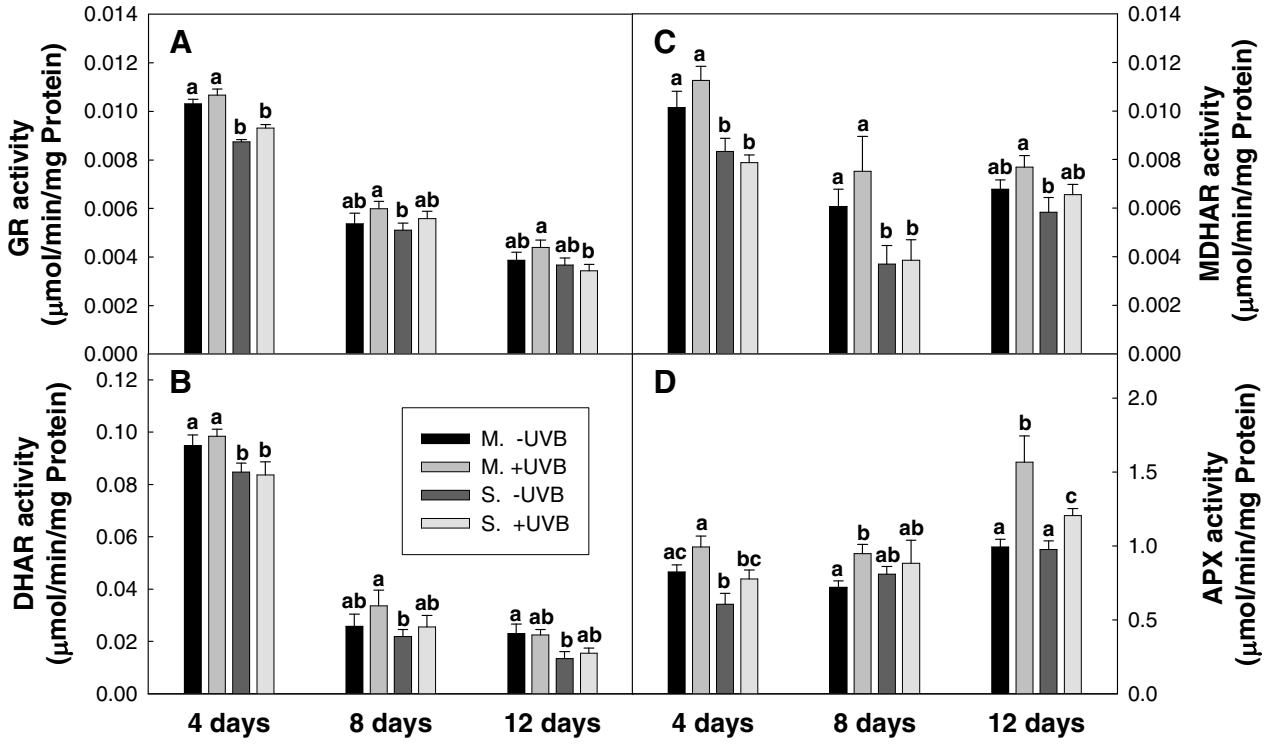

Fig. 2.5. The influence of UV-B radiation on activities of GR (A), DHAR (B), MDHAR (C), and APX(D) in the Clark standard and magenta lines of soybean following 4, 8, and 12 days exposure. Each bar is the mean ±SE (n=10) for each treatment. Bars with the same letter were not significantly different at $\alpha < 0.05$.

CHAPTER 3 SEPARATION AND IDENTIFICATION OF SOYBEAN LEAF PROTEINS BY TWO-DIMENSIONAL GEL ELECTROPHORESIS AND MASS SPECTROMETRY

ABSTRACT

To establish a proteomic reference map for soybean leaves, we separated and identified leaf proteins using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry (MS). Tryptic digests of 268 spots were subjected to peptide mass fingerprinting (PMF) by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS. Fifty-four protein spots were identified by searching NCBI nr and SwissProt databases using the Mascot search engine. Eighty-eight spots that were not identified by MALDI-TOF MS analysis were analyzed with liquid chromatography tandem mass spectrometry (LC-MS/MS), and eighty-two spots were identified by searching against the NCBI nr, SwissProt and expressed sequence tag (EST) databases. Taking into account the multiplicity of the spots, we identified a total of 79 unique proteins on our gel. The majority of the identified leaf proteins are involved in energy metabolism. The results indicate that 2D-PAGE, combined with MALDI-TOF MS and LC-MS/MS, is a sensitive and powerful technique for separation and identification of soybean leaf proteins. A summary of the identified proteins and their putative functions are discussed.

3.1. INTRODUCTION

The two key steps in classical proteomics are the separation of proteins and their subsequent identification. In a standard approach, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry (MS) are combined. Two-dimensional PAGE, in which proteins are separated according to their isoelectric point (pI) in the first dimension and molecular weight (Mr) in the second dimension, is still the preferred separation technique of many researchers in the global and comparative analysis of proteins. MS has essentially replaced the classical technique of Edman degradation in protein identification because it is more sensitive, can deal with protein mixtures, and offers much higher throughput. There are two main approaches to MS protein identification. In peptide mass fingerprinting (PMF), the unknown protein is digested with a protease of known specificity such as trypsin. By determining the masses of the resulting peptides, a mass map or mass fingerprint can be obtained. This mass map is then compared with predicted mass maps of proteins within the database. The tandem mass spectrometric (MS/MS) method relies on fragmentation of individual peptides to obtain sequence information.

A number of plant proteomic studies have recently been published. Some focused on organelle or subcellular proteomes such as the chloroplast (Lonosky et al., 2004; Ferro et al., 2003), the mitochondria (Bardel et al., 2002; Kruft et al., 2001;) or the ribosome (Yamaguchi et al., 2002; Yamaguchi et al., 2000), whereas others have focused on a specific tissue, such as *Arabidopsis* seeds (Gallardo et al., 2001), maize root (Chang et al., 2000) and maize leaves (Porubleva et al., 2001), soybean seed (Natarajan et al., 2005; Mooney and Thelen, 2004), pea leaves (Schiltz et al., 2004)

and legume barrel medic roots (Mathesius et al., 2001). Large-scale projects to identify proteins from multiple tissues of the barrel medic (Watson et al., 2003), rice (Komatsu et al., 2004) and *Arabidopsis* (Giavalisco et al., 2005) have also been reported.

Soybean, *Glycine max* (*G. max*), provides an important source of protein for human food and for the animal industry and has been the dominant oilseed produced since the 1960s. So far, no protein reference map has been reported for soybean leaves to our knowledge. As a first step to study stress physiology of soybean, we separated and identified soybean leaf proteins from normal plants. We describe here the extraction and separation of soybean leaf proteins on 2D-PAGE gels and identification of proteins using both matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS and liquid chromatography tandem mass spectrometry (LC-MS/MS). The Mascot search engine was used to search against NCBI nr, SwissProt and EST databases for protein identification.

3.2. MATERIALS AND METHODS

3.2.1. Plant material

The seeds of soybean cultivar Clark were grown on horticultural vermiculite in 1-L pots in the greenhouse at the University of Maryland, College Park, MD. Plants were transferred to the field site at the USDA, Beltsville Southfarm just before the cotyledons emerged, and grown under full sunlight. Plants were watered and rotated daily, and fertilized with Hoagland solution every 3 days. Leaflet blades from the first

trifoliates were harvested when they were 12 days old. Four biological replicate samples were used for protein extraction and 2D-PAGE analysis. Samples were immediately frozen in liquid nitrogen, and stored at -80 ^{o}C prior to analysis.

3.2.2. Protein extraction

Frozen samples were ground in a mortar with liquid nitrogen and incubated with 10% trichloroacetic acid (TCA) and 0.07% 2-mercaptoethanol in acetone for 1 hr at -20 ^{o}C. The precipitated proteins were pelleted and washed with ice-cold acetone containing 0.07% 2-mercaptoethanol to remove pigments and lipids until the supernatant was colorless. The pellet was vacuum dried, resuspended in resolubilization solution (9M urea, 1% CHAPS, 1% DTT, 1% pharmalyte) and sonicated to extract proteins. Insoluble tissue was removed by centrifugation at 21,000 X g for 30 min at 4 ^{o}C. Protein concentration was determined according to Bradford (1976) using a commercial dye reagent (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin (BSA) as a standard.

3.2.3. 2D-PAGE

An IPGPhor apparatus (GE Healthcare, Piscataway, NJ) was used for isoelectric focusing (IEF) with immobilized pH gradient (IPG) strips (pH 3.0 - 10.0, linear gradient, 13 cm). The IPG strips were rehydrated 12 hrs with 250µL rehydration buffer (8M urea, 2% CHAPS, 0.5% pharmalyte, 0.002% bromophenol blue) containing 350 µg proteins. The voltage settings for IEF was 500 V for 1 hr, 1000 V for 1 hr, 5000 V for 1 hr, and 8000 V to a total 46.86 kVh. Following

electrophoresis, the protein in the strips was denatured with equilibration buffer (50mM Tris-HCl pH 8.8, 6M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 1% DTT) and then incubated with the same buffer containing 2.5% iodoacetamide instead of DTT for 30 min at room temperature. The second dimension electrophoresis was performed on a 12.5% gel using a Hoefer SE 600 Ruby electrophoresis unit (GE Healthcare, Piscataway, NJ). The gels were stained with Coomassie brilliant blue (CBB) G-250 (Newsholme et al., 2000) and scanned using a Personal Densitometer SI (GE Healthcare, Piscataway, NJ).

3.2.4. In-gel digestion of protein spots

Protein digestion was performed as described previously (Natarajan et al., 2005). Spots were excised from the stained gel and washed with 50% acetonitrile (ACN) containing 25 mM ammonium bicarbonate to remove the dye. The gel plug was dehydrated with 100% ACN, and was dried under vacuum and incubated overnight at 37 ^{o}C with 20 uL of 10 ug/mL porcine trypsin (modified porcine trypsin, sequencing grade, Promega, Madison, WI) in 20 mM ammonium bicarbonate. The resulting tryptic fragments were eluted by diffusion into 50% ACN and 0.5% trifluoroacetic acid (TFA). A sonic bath was used to facilitate the diffusion. The extract was vacuum dried and the pellet was dissolved in 50% ACN and 0.1% TFA.

3.2.5. Mass spectrometry

For PMF a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, Framingham, MA) operated in positive ion reflector mode was used to

analyze tryptic peptides. Samples were co-crystallized with α-cyanohydroxycinnamic acid matrix, and spectra were acquired with 50 shots of a 337 nm Nitrogen Laser operating at 20 Hz. Spectra were calibrated using the trypsin autolysis peaks at m/z 842.51 and 2,211.10 as internal standards. For MS/MS a Thermo Finnigan LCQ Deca XP plus Ion Trap mass spectrometer was used to analyze proteins that were not positively identified by MALDI-TOF MS. Peptides were separated on a reverse phase column using a 30 min gradient of 5% to 60% ACN in water with 0.1 % formic acid. The instrument was operated with a duty cycle that acquired MS/MS spectra on the three most abundant ions identified by a survey scan from 300-2000 Da. Dynamic exclusion was employed to prevent the continuous analysis of the same ions. Once two MS/MS spectra of any given ion had been acquired, the parent mass was placed on an exclusion list for the duration of 1.5 min. The raw data were processed by Sequest to generate DTA files for database searching. The merge.pl script from Matrix Science was used to convert multiple Sequest DTA files into a single mascot generic file suitable for searching in Mascot.

3.2.6. Data analysis

Protein identification was performed using the Mascot search engine, which uses a probability based scoring system (Perkins et al., 1999). NCBI non-redundant and SwissProt databases were selected as the primary databases to be searched. For LC-MS/MS, if the primary databases did not yield identity, the "EST_others" database was queried. The following parameters were used for database searches with MALDI-TOF PMF data: monoisotopic mass, 25 ppm mass accuracy, trypsin as digesting enzyme with

1 missed cleavage allowed, carbamidomethylation of cysteine as a fixed modification, oxidation of methionine, N-terminal pyroglutamic acid from glutamic acid or glutamine as allowable variable modifications. For database searches with MS/MS spectra, the following parameters were used: monoisotopic mass; 1.5 Da peptide and MS/MS mass tolerance; peptide charge of +1, +2, or +3; trypsin as digesting enzyme with 1 missed cleavage allowed; carbamidomethylation of cysteine as a fixed modification; oxidation of methionine, N-terminal pyroglutamic acid from glutamic acid or glutamine as allowable variable modifications. Taxonomy was limited to green plants for both MALDI and MS/MS ion searches. For MALDI-TOF-MS data to qualify as a positive identification, a protein's score had to equal or exceed the minimum significant score of sixty-four for NCBI nr or fifty-five for SwissProt database searching. Positive identifications of proteins by MS/MS analysis required a minimum of two unique peptides, with at least one peptide having a significant ion score.

3.3. RESULTS AND DISCUSSION

3.3.1. Separation of soybean leaf proteins

A previously described protein extraction protocol using acetone/TCA precipitation was employed for the extraction of protein from soybean leaves (Natarajan et al., 2005). The proteins separated by 2D-PAGE were visualized by CBB G-250 staining, which is MS compatible and allows reproducible protein detection. The resulting 2D-PAGE images, which were reproduced from four independent biological experiments, constitute the reference gel images. The simplicity of the

protocol favors the reproducibility of the protein separation. A representative 2D-PAGE protein pattern of soybean leaf is presented in Fig. 3.1. The dynamic range of protein accumulation is very large; this is a problem for leaf proteomic analysis because the preponderance of ribulose bisphosphate carboxylase/oxygenase (Rubisco) masks the detection of other proteins (Watson et al., 2003; Wilson et al., 2002). However, in our system a significant number of proteins were clearly separated and identified despite the predominance of Rubisco in the central portion of the gels.

3.3.2. Identification of separated proteins

To qualitatively survey the proteins visualized by 2D-PAGE, a total of 268 protein spots were excised from the 2D-PAGE gels and digested with the trypsin. The peptide fragments were extracted and analyzed by MALDI-TOF MS. Typically, high-quality MALDI-TOF MS peptide mass maps were obtained. Of the 268 protein spots processed, 54 proteins were successfully identified by querying NCBI nr and SwissProt databases using the Mascot search engine. The results are listed in Table 3.1. Data in Table 3.1 include an assigned protein spot number, theoretical pI and Mr, protein identity and its original species, number of peptides matched, percentage sequence coverage, MOWSE score, database searched, identification method and accession number of the best match. Although MALDI-TOF MS analysis is easy to automate and allows high throughput analysis, protein identification relies solely on the accurate matching of the peptide mass, and it is very difficult to correctly identify proteins if there is a protein mixture. Also the database must contain enough of the protein sequence to compare with the experimentally derived mass map.

LC-MS/MS analysis of 88 spots that were not identified by MALDI-TOF MS allowed the identification of 82 spots, searching against the NCBI nr, SwissProt and EST-others databases. The identifications are listed in Table 3.1. The efficiency of the identification is higher than that in a proteomic study of rice where 77% of proteins were identified (Lin et al., 2005). An example of an LC-MS/MS spectrum obtained from spot 4 is presented in Fig. 3.2. The tandem mass spectrometric method is technically more complex and less scalable than MALDI fingerprinting. Its main advantage is that sequence information derived from several peptides is much more specific for the protein than a list of peptide masses. A short region of local identity which spans two or more consecutive tryptic cleavage sites may enable identification, even when the remainder of the sequence is divergent (Newton et al., 2004; Pandey and Mann, 2000). In addition, the fragmentation data can be used to search nucleotide databases such as db EST as well as the protein databases. Most of the 136 identified protein spots were identified by searching against the NCBI nr database and contained only one protein, nine spots contained two different proteins (spot 6, 13, 35, 48, 63, 76, 116, 123, and 135). Sixteen spots were identified from EST_others database, and 13 spots were identified from the SwissProt database. Only 36% of the identifications came from *G. max* or *Glycine soja* (*G. soja*) species.

The theoretical and experimental Mrs and pIs matched closely for 79 of the 136 identified spots. We used the BLAST tool in ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (http://us.expasy.org/) for annotation and found that many of the proteins identified have a signal peptide. We calculated the theoretical pI/Mr of these proteins without the signal sequence using the

compute pI/Mr tool and found that an additional 26 spots have matched Mrs and pIs. For example, the identification for spot 94 is photosystem (PS) II stability/assembly factor HCF136 precursor that has 403 amino acids, and pI/Mr of 6.79/44133. The first 78 amino acids are a signal peptide, so the theoretical pI/Mr of the rest of the peptide is 5.15/35843, which closely matches with our observed values. The pI/Mr discrepancy for the other spots might be due to different amino acid sequences from different species (spot 6, 79, 80, 81, 106, and 116), amino acid sequences derived from an EST that may not include complete or accurate sequences (spot 21, 22, 82 112, 133, and 134), or co- and/or post-translational modification (spot 13, 29, 86, 122, and 135).

Multiple spots for a single protein are commonly found on 2D gels (Giavalisco et al., 2005; Sarnighausen et al., 2004). In this study 32 proteins have multiple spots: T-protein of glycine cleavage system (GCV) (spot 3 - 6); Rubisco both large (spot 6, 23, 24, and 122) and small (spot 25 - 29) subunits; Rubisco activase (spot 30 - 35, 121, and 124); stem 28 kDa protein (spot 90 - 93); and many others. Several factors may be responsible for this phenomenon. The migration of proteins on a 2D-PAGE gel is very sensitive to small structural differences. These spots might be different isoforms derived from different genes of a multigene family. The complex genome of soybean is expected to contain multiple copies of many genes, and the distinct biophysical properties might be due to amino acid sequence differences in the different isoforms. Alternatively, one gene product may undergo different co- and/or post-translational modifications that affect its pI or/and Mr. The multiple spots corresponding to one protein could also be a consequence of artificial modification of proteins, such as carbamylation, during the extraction or separation procedure (Berven et al., 2003). However, appropriate

precautions were used to prevent artificial modifications and the multiple spots are highly reproducible. Therefore, it is unlikely that the multiple spots in this study are artifacts of protein damage during sample preparation. Taking into account the multiplicity of the spots, we identified a total of 79 unique proteins on our gel.

Based on bioinformatic protein sequence analysis, proteins with at least one transmembrane-spanning domain constitute approximately 20% of all proteins in eukaryotic genomes (Stevens and Arkin, 2000; Wallin and von Heijne, 1998). These proteins are often underrepresented on 2D-PAGE gels due to the tendency of the hydrophobic transmembrane regions to cause the proteins to precipitate, mainly during IEF (Santoni et al., 2000; Molloy et al., 1998). The observation of plant proteins in 2D-PAGE relative to their general average hydropathicity score has been discussed (Millar et al., 2001). Most proteins identified in this study were expected to be soluble proteins, given that TCA precipitation results in the loss of integral membrane proteins (Wang et al., 2000). Nevertheless, we found some spots that were identified as integral or peripheral membrane proteins, such as chlorophyll a/b binding protein (spot 63), oxygen-evolving enhancer (OEE) protein of PS II (spot 69 - 75), and subunits of the PS I complex (spot 64 - 67). These light-harvesting complexes of PS I and PS II are highly abundant in the thylakoid membranes of plant chloroplasts (Gomez et al., 2000). Therefore, a small fraction of these proteins was extracted during the sample preparation from the whole leaves.

3.3.3. Functional distribution of identified proteins

Identified proteins were classified according to their functions in the categories described by Bevan et al. (1998). In our study, more than 50% of the identified protein spots are involved in energy metabolism (Fig. 3.3). This category was divided into four subcategories: the pentose phosphate pathway, the glycolysis pathway/glyoxylate cycle/gluconeogenesis, electron transport, and photosynthesis. The most abundant proteins were also included in the energy category, and most of these proteins appeared as multiple spots. The primary function of leaves is energy harvesting, conversion, and storage. Therefore, it is not surprising that a significant number of abundant proteins in the soybean leaf proteome are involved in energy metabolism. The identified proteins nvolved in photosynthetic electronic transport were: subunits of PS I (spot 64 - 67) and PS II (spot 63, 68 - 75); Rieske FeS protein (spot 76); plastocyanin (spot 77); and ferredoxin (spot 61 and 62). Several enzymes responsible for carbon metabolism were also identified: rubisco (spot 6, 23 - 29, and 122); malate dehydrogenase (spot 49 and 50); sedoheptulose-1,7-biphosphatase (spot 36 and 112); phosphoglycerate kinase (spot 13, 45, and 46); glyceraldehyde-3-phosphate dehydrogenase (GADPH) (spot 41 - 44, and 125); triosephosphate isomerase (spot 51, 52, and 123); and transketolase (spot 39 and 40). Rubisco is the primary enzyme in photosynthetic carbon fixation and the likely rate-limiting factor for photosynthesis under light-saturated conditions and atmospheric CO_2 pressure (Makino et al., 1985). The ATPase consists of two parts: a hydrophobic membrane-bound portion called CF_0, and a soluble portion that sticks out into the stroma called CF_1. CF_1 consists of 5 different subunits: α-, β-, γ-, δ-, and ε-units (Taiz and

Zeiger, 2002). Only the soluble α- (spot 59 and 60), β- (spot 56 - 58), and ε- (spot 135) subunits of the ATPase complex were present on our 2D-PAGE gels.

About 16 % of the identified protein spots are involved in general metabolism. Most of these are involved in amino acid metabolism: glutamine synthetase (spot 11 - 13); glycine (serine) hydroxymethyltransferase (SHMT) (spot 16 - 19); alanine aminotransferase (spot 9 and 10); methionine synthase (spot 14); aspartate transaminase (spot 15); P- (spot 7, 8, and 120), H- (spot 1 and 2), and T-protein (spot 3 - 6) of the GCV. SHMT catalyzes the interconversion of serine and glycine. It is a key enzyme in the biosynthesis of purines, lipids, hormones and other compounds (Kopriva and Bauwe, 1995). The GCV catalyzes the degradation of glycine and is composed of four proteins: P, T, L and H proteins (Bourguignon et al., 1993). Unexpectedly, nitrite reductase (NR) was not detected in this study. NR mediates the ferredoxin-dependent reduction of nitrite to ammonium during primary nitrogen assimilation in plants (Hoff et al. 1994). Porubleva et al. (2001) also was uable to detect NR in maize leaves using the same method. The absence of this enzyme may be due to the extraction method or to the predominance of Rubisco which could mask the presence of NR.

Other identified proteins belong in the protein destination and storage category: chaperonin (spot 95, 96, 100, 113, and 135), HSP/HSC (heat shock protein / heat shock cognate) 70 and associated co-chaperones (spot 97, 98, 99, 101, and 130), stem 28 kDa protein (spot 90 - 93), cyclophilin (spot 76, 102, and 103), endopeptidase Clp (spot 105) and polyubiquitin (spot 104). HSPs are associated with protein folding, protein translocation across membranes, assembly of oligomeric proteins, modulation of receptor activities, mRNA protection, prevention of enzyme denaturation and their stress-induced

aggregation, and with post-stress ubiquitin and chaperonin-aided repair. Based on these functions, HSPs have been termed "molecular chaperones" (Leone et al., 2000; Georgopoulos and Welch, 1993). Stem 28 kDa glycoprotein is also known as vegetative storage protein A. It may function as a somatic storage protein during early seedling development and mainly accumulates in the stem of developing seedlings (Mason et al., 1988).

Additional identified protein spots are included in disease/defence category: ascorbate peroxidase (spot 109 and 110); catalase (spot 111); superoxide dismutase (spot 123 and 131); stress-induced protein SAM22 (spot 114); peroxiredoxin (spot 116 and 117); PR1 (spot 115); thaumatin-like protein (spot 133); and lectin (spot 132). Three signal transduction proteins were detected in our study: harpin-binding protein 1 (spot 63); P21 protein (spot 108); and G protein (spot 107). Other identified proteins are involved in protein synthesis (ribosomal protein, spot 83 - 85; elongation factor, spot 86 - 89), in transcription (RNA binding protein, spot 119 and 82), in ion transport (voltage-dependent anion-selective channel, spot 106), and in secondary metabolism (1-deoxy-D-xylulose 5-phosphate reductoisomerase, spot 118; chalcone reductase, spot 134). Only two identified protein have unknown function (spot 116 and 136). None of the identified proteins are in the cell growth/division, intracellular traffic, cell structure or transposon categories.

3.4. CONCLUSION

In summary, we separated soybean leaf proteins using 2D-PAGE and identified 136 protein spots with MALDI-TOF MS and LC-MS/MS. The broad

dynamic range of protein expression is one of the major difficulties in separation of soybean leaf proteins by 2D-PAGE. LC-MS/MS is a more powerful and sensitive way to obtain positive identifications from 2D-PAGE spots, although it is more laborious and more difficult to automate than MALDI-TOF MS analysis. Future studies foliar physiology will benefit from this proteome reference map of soybean leaves.

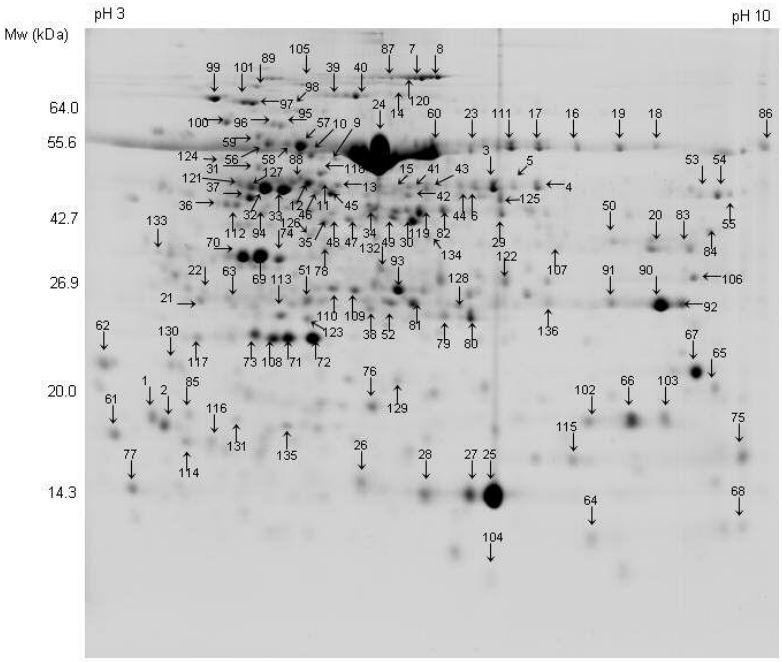

Fig 3.1. Coomassie stained 2D-PAGE gel image of separated soybean leaf proteins. Proteins were separated in the first dimension on a IPG strip pH 3.0 - 10.0 and in the second dimension on a 12.5% acrylamide SDS-gel. The numbered spots were identified and the derived data are presented in the Table 3.1.

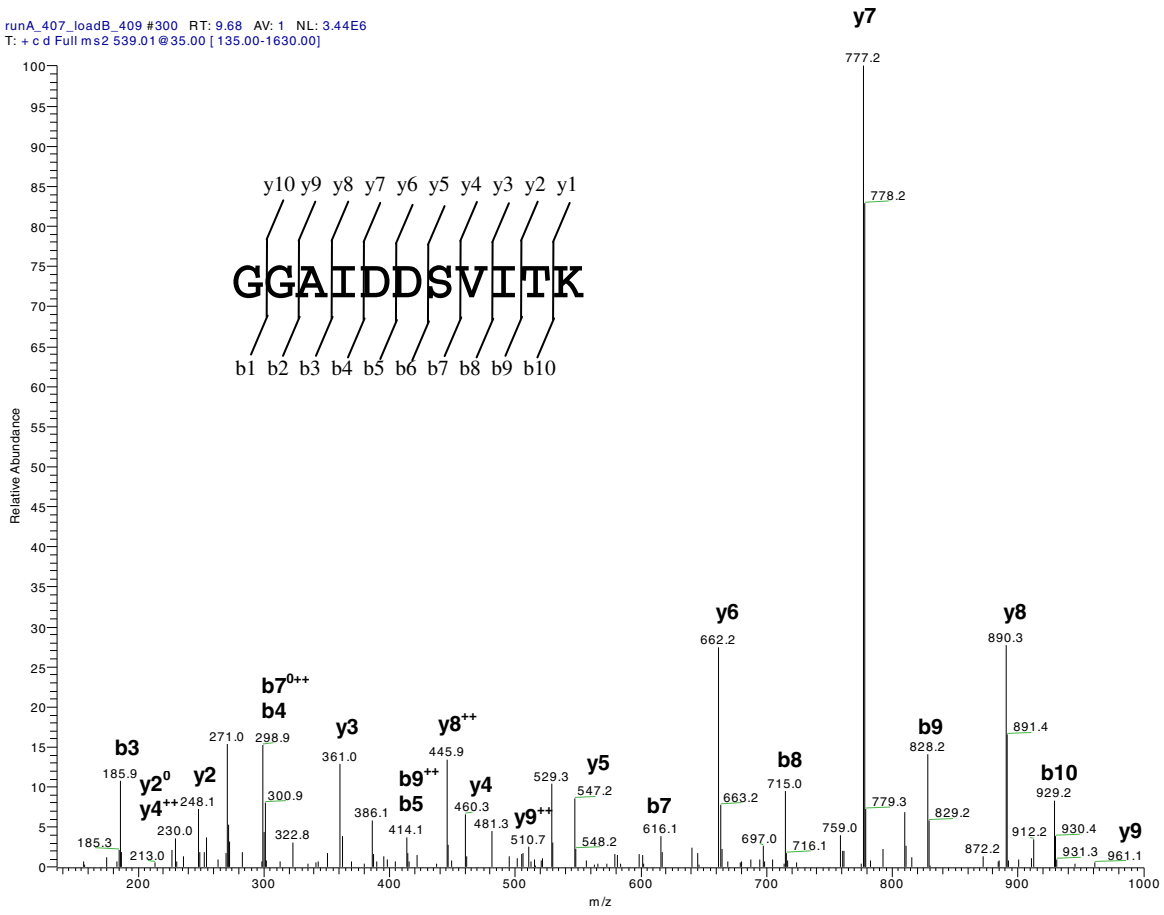

Fig. 3.2. MS/MS spectrum of one tryptic peptide of spot 4. The protein was identified as T-protein of the glycine cleavage system on the basis of finding ten unique tryptic peptides, eight with significant ion score, and total protein coverage of 25%.

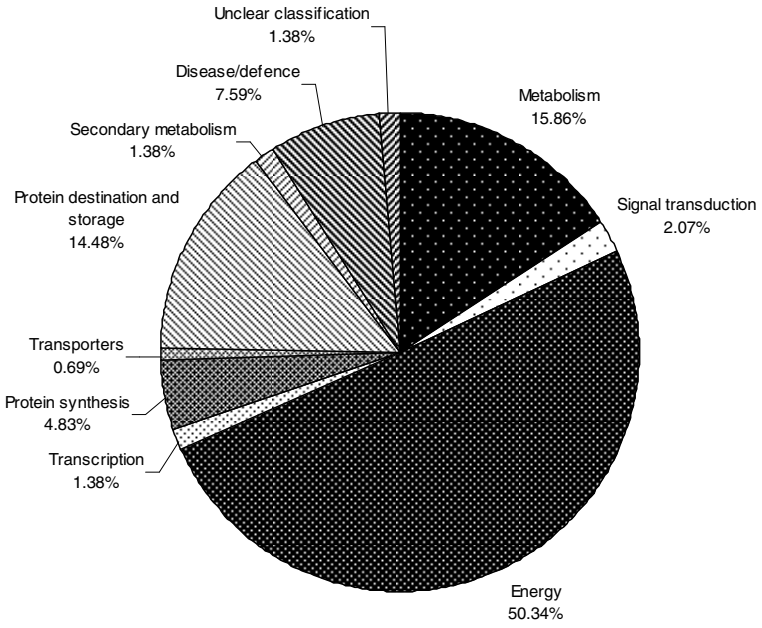

Fig 3.3. Assignment of the identified proteins to functional categories using the classification described by Bevan et al. (1998). A total of 136 spots representing 79 different proteins were classified. If a spot contained two proteins it was counted twice.

Table 3.1. Proteins identified from soybean leaves by MS

ID	Protein Identification [species]	T. Mr/pI	MO	PM	SC	Acce. No.	Databases
Functional category 1: Metabolism							
1	H-protein GCV [*Glycine soja*]	17744/4.59	65	2	10%	gil26045029	EST_others
2	H-protein GCV [*Glycine soja*]	17744/4.59	145	3	22%	gil26045029	EST_others
3	T-protein GCV [*Pisum sativum*]	44661/8.79	131	3	7%	gil407475	NCBInr
4	T-protein GCV [*Pisum sativum*]	44661/8.79	506	10	25%	gil407475	NCBInr
5*	T-protein GCV [*Pisum sativum*]	44656/8.79	65	8	25%	P49364	SwissProt
6	T-protein GCV [*Pisum sativum*]	44661/8.79	151	3	7%	gil407475	NCBInr
7	P-protein GCV [*Arabidopsis thaliana*]	114672/6.18	463	9	10%	gil3413705	NCBInr
8	P-protein GCV [*Arabidopsis thaliana*]	114672/6.18	514	12	12%	gil3413705	NCBInr
120	P-protein GCV [*Pisum sativum*]	115411/7.17	73	2	2%	gil20741	NCBInr
9	Alanine aminotransferase [*Arabidopsis thaliana*]	53780/6.49	164	4	9%	gil23297208	NCBInr
10	Alanine aminotransferase [*Arabidopsis thaliana*]	53780/6.49	128	3	7%	gil23297208	NCBInr
11*	Glutamine synthetase [*Glycine max*]	47948/6.73	83	12	35%	gil13877511	NCBInr
12*	Glutamine synthetase [*Glycine max*]	47948/6.73	70	9	21%	gil13877511	NCBInr
13	Glutamine synthetase [*Glycine max*]	47948/6.73	407	12	14%	gil13877511	NCBInr
14*	Methionine synthase [*Glycine max*]	84401/5.93	143	24	36%	gil33325957	NCBInr
15*	Aspartate transaminase [*Glycine max*]	50725/7.16	81	14	36%	gil485495	NCBInr
16*	SHMT [*Flaveria pringlei*]	57127/8.72	71	17	30%	gil437995	NCBInr
17	SHMT [*Flaveria pringlei*]	57068/8.8	234	4	10%	gil437997	NCBInr
18*	SHMT [*Solanum tuberosum*]	57224/8.40	55	19	29%	P50433	SwissProt
19	SHMT [*Arabidopsis thaliana*]	57534/8.4	157	2	5%	Q9SZJ5	SwissProt

Table 3.1. Continued

ID	Protein Identification [species]	T. Mr/pI	MO	PM	SC	Acce. No.	Databases
20*	Gamma-glutamyl hydrolase [*Glycine max*]	37824/6.08	92	9	29%	gil7488702	NCBInr
21	Ribose 5-phosphate isomerase [*Glycine max*]	19726/4.69	386	6	40%	gil15285625	EST_others
22	Ribose 5-phosphate isomerase [*Glycine max*]	19726/4.69	118	2	15%	gil15285625	EST_others
Functional category 2: Energy/Pentose phosphate							
23*	Rubisco rbcL [*Ophioglossum engelmannii*]	53034/5.96	104	15	33%	gil309636	NCBInr
24*	Rubisco rbcL [*Glycine max*]	52802/6.09	163	16	33%	gil3114769	NCBInr
6	Rubisco [*Lechenaultia heteromera*]	43938/6.36	152	4	10%	gil1304320	NCBInr
122	Rubisco rbcL [*Tribeles australis*]	52050/6.13	132	5	11%	gil9910002	NCBInr
25*	Rubisco rbcS2 [*Glycine max*]	20220/8.87	150	14	57%	gil10946377	NCBInr
26*	Rubisco rbcS2 [*Glycine max*]	20220/8.87	91	6	28%	gil10946377	NCBInr
27*	Rubisco rbcS2 [*Glycine max*]	20220/8.87	175	15	57%	gil10946377	NCBInr
28	Rubisco rbcS 4 [*Glycine max*]	20232/8.87	301	6	26%	gil132113	NCBInr
29*	Rubisco rbcS4 [*Glycine max*]	20232/8.87	94	10	56%	gil132113	NCBInr
30	Rubisco activase [*Datisca glomerata*]	41045/7.59	74	2	6%	gil3687652	NCBInr
31	Rubisco activase [*Chenopodium quinoa*]	47925/6.56	386	6	18%	gil21950712	NCBInr
32*	Rubisco activase [*Vigna radiata*]	48042/7.57	123	13	29%	gil10720249	NCBInr
33*	Rubisco activase [*Vigna radiata*]	48042/7.57	73	9	22%	gil10720249	NCBInr
34*	Rubisco activase [*Vigna radiata*]	48042/7.57	68	11	32%	gil10720249	NCBInr
35	Rubisco activase [*Deschampsia antarctica*]	47371/7.57	86	2	9%	gil32481063	NCBInr
121*	Rubisco activase [*Vigna radiata*]	48042/7.57	75	10	27%	gil8954287	NCBInr

Table 3.1. Continued

ID	Protein Identification [species]	T. Mr/pI	MO	PM	SC	Acce. No.	Databases
124	Rubisco activase [*Phaseolus vulgaris*]	48342/8.19	142	4	10%	gil3033513	NCBInr
36	Sedoheptulose-1,7-bisphosphatase [*Spinacia oleracea*]	42568/5.87	88	2	5%	gil2529376	NCBInr
112	Sedoheptulose-1,7-bisphosphatase [*Glycine max*]	23722/7.89	271	7	23%	gil31464866	EST_others
37*	Phosphoribulokinase [*Pisum sativum*]	39230/5.41	106	14	40%	gil1885326	NCBInr
127	Phosphoribulokinase [*Pisum sativum*]	39230/5.41	471	14	32%	gil1885326	NCBInr
38	Ribulose-phosphate 3-epimerase [*Spinacia oleracea*]	30632/8.23	233	2	12%	gil3264788	NCBInr
39	Transketolase [*Spinacia oleracea*]	80744/6.2	238	3	6%	gil2529342	NCBInr
40	Transketolase [*Spinacia oleracea*]	80744/6.2	259	3	6%	gil2529342	NCBInr
Functional category 2: Energy/Glycolysis/Glyoxylate cycle/Gluconeogensis							
41	GAPDH [*Capsicum annuum*]	34126/6.34	204	3	11%	gil18072799	NCBInr
42	GAPDH [*Capsicum annuum*]	34126/6.34	311	6	17%	gil18072799	NCBInr
43	GAPDH [*Pisum sativum*]	43597/8.8	294	4	14%	gil12159	NCBInr
44	GAPDH [*Nicotiana tabacum*]	42122/6.6	364	6	17%	gil170237	NCBInr
125	GAPDH [*Glycine max*]	43479/8.42	668	20	35%	gil77540210	NCBInr
45*	Phosphoglycerate kinase [*Nicotiana tabacum*]	50317/8.48	116	10	28%	gil1161600	NCBInr
46*	Phosphoglycerate kinase [*Nicotiana tabacum*]	50317/8.48	104	12	36%	gil1161600	NCBInr
13	Phosphoglycerate kinase precursor [*Solanum tuberosum*]	50594/7.68	232	4	9%	gil3328122	NCBInr
47	Plastidic aldolases [*Solanum tuberosum*]	38632/5.89	289	5	14%	gil1781348	NCBInr
35	Plastidic aldolases [*Solanum tuberosum*]	38632/5.89	307	5	18%	gil1781348	NCBInr
48	Fructose-bisphosphate aldolase [*Spinacia oleracea*]	42727/6.85	153	2	6%	P16096	SwissProt

Table 3.1. Continued

ID	Protein Identification [species]	T. Mr/pI	MO	PM	SC	Acce. No.	Databases
49*	Malate dehydrogenase [*Mesembryanthemum crystallinum*]	35817/6.00	66	7	31%	O24047	SwissProt
50	Malate dehydrogenase 2, glyoxysomal [*Brassica napus*]	38043/8.14	214	2	9%	gil4995091	NCBInr
51*	Triosephosphate isomerase [*Fragaria x ananassa*]	33505/7.64	76	11	37%	gil7650502	NCBInr
52*	Triosephosphate isomerase [*Glycine max*]	27441/5.87	108	10	51%	gil48773765	NCBInr
123	Triosephosphate isomerase [*Fragaria* × *ananassa*]	33733/7.64	496	17	35%	gil7650502	NCBInr
53	Glycolate oxidase, peroxisomal [*Spinacia oleracea*]	40317/9.16	261	6	12%	gil121530	NCBInr
54	Glycolate oxidase [*Lens culinaris*]	40907/9.38	277	7	15%	gil228403	NCBInr
55*	Glycolate oxidase [*Mesembryanthemum crystallinum*]	40644/9.02	71	9	28%	gil1773330	NCBInr
	Functional category 2: Energy/Electron transport						
56*	ATPase beta subunit [*Oryza sativa (japonica cultivar-group)*]	45265/5.26	94	12	35%	gil56784992	NCBInr
57*	ATPase beta subunit [*Crossosoma californicum*]	52019/5.20	194	22	57%	gil14718020	NCBInr
58*	ATPase beta subunit [*Platytheca verticellata*]	51184/5.07	147	21	50%	gil7708546	NCBInr
59*	ATPase alpha subunit [*Lotus corniculatus var. japonicus*]	55803/5.22	136	13	33%	gil13358984	NCBInr
60*	ATPase alpha subunit [*Phaseolus vulgaris*]	55595/6.51	90	14	31%	gil169318	NCBInr
135	ATP synthase CF1 epsilon sub [*Glycine max*]	14801/5.41	291	13	41%	gil91214127	NCBInr
61	Ferredoxin I precursor [*Glycine max*]	18448/4.84	122	3	16%	gil5666556	EST_others
62	Ferredoxin-like protein [*Glycine max*]	22101/4.45	131	2	13%	gil23731638	EST_others

Table 3.1. Continued

ID	Protein Identification [species]	T. Mr/pI	MO	PM	SC	Acce. No.	Databases
126	Ferredoxin NADP reductase [*Arabidopsis thaliana*]	40643/8.32	171	5	16%	gil10177134	NCBInr
Functional category 2: Energy/Photosynthesis							
63	Chlorophyll a/b binding protein [*Arabidopsis thaliana*]	25036/5.12	103	2	6%	gil16374	NCBInr
64	PSI PsaN subunit [*Zea mays*]	12841/8.37	75	2	20%	gil2981214	NCBInr
65	PSI D2 subunit [*Nicotiana sylvestris*]	22467/9.78	106	2	13%	gil19748	NCBInr
66	PSI subunit IV A [*Glycine max*]	16368/9.08	143	4	13%	gil5606709	EST_others
67*	PSI subunit D precursor [*Solanum tuberosum*]	22849/9.63	72	11	44%	gil34787117	NCBInr
68	PSII 10 KD Polypeptide [*Glycine max*]	13753/9.73	113	2	18%	gil607356	EST_others
69*	PSII OEE protein 1 [*Pisum sativum*]	35100/6.25	91	9	27%	gil20621	NCBInr
70*	PSII OEE protein 1 [*Pisum sativum*]	35100/6.25	66	7	23%	P14226	SwissProt
71	PSII OEE protein 2 [*Pisum sativum*]	28201/8.29	169	6	14%	P16059	SwissProt
72	PSII OEE protein 2 [*Solanum tuberosum*]	28158/8.27	120	3	8%	gil1771778	NCBInr
73	PSII OEE protein 2 [*Glycine max*]	19825/4.81	200	6	28%	gil16995778	EST_others
74*	PSII OEE protein 3 [*Nicotiana tabacum*]	35377/5.89	85	9	31%	gil505482	NCBInr
75	PSII OEE protein 3 [*Lycopersicon esculentum*]	24557/9.64	85	3	15%	gil51457944	NCBInr
76	Chloroplast Rieske FeS protein [*Pisum sativum*]	24683/8.63	190	6	16%	gil20832	NCBInr
77	Plastocyanin [*Cucurbita pepo*]	10544/4.34	63	3	42%	gil130265	NCBInr
78	Ferredoxin-NADP+ reductase [*Arabidopsis thaliana*]	40643/8.32	381	6	18%	gil20465661	NCBInr
48	Quinone oxidoreductase-like protein [*Arabidopsis thaliana*]	41132/8.46	234	4	8%	Q9ZUC1	SwissProt

Table 3.1. Continued

ID	Protein Identification [species]	T. Mr/pI	MO	PM	SC	Acce. No.	Databases
79	Carbonic anhydrase [*Vigna radiata*]	35804/7.59	305	8	20%	gil8954289	NCBInr
80	Carbonic anhydrase [*Vigna radiata*]	35804/7.59	292	8	25%	gil8954289	NCBInr
81	Carbonic anhydrase [*Vigna radiata*]	35804/7.59	267	7	14%	gil8954289	NCBInr
128	Carbonic anhydrase [*Vigna radiata*]	35804/7.59	64	2	6%	gil8954289	NCBInr
Functional category 4: Transcription							
82	Chloroplast mRNA-binding protein CSP41 precursor [*Glycine max*]	22707/5.31	169	6	21%	gil7639890	EST_others
119	RNA-binding protein [*Arabidopsis thaliana*]	42303/7.71	241	4	12%	gil3850621	NCBInr
Functional catoegory 5: Protein synthesis							
83	30S ribosomal protein S5 [*Arabidopsis thaliana*]	32682/8.99	128	2	8%	gil21593322	NCBInr
84	50S Ribosomal protein L1 [*Glycine soja*]	29084/9.43	119	2	9%	gil26046597	NCBInr
85	50S Ribosomal protein L12 [*Glycine max*]	16769/4.64	467	6	53%	gil22927916	EST_others
86	Elongation (EF-1a) [*Glycine max*]	49689/9.14	264	7	17%	gil18765	NCBInr
87*	Elongation (EF-2) [*Beta vulgaris*]	94708/5.93	56	14	19%	O23755	SwissProt
88*	Translation elongation factor-TU [*Glycine max*]	52177/6.21	170	22	55%	gil18776	NCBInr
89	Translation elongation factor G [*Glycine max*]	77866/5.04	465	9	14%	gil402753	NCBInr
Functional catoegory 6: Protein destination and storage							
90*	Stem 28 kDa protein [*Glycine max*]	29218/8.75	74	8	29%	gil169898	NCBInr
91*	Stem 28 kDa protein [*Glycine max*]	29218/8.75	82	9	33%	gil169898	NCBInr
92*	Stem 28 kDa protein [*Glycine max*]	29218/8.75	61	7	30%	P15490	SwissProt
93	Vegetative storage protein, precursor [*Glycine max*]	29433/6.72	217	4	16%	gil72303	NCBInr

Table 3.1. Continued

ID	Protein Identification [species]	T. Mr/pI	MO	PM	SC	Acce. No.	Databases
94*	PS II stability/assembly factor HCF136, [*Arabidopsis thaliana*]	44133/6.79	61	10	28%	O82660	SwissProt
95*	Chaperonin precursor [*Pisum sativum*]	63287/5.85	105	10	24%	gil806808	NCBInr
96*	Chaperonin precursor [*Pisum sativum*]	63287/5.85	110	10	24%	gil806808	NCBInr
113	chaperonin 2 [*Glycine max*]	24022/5.44	249	4	33%	gil31465298	EST_others
97*	ER HSC70-cognate binding protein precursor [*Glycine max*]	73822/5.15	88	15	23%	gil2642238	NCBInr
98*	70 kDa heat shock protein [*Phaseolus vulgaris*]	72721/5.95	87	19	27%	gil22636	NCBInr
99*	Heat shock protein 70 [*Cucumis sativus*]	75480/5.15	164	16	25%	gil1143427	NCBInr
100*	Chaperonin groEL [*Ricinus communis*]	52461/4.77	127	12	32%	gil72958	NCBInr
101*	ER HSC70-cognate binding protein precursor [*Glycine max*]	73822/5.15	92	14	21%	gil2642238	NCBInr
135	60 kDa chaperonin [*Ricinus communis*]	52461/4.77	186	5	9%	gil134101	NCBInr
130	Copper chaperone homolog [*Glycine max*]	13753/4.66	92	3	20%	gil6525011	NCBInr
129	Metalloproteinase [*Glycine max*]	19001/5.82	135	5	19%	gil384337	NCBInr
76	Cyclophilin [*Oryza sativa (japonica cultivar-group)*]	25273/8.05	123	3	23%	gil34902534	NCBInr
102	Cyclophilin [*Phaseolus vulgaris*]	18376/8.36	265	7	33%	gil829119	NCBInr
103	Cyclophilin [*Glycine max*]	18395/8.7	174	2	15%	gil17981611	NCBInr
104*	Polyubiquitin 1 [*Phaseolus vulgaris*]	6547/6.51	67	8	63%	gil33327284	NCBInr
105*	Endopeptidase Clp ATP-binding chain cd4B [*Lycopersicon esculentum*]	102463/5.86	102	29	30%	gil9758239	NCBInr

Table 3.1. Continued

ID	Protein Identification [species]	T. Mr/pI	MO	PM	SC	Acce. No.	Databases
Functional catoegory 7: Transporters							
106	Voltage-dependent anion-selective channel [*Lotus corniculatus var. japonicus*]	29696/8.57	93	2	8%	gil36957183	NCBInr
Functional catoegory 10: Signal transduction							
63	Harpin binding protein 1 [*Glycine max*]	28471/7.88	131	3	10%	gil38679315	NCBInr
107*	Guanine nucleotide-binding protein beta subunit-like protein [*Glycine max*]	35985/7.62	70	11	40%	gil1256608	NCBInr
108	P21 protein [*Glycine max*]	22365/4.84	146	3	16%	P25096	SwissProt
Functional catoegory 11: Disease/defence							
109*	Ascorbate peroxidase 2 [*Glycine max*]	27180/5.65	154	12	58%	gil1336082	NCBInr
110*	Ascorbate peroxidase 2 [*Glycine max*]	27180/5.65	71	8	41%	gil1336082	NCBInr
111*	Catalase [*Glycine max*]	57043/6.80	123	15	32%	gil2661021	NCBInr
114	Stress-induced protein SAM22 [*Glycine max*]	16762/4.69	282	4	31%	P26987	SwissProt
115	PR1A precursor [*Glycine max*]	18108/8.20	221	4	25%	gil13479525	EST_others
116	Peroxiredoxin [*Hyacinthus orientalis*]	14120/5.43	98	2	21%	gil42565527	NCBInr
117	Peroxiredoxin [*Phaseolus vulgaris*]	28776/5.17	232	6	25%	gil11558244	NCBInr
123	Superoxide dismutase [*Glycine max*]	27881/5.60	308	11	27%	gil134646	NCBInr
131	Cu-Zn-superoxide dismutase [*Pinus pinaster*]	22328/6.11	103	2	13%	gil16798638	NCBInr
132	Lectin [*Glycine max*]	22568/9.59	155	4	18%	gil81238245	NCBInr
133	Thaumatin-like protein precursor [*Glycine max*]	17425/4.12	105	2	19%	gil15000431	EST_others
Functional catoegory 20: Secondary metabolism							

Table 3.1. Continued

ID	Protein Identification [species]	T. Mr/pI	MO	PM	SC	Acce. No.	Databases
118*	1-deoxy-D-xylulose 5-phosphate reductoisomerase [*Pueraria montana var. lobata*]	50808/5.83	87	12	28%	gil35187000	NCBInr
134	Chalcone reductase [*Glycine max*]	23143/8.63	81	2	10%	gil7588574	EST_others
Functional catoegory 12: Unclear classfication							
116	Hypothetical protein [*Oryza sativa (japonica cultivar-group)*]	24917/7.49	95	2	9%	gil22165076	NCBInr
136	Putative protein [*Arabidopsis thaliana*]	27880/8.31	93	3	10%	gil7269238	NCBInr

The data were classified according to protein function described by Bevan et al. (1998) and included an assigned spot number (Fig. 3.1). ID: Spot ID (* indicates the spot identified by MALDI-TOF, otherwise by LC-MS/MS); T. Mr/pI: theoretical molecular weight and pI; MO: MOWSE score; PM: the number of peptides matched; SC: the percentage of sequence coverage; Acce. No.: Accession number; ID method: identification method. The assigned protein of the best match was given with the species in which it has been identified and its accession number.

CHAPTER 4 IMPACT OF SOLAR ULTRAVIOLET-B ON THE PROTEOME IN SOYBEAN LINES DIFFERING IN FLAVONOID CONTENTS

ABSTRACT

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was used to systematically investigate the impact of solar ultraviolet-B (UV-B) radiation on the soybean leaf proteome. Two isolines of the Clark cultivar, the standard line with moderate levels of flavonoids and the magenta line with reduced flavonoids, were grown in the field with or without natural levels of UV-B. The first trifoliates were harvested for proteomic analysis after twelve day exposure. More than 300 protein spots were reproducibly resolved and detected on each gel. Statistical analysis showed that 67 protein spots were significantly ($P < 0.05$) modified by solar UV-B. Many more spots were affected by UV-B in the magenta line than in the standard line. Another 12 protein spots were not altered by UV-B but showed significantly ($P < 0.05$) different accumulations between the two lines, and for most spots the line-specific differences were also observed under UV-B exclusion. Most of the differentially accumulated spots were identified by mass spectrometry. The proteins were quite diverse, and were involved in metabolism, energy, protein destination/storage, protein synthesis, disease/defense, transcription, and secondary metabolism. The results suggest that high levels of flavonoids lead to a reduction in UV-B sensitivity at the proteomic level.

4.1. INTRODUCTION

Increases in chlorofluorocarbons in the atmosphere have depleted the earth's stratospheric ozone layer, leading to an increase in the levels of ultraviolet-B (UV-B: 280-320 nm) radiation that reaches the earth's surface (Madronich et al., 1998; Gleason et al., 1993; Blumthaler and Amback, 1990). Although UV-B radiation has important regulatory and photomorphogenic roles (Ballare et al., 1995), excessive UV-B radiation is clearly harmful. In general, a high level of UV-B causes reduced photosynthesis and growth (Germ et al., 2005; Ruhland et al., 2005), oxidative damages (Yannarelli et al., 2006ab), and damage to DNA (Bray and West, 2005).

Plants possess an array of adaptive responses to UV-B that allow them to prevent, mitigate or repair UV-B damage. We still do not have a complete understanding of the molecular basis of these responses, but they generally are the result of signal perception by receptor molecules and transduction of a response signal to the cellular machinery, a part of which may regulate gene expression (Ballare, 2003). Many researchers have studied the effects of UV-B on gene expression and have identified a number of UV-B responsive genes in plants. For example, photosynthetic genes may be down-regulated (A-H-Mackerness et al., 2001; Jordan et al., 1998; Surplus et al., 1998), while pathogenesis-related genes, the defencin gene (PDF 1, 2) (A-H-Mackerness et al., 2001, 1999), genes for flavonoid biosynthesis (A-H-Mackerness et al., 2001; Chappell and Hahlbrock, 1984) and antioxidant enzymes (A-H-Mackerness et al., 1998; Willekens et al., 1994) may be up-regulated by UV-B. Using microarray analysis, Casati and Walbot (2004) and Ulm et al. (2004) identified more than one hundred UV-B responsive genes in maize and *Arabidopsis*, respectively. However, most studies were conducted indoors

under artificial conditions with unrealistically high UV-B radiation as well as low ultraviolet-A (UV-A: 320-400 nm) and photosynthetically active radiation (PAR: 400-700 nm). Differences in levels of UV-A and PAR levels in field and controlled conditions may lead to differences in the magnitude or the direction of the responses of plants to UV-B radiation (e.g. Krizek, 2004). Casati and Walbot (2003) examined the response of gene expression in maize to solar UV-B under field conditions, and found several photosynthesis-associated genes were down-regulated and antioxidant-associated genes were up-regulated. Also, the genes involved in fatty acid metabolism and oxylipin biosynthesis were up-regulated by solar UV-B (Izaguirre et al., 2003). These results are the most comprehensive data currently available on the effects of solar UV-B on plant gene expression.

However, these studies only looked at mRNA levels, which may not necessarily translate into the quantity and quality of the final gene products, i.e. the proteins. There is a loose correlation between mRNA and protein levels, especially for chloroplast genes, which are usually controlled at the post-transcriptional level (A-H-Mackerness et al., 1997; Jordan et al., 1992). Moreover, many proteins undergo post-translational modifications (PTM) such as removal of signal peptides, phosphorylation and glycosylation, which are extremely important for protein activities and subcellular localizations. Therefore, changes at the mRNA level alone may not adequately assess the response to UV-B, and it is necessary to study the effects of UV-B at the protein level. There has been only limited research on the effects of UV-B on proteins, and most of this research focused on a single protein, such as PR-1 (Green and Fluhr, 1995), glutathione

reductase, ascorbate peroxidase, superoxide dismutase (Rao et al., 1996) or nitrite reductase (Migge et al., 1998).

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is a sensitive and powerful technique for resolving hundreds of proteins in parallel. Combined with mass spectrometry (MS), it allows rapid and reliable protein identification and can provide information about abundance and PTM. In recent years, proteomic-based technologies have been successfully applied to the systematic study of the proteomic responses in many plant species to a wide range of abiotic stresses, including drought (Pinheiro et al., 2005), nutrition deficiency (Alves et al., 2006), temperature (Yan et al., 2006; Sule et al., 2004), oxidative stress (Wang et al., 2004), herbicide (Castro et al., 2005), wound (Shen et al., 2003), anoxia (Chang et al., 2000), salt (Yan et al., 2005) and heavy metal (Labra et al., 2006). Casati et al. (2005) have used proteomic technologies to investigate the effects of UV-B on the proteome of the maize leaf. They found that UV-B radiation affected the accumulation of 178 protein spots and phosphorylated pyruvate phosphate dikinase.

Soybean (*Glycine max*) provides an important source of protein for human consumption and the animal industry and it has been the dominant oilseed produced since the 1960s. Soybean genotypes exhibit a wide range in sensitivity to UV-B radiation, due in part to differences in flavonoid content (Middleton and Teramura, 1993; Reed et al., 1992). The purpose of this UV-B exclusion study was to systematically examine the effects of solar UV-B on the soybean leaf proteome and to investigate whether flavonoids afforded protection against solar UV-B under field conditions. Two soybean isolines with different flavonoid content were used: the Clark standard line that produces

moderate levels of flavonol glycoside, and the Clark magenta line that has reduced flavonol glycoside levels (Buzzell et al., 1977).

4.2. MATERIALS AND METHODS

4.2.1. Plant materials and experiment design

Seeds of the standard and magenta isolines of the Clark cultivar of soybean were planted in pots in a greenhouse at the University of Maryland (College Park, MD) and allowed to germinate for 3 days. Following this period the plants were moved to the USDA South farm (Beltsville, MD) where they were separated into two UV-B treatment regimes. Half of the plants were placed inside either of two open-ended exclusion shelters made of polyester (DuPont, Circleville, OH, USA), which absorbs almost all solar radiation below 316 nm. The remaining plants were placed under another two shelters covered by clear Teflon (DuPont, Circleville, OH, USA), which is virtually transparent to solar UV radiation. The materials are similar in transmission properties in the UV-A and PAR wavelength. The plants beneath the polyester filters received very little UV-B radiation and served as controls for seasonal changes in temperature and PAR, etc. Plants were rotated every day and watered to minimize the occurrence of drought stress. The first trifoliates appeared on July 4th, 2005 and leaflet blades were harvested after 12 days from 3-5 plants for each sample. Five independent samples were harvested for each treatment replicate. The harvested samples were immediately frozen in liquid nitrogen, and then stored at -80^{o}C prior to analysis.

4.2.2. Protein extraction and 2D-PAGE

Frozen samples were ground with liquid nitrogen and incubated with 10% trichloroacetic acid (TCA) and 0.07% 2-mercaptoethanol in acetone for 1 hr at -20^{o}C. The precipitated proteins were pelleted and washed with ice-cold acetone containing 0.07% 2-mercaptoethanol to remove pigments and lipids until the supernatant was colorless. The pellet was vacuum dried, resuspended in resolubilization solution (9M urea, 1% CHAPS, 1% DTT, 1% pharmalyte) and sonicated to extract proteins. Insoluble tissue was removed by centrifugation at 21,000 X g for 20 min. Protein concentration was determined according to Bradford (1976) using a commercial dye reagent (Bio-Rad Laboratories, Hercules, CA) with BSA as a standard.

An IPGPhor apparatus (GE Healthcare, Piscataway, NJ) was used for isoelectric focusing (IEF) with immobilized pH gradient (IPG) strips (pH 3.0 - 10.0, linear gradient, 13 cm). The IPG strips were rehydrated for 12 hrs with 250µL rehydration buffer (8M urea, 2% CHAPS, 0.5% pharmalyte, 0.002% bromophenol blue) containing 350 µg proteins. The voltage settings for IEF were 500 V for 1 hr, 1000 V for 1 hr, 5000 V for 1 hr, and 8000 V to a total 46.86 kVh. Following electrophoresis, the protein in the strips was denatured with equilibration buffer (50mM Tris-HCl pH 8.8, 6M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 1% DTT) and then incubated with the same buffer containing 2.5% iodoacetamide instead of DTT for 30 min at room temperature. The second dimension electrophoresis was performed on a 12.5% gel using a Hoefer SE 600 Ruby electrophoresis unit (GE Healthcare, Piscataway, NJ). The gels were stained with Coomassie brilliant blue (CBB) G-250 (Newsholme et al., 2000) and scanned using a Personal Densitometer SI (GE Healthcare, Piscataway, NJ).

4.2.3. Image acquisition and data analysis

Densitometry images were analyzed with Image Master 2D-Elite (version 4.01) (GE Healthcare, Piscataway, NJ) software. Image analysis included the following procedures: spot detection, spot measurement, background subtraction, and spot matching. Only spots that were detected on all the five replicate gels were further analyzed. To correct the variability due to CBB staining, the spot volumes were normalized as a percentage of the total volume of all spots on the gel. The General Linear Model procedure (SAS PC version 6.04, SAS Institute, Inc., Cary, NC) was used for the different analysis of variance (ANOVA) to test for main effects of UV-B, lines, time and their interactions.

4.3. RESULTS

More than 300 protein spots were clearly separated and detected by 2D-PAGE despite the predominance of ribulose bisphosphate carboxylase/oxygenase (Rubisco). The 2D-PAGE gels were reproducible and had well separated spots, although as is frequently observed with proteome analysis, the dynamic range of protein accumulation was very large (Watson et al., 2003; Wilson et al., 2002). A representative gel image is presented in Fig. 4.1. Only spots that were significantly altered changed by UV-B or those that differed between the two lines were further analyzed. A total of 79 spots were selected, and no interaction between UV-B and line was detected for these 79 spots. The statistical data for the 79 spots are listed in Table 4.1, and magnified regions of several differentially accumulated proteins are presented in Fig. 4.2.

The 79 selected spots were divided into three groups. Spots in Group I included those that were increased by solar UV-B; this group had 31 spots (spot 1 - 31; Fig. 4.1; Table 4.1). Group II included 36 spots that were decreased by solar UV-B (spot 32 - 67; Fig. 4.1; Table 4.1). Group III included 12 spots that had different intensity between the two lines but were not altered by UV-B (spot 68 - 79; Fig. 4.1; Table 4.1). The accumulations of a total of 67 spots were changed by solar UV-B, and the total number of spots that increased in abundance was similar to the number of spots that decreased.

In group I, only 4 spots (spot 5, 27, 29, and 30) were increased in both lines. Twenty-three spots (spot 2, 4, 6 - 9, 11 - 16, 18 - 26, 28, 31) were increased only in the magenta line and 4 spots (spot 1, 3, 10, and 17) increased only in the standard line (Table 4.1). In group II, 14 spots (spot 32, 38 - 40, 44, 45, 47, 49, 51, 56-59, 61) were decreased in both lines, and 16 spots (spot 35 - 37, 41 - 43, 46, 48, 50, 52, 54, 55, 60, 64-66) were decreased only in the magenta line and, whereas 6 spots (spot 33, 34, 53, 62, 63, 67) decreased only in the standard line (Table 4.1). Many more proteins were responsive to solar UV-B in the magenta line than in the standard line. In group III, 11 spots (spot 68 - 70, 72 - 79) showed different intensities between the two lines even with no UV-B treatment (Table 4.1).

4.4. DISCUSSION

4.4.1. Impacts of solar UV-B and flavonoids on protein accumulation

In this study, the accumulation of 67 protein spots appears to be changed by solar UV-B, with the total number of spots increasing in abundance similar to the number of

spots decreasing. Casati et al. (2005) found that 178 maize leaf protein spots were altered by UV-B radiation, and that more protein spots increased than decreased after UV-B exposure. This disparity may be due to the dissimilar species used, or to the different conditions used in the experiments. The maize experiments were conducted using both field and greenhouse conditions and the field experiments included both exclusion and supplemental UV-B studies (Casati et al., 2005). In the present study, only UV-B exclusion was examined in the field.

More proteins were responsive to solar UV-B in the magenta line than in the standard line. Plants have evolved several mechanisms to cope with UV-B damage. One of the most important mechanisms is screening out UV-B radiation by accumulation of flavonoids in the leaf epidermis (Schmelzer et al., 1988; Robberecht and Caldwell, 1983). The increased response of the magenta line to UV-B may be related to its reduced flavonoid content. Of the group III spots, 11 spots (spot 68 - 70, 72 - 79) showed different intensities between the two lines even without UV-B treatment (Table 4.1). This might be due to the high UV-A and PAR levels in the solar radiation and/or oxidative stress caused by other stress factors such as high temperature. While flavonoids provide UV-B protection by absorbing radiation in UV-B region, they also are effective UV-A absorbers (Markstädter et al., 2001). Although it is less damaging on a photon basis than UV-B, UV-A comprises a much larger portion of the solar radiation than does UV-B. Also UV-A is able to penetrate to greater depths within the leaf than UV-B (Liakoura et al., 2003). The greater responses under UV-B exclusion in the magenta line could be related to its inability to synthesize flavonoids. Irradiation of the magenta line with solar UV radiation could have resulted in increased flux of UV-A

through the more transparent epidermis reaching mesophyll cells, as compared to the standard line capable of synthesizing flavonoids. Also, because flavonoids can act as antioxidants (Peng et al., 2003), their absence in the magenta line could also lead to greater oxidative stress, and this hypothesis is supported by evidence for greater oxidative responses in the magenta line (Fig. 2.1).

Taking into account the multiplicity of spots, we detected 47 unique proteins modified by UV-B radiation (Table 4.1). In maize, Casati et al. (2005) reported that 14 proteins that were represented with multiple spots showed opposite regulation by UV-B radiation. For example, three spots of the Rubisco large subunit were increased while another two spots of Rubisco large subunit were decreased by UV-B radiation (Casati et al., 2005). Similar results were observed for Rubisco activase and glycine cleavage system (GCV) P-protein in the present study. One spot (spot 3) of Rubisco activase was increased by UV-B, while another 3 spots (spot 44 - 46) of this protein were decreased. In the present study, most of the spots contained only one protein, but 3 spots contained two different proteins (spot 6: triosephosphate isomerase and superoxide dismutase; spot 7: ATP synthase CF1 epsilon subunit and 60 kDa chaperonin alpha subunit; spot 40: phosphoglycerate kinase and glutamine synthetase). UV-B may regulate one or both of the proteins in each spot; although at this stage of our investigation we could not distinguish between these possibilities.

4.4.2. Functional analyses of proteins responsive to UV-B

The identified proteins in this study were classified according to the functional categories described by Bevan et al. (1998) (Table 4.2). The proteins are quite diverse

and are involved in metabolism, energy, protein destination/storage, disease/defense, transcription, protein synthesis, and secondary metabolism. The functional group with the largest number of protein spots modulated by UV-B was the energy category. In this category, 30 protein spots were altered by UV-B exposure, 14 increased while 16 decreased (Table 4.1 and 4.2). Nine spots were identified as photosystem (PS) II oxygen-evolving enhancer (OEE) (spot 12 - 16) or subunits of PS I (spot 8 - 11), and all were enhanced by solar UV-B. Only one protein spot (spot 7: epsilon subunit of ATP synthase CF1) is involved in electron transport, and this spot was also increased by UV-B. Using microarray technology, Izaguirre et al. (2003) found that most genes encoding PS polypeptides were down-regulated by solar UV-B, but one gene, encoding the PS II OEE 23-kDa polypeptide, was up-regulated. Interestingly, all the responsive proteins related to PS were increased by solar UV-B in this study.

The other spots affected by UV-B in the energy category were identified as enzymes involved in primary carbon metabolism. Most of the spots, identified as Rubisco activase (spot 44 - 46), Rubisco small subunit (spot 42 and 43), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (spot 48 - 51), phosphoglycerate kinase (spot 40 and 52), phosphoribulokinase (spot 41 and 47) or other enzymes (spot 53 - 55), were decreased by UV-B radiation (Table 4.1 and 4.2). In addition to catalyzing reactions in the Calvin cycle, GAPDH is also reported to have protein kinase activity (Duclos-Vallee et al., 1998), to bind RNA (Nagy and Rigby, 1995), and to increase ribozyme (Sioud and Jespersen, 1996) and phosphotransferase activities (Engel et al., 1998). Other environmental stresses have been reported to increase GAPDH level (Chang et al., 2000; Yang et al., 1993; Russell et al., 1990), however, four spots of GAPDH were decreased

by UV-B in the present study. This inconsistency may be due to the multiple isoforms of GAPDH. Izaguirre et al. (2003) also found that some genes for Calvin cycle enzymes were down-regulated by solar UV-B. It is reported that the inhibition of UV-B on photosynthesis is associated with enzymatic, rather than PS II limitations (Keiller et al., 2003; Keiller and Holmes, 2001; Kolb et al., 2001; Xiong and Day, 2001). Sullivan and Teramura (1990) found that UV-induced reductions involving PSs in soybean occurred only at high internal CO_2 levels, suggesting that UV-B could lead to increased substrate limitations on photosynthesis. Also, some studies indicated that UV-B could reduce the carbohydrate levels in plants (Pancotto et al., 2005; Zhao et al., 2005; Quaggiotti et al., 2004; Ghisi et al., 2002). Consistent with these results, the enzymes involved in CO_2 assimilation were suppressed by solar UV-B in this study. The remaining spots (spot 3: Rubisco activase; spot 4: Rubisco large subunit; spot 5: ribulose-phosphate 3-epimerase; spot 6: triosephosphate isomerase) were increased by UV-B. Spot 4 is only a fragment of Rubisco large subunit because its sequence only matches the first half part of Rubisco and it has very low Mr. Therefore, an increase in spot 4 most probably indicates increased degradation of the Rubisco large subunit.

Increases in proteins related to PS and electron transport may lead to greater reducing power. However, ambient levels of UV-B may limit CO_2 fixation and reduce the regeneration of $NADP^+$ and ribulose bisphosphate, because the enzymes involved in the Calvin cycle were decreased. For example, GAPDH and phosphoglycerate kinase both are key enzymes in the regeneration of ribulose bisphosphate and $NADP^+$. Therefore, the photosynthetic electron transport chain may be over reduced, leading to the formation of superoxide radicals and singlet oxygen (Asada, 1999; Foyer et al.,

1994ab). We have found that solar UV-B radiation did cause oxidative stress in the two soybean lines.

The metabolism category includes 11 spots modified by UV-B (Table 4.2). Two of them (spot 1: GCV T- protein; spot 2: gamma-glutamyl hydrolase) increased in abundance, while 9 spots, identified as glutamine synthetase (spot 38 - 40), alanine aminotransferase (spot 35), GCV T- or P-protein (spot 32 - 34), and serine hydroxymethyltransferase (SHMT) (spot 36 and 37), were decreased by solar UV-B. These enzymes are involved in nitrogen metabolism. Glutamine synthetase catalyzes the assimilation of ammonium to glutamine using glutamic acid as its substrate (Chen and Silflow, 1996). Down-regulation of this enzyme under stress conditions has been reported, and this may be a protective mechanism because nitric oxide, an intermediate of nitrogen assimilation, is an active radical (Wang et al., 2004). It has been reported that UV-B can decrease nitrogen assimilation in some plants (Pancotto et al., 2005; Quaggiotti et al., 2004; Ghisi et al., 2002; Takeuchi et al., 2002; Balakumar et al., 1999). The down-regulation of enzymes involved in primary nitrogen and carbon metabolism indicates redirection of carbon and nitrogen resources into other pathways, such as those involved in repair or protection processes.

The protein destination and storage category includes 12 spots altered by solar UV-B (Table 4.2). Several spots (spot 57 - 61) of chaperonin and associated co-chaperones were decreased by solar UV-B. Other spots, identified as 60 kDa chaperonin (spot 7), vegetative storage protein (spot 20 and 23), chaperonin 2 (spot 21), copper chaperone homolog (spot 24), and cyclophilin (spot 22), were increased by UV-B radiation. Chaperones are proteins whose function is to assist other proteins in achieving

proper folding, and have been shown to accumulate in plants in response to many stresses (Yan et al., 2006; Wang et al., 2004). In this study, some chaperone spots (spot 7, 21, and 24) were increased while others (spot 57 - 61) were decreased by UV-B, and similar results were observed under UV-B stress by Casati et al. (2005). These results can be explained by the varied functions of chaperones. Some chaperones act to repair the potential damage caused by misfolding; some are involved in folding newly made proteins as they are extruded from the ribosome; and others are involved in transport across membranes (Leone et al., 2000; Georgopoulos and Welch, 1993).

Only six protein spots involved in the disease and defense category were altered by UV-B radiation (Table 4.2). Two of them, identified as catalase (spot 63) and peroxiredoxin (spot 64), were decreased by UV-B. Four spots, identified as ascorbate peroxidase (spot 26), superoxide dismutase (spot 6 and 25), and lectin (spot 27), were increased by UV-B. All six of these proteins, except lectin, are enzymes that destroy active oxygen species and are usually increased under stress conditions (Yan et al., 2006; Wang et al., 2004). In this study solar UV-B increased the total activity of ascorbate peroxidase, decreased the total activity of superoxide dismutase, and had no effect on catalase activity (Fig. 2.4 and 2.5). However, these measurements of total enzymatic activity may not reflect changes in the isoforms of the same enzyme. Our results suggest that changes in enzyme activity may not be accompanied by changes in protein quantity.

Only two spots in the secondary metabolism category (spot 65: 1-deoxy-D-xylulose 5-phosphate reductoisomerase; spot 66: chalcone reductase) were responsive to solar UV-B (Table 4.2). These two spots were decreased by solar UV-B in the magenta line but not in the standard line. Increases in leaf flavonoid concentration are the most

consistent response to supplemental UV-B (Searles et al., 2001). However, increases in proteins involved in the flavonoid biosynthetic pathway were not detected in the present study. Proteins involved in flavonoid synthesis may be in too low of abundance to be detected on these gels, because more than one hundred protein spots were identified and no other proteins associated with secondary metabolism were found.

One spot (spot 17) responsive to solar UV-B in the transcription category was identified as RNA-binding protein, and its abundance was increased by UV-B. The protein synthesis category had 3 spots that were altered by solar UV-B. Two of them (spot 18: 50S ribosomal protein; spot 19: 30S ribosomal protein) were increased in abundance in response to UV-B, while spot 56, translation elongation factor, decreased in abundance.

4.5 CONCLUSION

In summary, our results indicate that proteins related to the photosynthetic photosystems increased in abundance, while enzymes involved in primary carbon and nitrogen metabolism decreased, and substantiate that flavonoids act as screening and antioxidant compounds in protecting plants from UV-B radiation. No effects on proteins involved in signal transduction were detected, possibly because many of the proteins involved in the signal transduction occur in too low abundance to be detected in crude extracts, or because membrane proteins are usually under-represented on 2D-PAGE gels. This study provides new insights into the responses in the soybean leaf to solar UV-B radiation. Further studies are needed to better understand the molecular basis of the UV-B response in soybean.

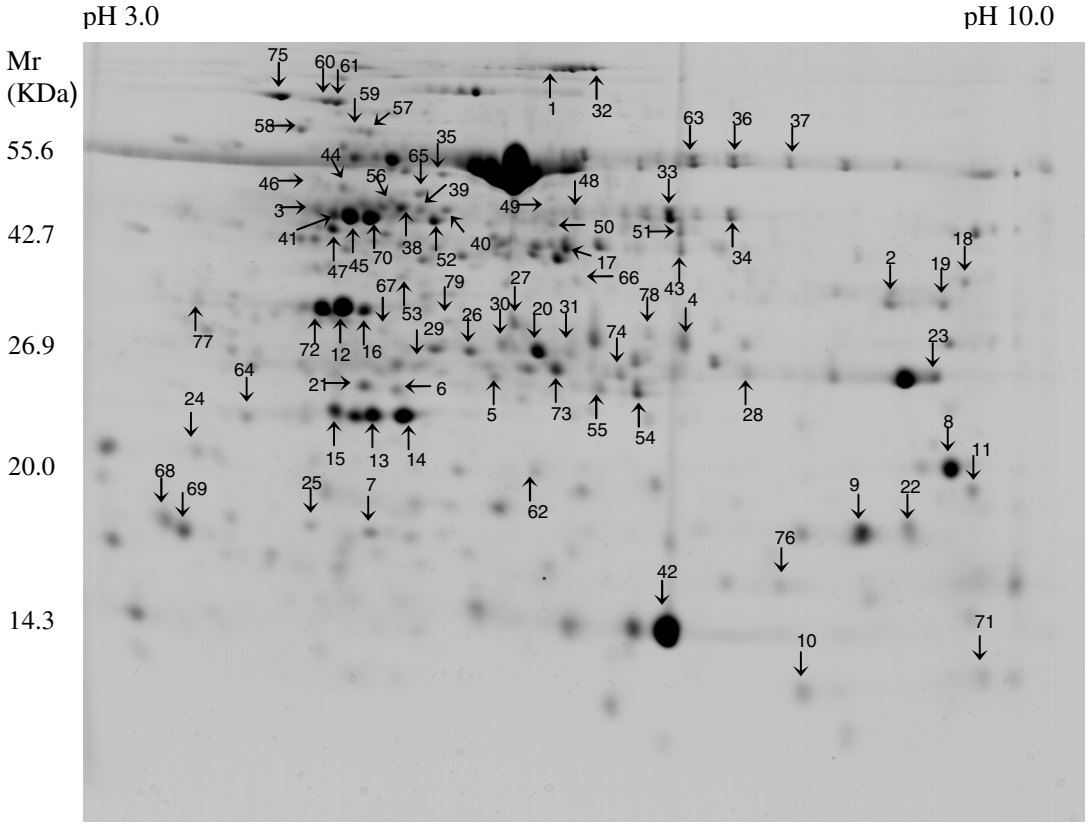

Fig. 4.1 Representative 2-D PAGE gel image of soybean leaves (cv. Clark, standard line) grown under Teflon shelter. Arrows indicate protein spots accumulated differently between different UV-B treatments or between different lines ($P < 0.05$). Numbers correlate with protein identifications and statistical analysis results listed in Table 4.1 and 4.2.

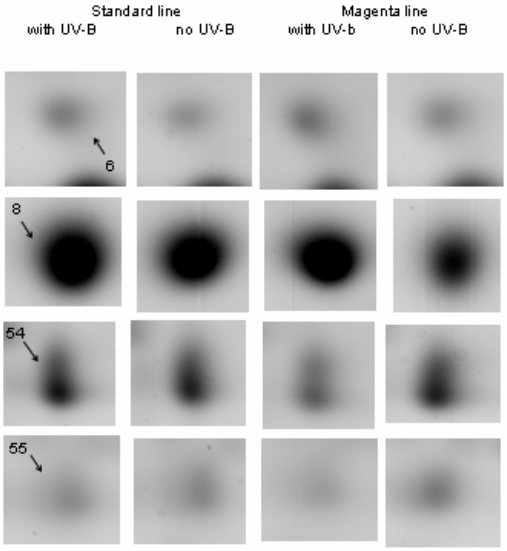

Fig. 4.2 Magnified regions of several differentially accumulated proteins in two lines of soybean growing under different UV-B conditions. Numbers correlate with statistical analysis results listed in Table 4.1 and 4.2.

Table 4.1. Relative abundance of differentially accumulated protein spots.

ID	Protein	Relative abundance* (Mean ±SD)			
		Standard line		Magenta line	
		With UV-B	No UV-B	With UV-B	No UV-B
	Group I: Spots increased by solar UV-B				
1	GCV P-protein	0.310±0.03[a]	0.256±0.02[b]	0.247±0.01[b]	0.239±0.01[b]
2	Gamma-glutamyl hydrolase	0.363±0.01[a]	0.340±0.02[a]	0.366±0.02[a]	0.294±0.02[b]
3	Rubisco activase	0.440±0.02[a]	0.361±0.03[b]	0.434±0.04[ab]	0.375±0.02[b]
4	Rubisco rbcL	0.379±0.03[a]	0.363±0.03[a]	0.451±0.02[b]	0.354±0.02[a]
5	Ribulose-phosphate 3-epimerase	0.361±0.02[a]	0.252±0.02[b]	0.333±0.02[a]	0.260±0.01[b]
6	Triosephosphate isomerase	0.236±0.01[a]	0.216±0.01[a]	0.266±0.01[b]	0.217±0.01[a]
7	Superoxide dismutase ATP synthase CF1 60 kDa chaperonin	0.190±0.02[a]	0.161±0.01[a]	0.276±0.02[b]	0.170±0.01[a]
8	PSI subunit D	1.411±0.04[a]	1.401±0.06[a]	1.346±0.06[a]	0.933±0.05[b]
9	PSI subunit IV A	1.167±0.04[a]	1.076±0.09[a]	1.152±0.04[a]	0.915±0.04[b]
10	PSI PsaN subunit	0.148±0.02[a]	0.098±0.02[b]	0.128±0.01[ab]	0.090±0.01[b]
11	PSI D2 subunit	0.154±0.02[a]	0.141±0.01[a]	0.157±0.02[a]	0.089±0.01[b]
12	PSII OEE protein 1	2.487±0.07[a]	2.405±0.04[a]	2.311±0.04[a]	2.154±0.03[b]
13	PSII OEE protein 2	1.162±0.04[ab]	1.080±0.03[a]	1.204±0.02[b]	0.993±0.02[c]
14	PSII OEE protein 2	1.627±0.07[a]	1.622±0.06[a]	1.764±0.04[a]	1.472±0.04[b]
15	PSII OEE protein 2	0.688±0.03[ab]	0.633±0.02[ac]	0.708±0.03[b]	0.599±0.01[c]
16	PSII OEE protein 3	0.577±0.03[a]	0.554±0.02[a]	0.579±0.02[a]	0.494±0.01[b]
17	RNA-binding protein	0.694±0.03[a]	0.630±0.04[b]	0.705±0.03[a]	0.657±0.02[ab]
18	50S ribosomal protein	0.137±0.01[a]	0.136±0.01[a]	0.140±0.01[a]	0.106±0.01[b]
19	30S ribosomal protein	0.235±0.01[ab]	0.215±0.01[a]	0.259±0.01[b]	0.185±0.01[c]
20	Vegetative storage protein	1.042±0.04[a]	0.921±0.13[a]	1.045±0.05[a]	0.713±0.02[b]
21	Chaperonin 2	0.364±0.01[ab]	0.346±0.01[ac]	0.392±0.01[b]	0.334±0.01[c]
22	Cyclophilin	0.280±0.02[a]	0.242±0.03[ab]	0.257±0.02[a]	0.182±0.02[b]
23	Vegetative storage protein	0.530±0.02[a]	0.467±0.06[a]	0.465±0.04[a]	0.353±0.03[b]
24	Copper chaperone homolog	0.094±0.01[a]	0.072±0.01[a]	0.100±0.01[a]	0.052±0.01[b]
25	Cu-Zn-superoxide dismutase	0.089±0.01[ab]	0.072±0.01[a]	0.110±0.01[b]	0.079±0.01[a]
26	Ascorbate peroxidase	0.421±0.01[a]	0.434±0.02[a]	0.439±0.01[a]	0.351±0.01[b]
27	Lectin	0.318±0.02[a]	0.246±0.03[b]	0.297±0.02[ab]	0.194±0.01[c]
28	Putative protein	0.160±0.01[a]	0.132±0.03[a]	0.138±0.01[a]	0.093±0.01[b]
29	Unidentified	0.188±0.01[a]	0.142±0.01[b]	0.196±0.01[a]	0.145±0.01[b]
30	Unidentified	0.398±0.02[a]	0.310±0.03[b]	0.431±0.01[a]	0.239±0.01[c]
31	Unidentified	0.211±0.01[a]	0.196±0.03[a]	0.217±0.01[a]	0.143±0.01[b]
	Group II: Spots decreased by solar UV-B				
32	GCV P-protein	0.303±0.02[a]	0.438±0.05[b]	0.255±0.01[c]	0.345±0.03[ab]
33	GCV T-protein	0.677±0.04[a]	0.778±0.04[b]	0.585±0.02[c]	0.642±0.02[ac]
34	GCV T-protein	0.292±0.02[a]	0.337±0.01[b]	0.260±0.01[a]	0.287±0.01[a]

Table 4.1. Continued

ID	Protein	Relative abundance* (Mean ±SD)			
		Standard line		Magenta line	
		With UV-B	No UV-B	With UV-B	No UV-B
35	Alanine aminotransferase	0.074±0.01[ab]	0.101±0.02[a]	0.060±0.01[b]	0.098±0.01[ac]
36	SHMT	0.511±0.03[a]	0.526±0.01[a]	0.510±0.02[a]	0.586±0.03[b]
37	SHMT	0.224±0.01[a]	0.216±0.02[a]	0.272±0.01[b]	0.315±0.01[c]
38	Glutamine synthetase	0.632±0.03[a]	0.774±0.04[b]	0.586±0.02[a]	0.704±0.02[c]
39	Glutamine synthetase	0.185±0.01[a]	0.221±0.02[b]	0.155±0.01[c]	0.193±0.01[ab]
40	Phosphoglycerate kinase Glutamine synthetase	0.145±0.03[a]	0.238±0.03[b]	0.132±0.01[a]	0.206±0.01[b]
41	Phosphoribulokinase	0.460±0.02[a]	0.466±0.03[a]	0.384±0.02[b]	0.434±0.01[a]
42	Rubisco rbcS2	5.306±0.22[a]	5.622±0.18[a]	5.853±0.20[a]	6.549±0.13[b]
43	Rubisco rbcS4	0.280±0.01[a]	0.306±0.01[ab]	0.293±0.01[a]	0.330±0.02[b]
44	Rubisco activase	0.142±0.02[a]	0.233±0.01[b]	0.136±0.01[a]	0.189±0.01[c]
45	Rubisco activase	1.315±0.13[a]	1.699±0.04[b]	0.970±0.03[c]	1.273±0.05[a]
46	Rubisco activase	0.050±0.01[ab]	0.069±0.01[ac]	0.047±0.01[b]	0.071±0.01[c]
47	Phosphoribulokinase	0.444±0.04[a]	0.676±0.02[b]	0.429±0.01[a]	0.643±0.02[b]
48	GAPDH	0.345±0.03[a]	0.407±0.03[ab]	0.356±0.02[a]	0.431±0.02[b]
49	GAPDH	0.103±0.02[a]	0.160±0.02[b]	0.091±0.01[a]	0.148±0.01[b]
50	GAPDH	0.117±0.02[ab]	0.153±0.01[ac]	0.108±0.01[b]	0.159±0.01[c]
51	GAPDH	0.341±0.02[a]	0.429±0.02[b]	0.346±0.03[a]	0.505±0.02[c]
52	Phosphoglycerate kinase	0.502±0.02[a]	0.527±0.03[a]	0.536±0.01[a]	0.619±0.02[b]
53	Ferredoxin NADP reductase	0.165±0.01[a]	0.204±0.01[b]	0.170±0.01[a]	0.176±0.01[a]
54	Carbonic anhydrase	0.679±0.04	0.695±0.04[a]	0.640±0.05[a]	0.832±0.05[b]
55	Carbonic anhydrase	0.298±0.02[ab]	0.330±0.02[a]	0.269±0.02[b]	0.384±0.018[c2]
56	Translation elongation factor	0.133±0.01[a]	0.178±0.01[b]	0.124±0.02	0.202±0.01[c]
57	Chaperonin precursor	0.179±0.01[a]	0.214±0.01[b]	0.170±0.01[a]	0.231±0.01[b]
58	Chaperonin groEL	0.301±0.02[a]	0.353±0.01[b]	0.262±0.02[a]	0.352±0.01[b]
59	Chaperonin precursor	0.121±0.01[a]	0.161±0.01[b]	0.095±0.01[c]	0.153±0.01[b]
60	ER HSC70-cognate binding protein	0.350±0.02[a]	0.383±0.02[a]	0.298±0.02[b]	0.361±0.02[a]
61	ER HSC70-cognate binding protein	0.374±0.01[a]	0.466±0.01[b]	0.337±0.01[c]	0.428±0.01[d]
62	Metalloproteinase	0.103±0.01[a]	0.134±0.01[b]	0.093±0.01[a]	0.080±0.01[a]
63	Catalase	0.508±0.03[a]	0.691±0.06[b]	0.502±0.03[a]	0.526±0.02[a]
64	Peroxiredoxin	0.232±0.03[a]	0.246±0.01[a]	0.240±0.02[a]	0.307±0.01[b]
65	1-deoxy-D-xylulose 5-phosphate reductoisomerase	0.277±0.01[a]	0.269±0.02[a]	0.234±0.01[b]	0.298±0.01[a]
66	Chalcone reductase	0.106±0.01[a]	0.120±0.01[a]	0.087±0.01[b]	0.110±0.01[a]
67	Unidentified	0.074±0.01[a]	0.095±0.01[b]	0.069±0.01[a]	0.063±0.01[a]
Group III: Spots not altered by UV-B but had different accumulations between the two lines					
68	GCV H-protein	0.223±0.03[ab]	0.266±0.02[a]	0.200±0.03[b]	0.193±0.01[b]
69	GCV H-protein	0.427±0.04[ab]	0.461±0.02[a]	0.356±0.04[b]	0.348±0.01[b]

Table 4.1. Continued

ID	Protein	Relative abundance* (Mean ±SD)			
		Standard line		Magenta line	
		With UV-B	No UV-B	With UV-B	No UV-B
70	Rubisco activase	1.326±0.08[a]	1.466±0.05[a]	1.039±0.04[b]	1.156±0.05[b]
71	PSII 10 KD peptide	0.095±0.02[a]	0.165±0.03[ab]	0.210±0.04[b]	0.237±0.03[b]
72	PSII OEE protein 1	1.440±0.05[a]	1.396±0.02[ab]	1.315±0.06[bc]	1.209±0.03[c]
73	Carbonic anhydrase	0.576±0.03[a]	0.527±0.04[a]	0.458±0.02[b]	0.450±0.02[b]
74	Carbonic anhydrase	0.173±0.01[a]	0.167±0.02[a]	0.127±0.02[b]	0.107±0.01[b]
75	Heat shock protein 70	0.819±0.04[a]	0.839±0.02[a]	0.872±0.04[ab]	0.976±0.05[b]
76	PR1 A precursor	0.133±0.01[ab]	0.187±0.02[a]	0.115±0.02[bc]	0.084±0.01[c]
77	Thaumatin-like protein	0.146±0.01[a]	0.192±0.03[a]	0.306±0.03[b]	0.285±0.02[b]
78	Unidentified	0.108±0.01[a]	0.120±0.01[a]	0.074±0.01[b]	0.074±0.01[b]
79	Unidentified	0.082±0.01[ab]	0.086±0.01[a]	0.061±0.01[b]	0.041±0.01[b]

* Different letters indicate significantly different means separated by least significant difference ($P \leq 0.05$). Spot numbers (ID) correspond to Fig. 4.1.

Table 4.2. Functional distribution of protein spots responsive to UV-B

UV-B Regulation	Protein Spot number *							
	Energy	Metabolism	Protein destination storage	Transcription	Protein synthesis	Disease defence	Secondary metabolism	Unclear
Up	3-16	1, 2	7, 20-24	17	18, 19	6, 25-27		28-31
Down	40-55	32-40	57-62		56	63, 64	65, 66	67

Protein spots were grouped according to the functional categories described by Bevan et al. (1998). * Spot numbers correspond to Fig. 4.1.

CHAPTER 5: OVERALL CONCLUSIONS

Even though a number of studies have been conducted on the response of plants to UV-B, we still lack a very detailed understanding of many of the mechanisms of damage by UV-B and the protective mechanisms that have evolved in terrestrial plants. For example, many studies have shown that UV-B increases lipid peroxidation (Yannarelli et al., 2006a; Hideg et al., 2003; Costa et al., 2002; Alexieva et al., 2001). However, all these experiments were conducted in growth chamber or greenhouse under artificial conditions, and there are questions as to the applicability of indoor studies to field conditions. In field supplementation studies, UV-B increased lipid peroxidation in buckthorn (Yang et al., 2005) and *Picea aspirate* (Yao and Liu, 2007), but its effect varied among soybean cultivars (Yanqun et al., 2003). This study indicates that ambient solar UV-B radiation increased lipid peroxidation and oxidative stress in soybean.

The production of active oxygen species (AOS) as a result of exposure to UV-B is one possible means of damage by UV-B. However, there is very limited information about how plants irradiated with UV-B generate AOS. UV-B exposure enhanced NADPH-oxidase in *Arabidopsis* (Rao et al., 1996), and the involvement of NADPH-oxidase in H_2O_2 generation has been demonstrated in plants (Rao et al., 1996; Moller and Lim, 1986). However, Wang et al. (2006) reported that AOS induced by UV-B was not from NADPH-oxidase. This study suggest that the photosynthetic electron transport chain may be an AOS source under solar UV-B radiation, because the proteins related to photosystems were increased while enzymes involved in the Calvin cycle were decreased. This could cause the photosynthetic electron transport chain to be

overreduced and generate superoxide radicals and singlet oxygen (Asada, 1999; Foyer et al., 1994ab). Also H_2O_2 content was not altered by solar UV-B. Therefore, the increased lipid peroxidation may be caused by superoxide radicals and/or singlet oxygen rather than H_2O_2.

Solar UV-B radiation manipulated AOS metabolism mainly by decreasing SOD activity and increasing the activities of APX, CAT and GR. This resulted in decreased AsA and increased DHA. This study and others (Carletti et al., 2003; Rao et al., 1996; Strid, 1993) suggest that peroxidase-related enzymes like APX, rather than SOD, may have an important role in the control of AOS under varying UV-B conditions. However, only the total activities of these enzymes were determined in this study and total activities may not adequately reflect compartment-specific changes. UV-B could also differentially regulate enzyme isoforms (Yannarelli et al., 2006b; Rao et al., 1996; Willekens et al., 1994; Murali et al., 1988), leading to alterations in AOS metabolism that may not be detected in studies of the proteome. Further studies are needed to examine the response of different enzyme isoforms to solar UV-B radiation.

Many attenuation studies indicate that ambient solar UV-B inhibits photosynthesis (Albert et al., 2005; Ruhland et al., 2005; Xiong et al., 2002; Kolb et al., 2001; Xiong and Day, 2001; Krause et al., 1999; Lingakumar et al., 1999). Although photosynthesis was not examined in this study, our results suggest that ambient solar UV-B radiation may reduce photosynthesis, through enzymatic, rather than photosystem, limitations, because the proteins related to the photosystems were increased in abundance while enzymes involved in the Calvin cycle were decreased. Consistent with our results, other studies (Keiller et al., 2003; Keiller and Holmes, 2001; Kolb et al., 2001; Xiong and

Day, 2001; Allen et al., 1998) also reported that impairments in photosynthesis were associated with light-independent enzymatic, rather than photosystem II, limitations. Therefore the frequently reported effect of UV-B on photosystem II may not be the only mechanism by which UV-B limits photosynthetic carbon assimilation.

Alterations in nitrogen metabolism by UV-B could be another potential impact of exposure to UV-B radiation. Down-regulation of nitrogen assimilation by UV-B in plants has been demonstrated by other researchers (Pancotto et al., 2005; Quaggiotti et al., 2004; Ghisi et al., 2002; Takeuchi et al., 2002; Balakumar et al., 1999). Our results also suggest that primary nitrogen metabolism could be decreased by solar UV-B, because the accumulation of enzymes involved in nitrogen assimilation was decreased under solar UV-B radiation. This may be a protective mechanism because nitric oxide, an intermediate of nitrogen assimilation, is an active radical (Wang et al., 2004). Therefore, down regulation of nitrogen assimilation could reduce the generation of this radical. In general, the down-regulation of primary nitrogen and carbon metabolism could indicate a redirection of carbon and nitrogen resources into other pathways such as those involved in repair or protection processes. Also reduction in primary metabolism could have other impacts on overall plant performance such as slower growth.

In addition to production of the radical scavenging system and possible metabolic down regulation, plants have evolved several other mechanisms to cope with UV-B damage. One of the most important mechanisms is screening out UV-B radiation by accumulation of flavonoids in the leaf epidermis (Schmelzer et al., 1988; Robberecht and Caldwell, 1983). Increases in leaf flavonoid concentrations are the most consistent response to supplemental UV-B (Searles et al., 2001). However, increases in proteins

involved in the flavonoid biosynthetic pathway were not detected in the present study. Proteins involved in flavonoid synthesis may be in too low abundance to be detected on these gels, because more than one hundred protein spots were identified and only two protein spots were found to be associated with secondary metabolism. Also in the present study, only twelve-day-old leaves were examined for proteome. It is possible that other factors such as UV-A, or PAR in the field may have altered these proteins and masked any single effects of UV-B. Further extensive studies are needed to better understand the response of flavonoid biosynthesis to environmental factors.

Even though no increases in proteins involved in flavonoid synthesis were found in this study, this study supports the evidence that suggests that flavonoids act as screening compounds and antioxidants in protecting plants from UV-B radiation. This is based on the findings that the magenta line, with reduced flavonoid levels, had greater oxidative and proteomic responses than the standard line and this was not affected by UV treatment. The differences between the lines could have been due to unknown genetic differences in the lines or pleiotropic responses. However, if these lines are genetically identical except for flavonol contents, then this suggests that high UV-A, PAR, temperature, or other environmental factors may have differentially impacted the proteome and AOS metabolism in the field. Since flavonoids are also effective UV-A absorbers and antioxidants the magenta line, in addition to increased UV-B exposure, would have also been exposed to higher levels of UV-A in the mesophyll than the standard line and thus could be more effected by UV-A then the normal Clark line. The role of UV-A and other environmental factors in producing AOS and proteomic response needs to be studied more extensively in field conditions.

In summary, our studies indicate that ambient solar UV-B radiation caused oxidative stress in the soybean leaf and that the AOS may be generated through the electron transport chain in the chloroplast. Also proteome analysis indicated that UV-B decreased primary carbon and nitrogen metabolism by enzymatic limitations. As expected, the presence of flavonoids in the normal compared to the flavonoid deficient line modified these responses. This study also suggests that solar UV-A and other environmental factors could modify the proteome and AOS metabolism independent of UV exposure. Further studies are needed to better understand the molecular basis of the UV-B response and to elucidate the effect of solar UV-A radiation and other environmental factors on the proteome and AOS metabolism.

APPENDIX PICTURE OF THE SHELTERS IN THE FIELD

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