Principal Investigator: Bob B. Buchanan
Student: Ruixuan Zhang, Genetics and Plant Biology Major
Supply and Expense Amount Requested: $2,000
Project Title: ***Probing the Long-Term Viability of Ancient Chinese Sacred Lotus Seeds: Special Redox Regulation of Cotyledon and Embryonic Axis Proteins***

Abstract

A class of regulatory proteins, thioredoxin (Trx) *h*, with an active disulfide redox site catalyzes reversible reduction-oxidation (redox) regulation of many disulfide proteins of seeds. Studies show that in the oxidative deactivation of many seed proteins, sulfhydryl (-SH) groups of two cysteine amino acid residues form a disulfide bond (S-S)[1]. During seed maturation, storage proteins are stabilized via oxidation to form S-S bonds; in germination these proteins are activated via reduction [2(-SH)][2] (Appendix, Fig. 1). My specific interests are to study the redox status of proteins in the cotyledon and the embryo in ancient Chinese sacred lotus (*Nelumbo nucifera*) seeds collected from Liaoning, China. Although >1200 years old, the seeds still have a green, fleshy embryonic axis and are able to germinate. Since proteins in the cotyledon provide the main nitrogen reserves and embryonic axis proteins must be active in order to sustain the embryo, their redox status may be significantly different in ancient sacred lotus (SL) seeds. Hence, characterizing these proteins with respect to their function and redox regulation is key to understanding the seeds' longevity. To decipher this phenomenon, I will use *in vitro* and *in vivo* reduction assays on cotyledon and embryonic extracts to identify proteins that are redox-regulated by Trx and track their redox status in different aged seeds. A technique to label reduced proteins specifically, coupled with conventional two-dimensional gel electrophoresis, separates individual proteins, which can then be sequenced[3]. Knowledge of SL seed proteins' redox status and their regulation may help understand parallel regulatory mechanisms in other plants and humans[4], since in humans, Trx homologues serve as antioxidants to retain redox balance in cells[5].

Background and Justification

In cereal grains, like barley and wheat, mature seeds contain an embryo, the rudimentary plant resulting from a fertilized egg, and the endosperm, the nutritive tissue with storage reserves surrounding the embryo. Seed germination induces activated proteases in seeds to break-down (hydrolyze) storage proteins to nourish the embryo[6]. Proteins in both structures become biochemically inactive during maturation, desiccation and dormancy[1], which helps protect them from degradation/denaturation due to stresses on the system during these processes. And, as seeds age, proteins become progressively more oxidized[7]. Storage reserve proteins, lipids and carbohydrates are garnered in cereal (monocots) endosperm and the cotyledons of SL (a dicot). During germination, these reserves are mobilized to perform metabolic processes and hydrolyzed to provide energy and building blocks for embryo development[8,9]. Proteins in the cotyledon and embryonic axis, the major axis of polarity in the embryo, play major roles during germination. They are (1) reduced to become active in mobilizing and utilizing cotyledon resources and (2) solubilized by reduction and hydrolyzed to provide nitrogen, in the form of amino acids, for seedling development and growth.[1]

Activation and deactivation of seed storage proteins are regulated by redox via the Trx *h* system (Fig. 2) in the cotyledon, endosperm and embryo. Trxs are small, ~12kDa proteins, that are widely, if not universally, found in plants, animals and bacteria. This system is most complex in land plants. All Trxs, including *h*-type, contain two catalytically-active, cysteinyl residues in reduced form[10]. The availability of two -SH groups in its cysteine residues enables Trx to reduce disulfide bond (S-S) residues of participating cysteines in a polypeptide chain via two sequential thiol-disulfide exchange reactions (Fig. 3)[11]. Oxidized seed proteins are resistant to proteolysis but can be activated via reduction to the sulfhydryl or -SH state. In the Trx *h* system, NADPH reduces oxidized Trx via NADPH/Trx reductase. NADPH can also reduce the oxidized form of glutathione (GSSG) via glutathione reductase (GR), a tripeptide cellular reducing agent, yielding its reduced form (GSH). Alternatively, GSH can reduce glutaredoxin (Grx) which is a also a small disulfide reductase. Reduced Trx, GSH and Grx can reduce oxidized target proteins.[12,13,14] Because of Trx's ability to undergo reversible redox change, it often operates as a metabolic regulator[14] in processes, such as seed germination and photosynthesis[7,10]; thus it has become of interest for its potential in regulating proteins of the embryonic axis and cotyledon in ancient Chinese SL seeds.

Anatomically SL, *Nelumbo nucifera* (Fig. 4), bears closer resemblance to many monocotyledonous (having a single cotyledon) cereals, like rice, wheat and barley, than eudicotyledons (seeds having two cotyledons) in terms of leaf venation, endosperm and root structure. Some biologists suggest the two large cotyledons of SL seeds derived from cleavage of a single cotyledon, which implies that the plant could be a monocotyledon[15]. Preliminary results from our experiments on modern sacred lotus seeds reveal that like rice and oats, the main storage proteins (~70%) in SL cotyledons are glutelins, proteins soluble in dilute acid and base.[16] Partially due to similarities to monocot cereals, it is reasonable to apply results from cereal grain biochemistry to studies of ancient SL seeds.

In my research I will use ancient SL seeds excavated from a dry Holocene lakebed in Xipaozi village, Liaoning Province in northeastern China; a project carried out in 1996 by an international group of scientists led by Dr. Jane Shen-Miller from UCLA. Progenies of ancient seeds that were germinated by Dr. Shen-Miller will be used initially to set up the Trx *h* system in SL seeds. The 60 recovered ancient seeds range in age and include ones >1,200 years old, as determined by ^{14}C dating of the seed coat[17]. In these seeds, the embryonic axis, flanked by two large cotyledons, surprisingly remains green in dried, opened modern SL seeds as well as in the intact, ancient SL seeds (Fig. 5). Despite their long-term quiescent state and centuries-old exposure to low-dosage gamma radiation, many ancient SL seeds germinate[18]. Dr. Shen-Miller and colleagues explained this by: (1) the protective role of heat-resistant proteins and a fruit wall (pericarp) nearly impervious to water, air and microbes that could damage the biochemical content of the embryo and cotyledon, and (2) active proteins, enzymes and DNA repair mechanisms[10].

A primary objective of my studies is to examine differences in redox status of cotyledon and embryonic proteins as SL seeds age. Indirect evidence suggests that, as seeds senesce, embryonic proteins become more reduced than cotyledon counterparts because they must remain active to maintain life in the embryo. Maintaining these proteins in their active, reduced state may prepare the embryo for germination, giving it a head start with an active protein supply. If this is correct, these active proteins likely function in biochemical pathways essential to SL seeds' viability. I will also follow the redox status of proteins during germination to confirm that storage proteins become progressively reduced and digested as seeds germinate. Ultimately, I

plan to determine which proteins are Trx targets. Knowledge of redox mechanisms and redox status of embryonic and cotyledon proteins in living SL seeds will aid in understanding how SL seeds remain viable for extended periods—or why some become nonviable and dysfunctional.

Objectives and Hypotheses

Much research on cereal grains has led to the conclusion that proteins and enzymes are active in their reduced (-SH HS-) form and inert in their oxidized (S-S) form. Therefore, it is likely that in contrast to other seeds (which have shorter life spans), sacred lotus seed proteins are especially redox regulated so that they remain more reduced or are retained in easily reducible forms in order for the seeds to exhibit prolonged viability. My hypothesis is that compared to the cotyledon proteins, embryonic proteins are more reduced or easily reducible. This observation would correlate with dormancy studies which reveal that cotyledon proteins remain as immobilized metabolic storage reserves and embryonic proteins must remain active to sustain the embryo. As the seeds senesce, cotyledon proteins are stabilized via increasing oxidation and proteins in the embryonic axis must remain reduced. Consequently, the difference in cotyledon and embryonic proteins' redox states should be proportional to the age of the sacred lotus seeds. In reference to several publications, [9, 19] I expect the activity and reduced form of Trx *h* to increase during germination. Imbibition may induce both the cotyledon and embryonic axis proteins to undergo activation by gradual reduction of disulfide bonds until reaching their peaks, followed by decreasing reduction of storage proteins due to their mobilization and breakdown needed to nourish the germinating embryo.

The reversible deactivation and activation of many proteins in the seed tissues is regulated by redox via a vast array of cellular reducing agents such as: Trx *h*, GSH and glutaredoxin (Grx). Since research in Professor Buchanan's laboratory focuses on thioredoxin (Trx) and its regulatory pathways in various land plants, my study proposes that thioredoxin *h* may have a crucial role in the special redox regulation of sacred lotus proteins. Ideally, these results will contribute to our understanding of the longevity of the ancient Chinese sacred lotus and possibly other seeds.

Methods

Beginning in Summer 2010 and continuing into the fall semester, I will conduct *in vitro* and *in vivo* reduction assays of proteins extracted from 1996 and 2004 *China Antique* variety of SL seeds, provided by Dr. Shen-Miller. These seeds are progenies of ancient *China Antique* SL seeds that were germinated by Dr. Shen-Miller. This analysis will identify proteins targeted by Trx *h* and another common cellular reductant, glutathione (GSH). Embryonic axis and cotyledon tissues will be separated and different classes of proteins will be sequentially extracted, based on their solubility in water and various aqueous solutions[16]. After extraction, each protein fraction will be subjected to a series of *in vitro* reductions using a nonphysiological reductant (DTT), Trx *h* and GSH systems and a negative control (Fig. 6). Monobromoibimane (mBBr), a thiol-reactive fluorescent probe, will mark reduced proteins (-SH) by their absorption at λ_{UV}=394. The fate of newly generated sulfhydryl groups will be followed on SDS/PAGE gels and class(es) of proteins exhibiting redox changes when Trx and GSH are applied will be identified. Trx-targeted and other reduced proteins will be isolated by conventional two-dimension polyacrylamide gel: isoelectric focusing/reducing SDS/PAGE and identified by amino acid sequencing using

proteomics—mass spectrometry. A similar procedure of *in vivo* reduction experiments will follow to assay the native redox state of proteins. A qualitative and quantitative analysis of protein bands from 1996 and 2004 SL seeds will be used to discern differences in the redox status of proteins from the cotyledon and embryonic axis.

Germination of modern lotus seeds and determination of the *in vivo* redox status of resident proteins will begin late in the fall and continue in the spring. Changes in the *in vivo* redox status of cotyledon and embryonic axis proteins will be tracked during germination. At select times post-water addition and imbibtion[16], embryonic axes and cotyledons will be excised and redox state of the proteins determined as described. Graphs of total Trx *h*-targeted storage proteins extracted and their relative reduction—fluorescence to total protein ratio--versus the time course of germination can determine if SL seed proteins undergo reduction during germination as in other plants. Similar experiments on available older seeds will reveal the extent to which reduction occurs in new vs. older seeds.

During Spring 2011, proteins will be extracted from *ancient* SL seeds provided by Dr. Shen-Miller; in-vivo reduction experiments will be repeated to assay their redox status relative to the younger (1996 and 2004) SL seeds components. I expect ancient SL seeds to have: embryonic proteins that, for the most part, have been maintained in their reduced state and more storage proteins that are maintained in their oxidized state. Provided enough ancient seed material is available, *in vitro* reduction assays will be used to examine relative rates of protein reduction in older and younger seeds. Outer dried pericarp will be removed and ^{14}C radioactively dated to relate SL seed age to redox states of the proteins. Ideally in the end, I shall use western blots to detect the presence of endogenous Trx *h*, GSH and Glutaredoxin (Grx) in SL seeds. Coupling these results with the cellular reductants' redox status allows us to gauge their enzymatic activities in ancient and germinating sacred lotus seeds.

Timeline

Fall 2010

September:	Extracting of 1996 and 2004 *China Antique* sacred lotus proteins. 1-dimensional gel analysis of *in vivo* reduction assay of proteins to determine and compare their redox status
October:	Conducting *in vitro* reduction assay of proteins to assess the presence of GSH and Trx target proteins
November:	Isolating and sending GSH and Trx target protein to USDA mass spectrometer facility for identification
December:	Commencing germination of modern sacred lotus seeds Assessing variations in proteins' redox status during select days of germination via *in vivo* reduction assay

Spring 2011

January:	Extracting ancient *China Antique* sacred lotus seeds' proteins Carbon-14 dating pericarps of ancient *China Antique* sacred lotus seeds
February:	Assessing and comparing redox status of proteins from various aged sacred lotus seeds via *in vivo* reduction assays
March:	Using western blots to identify endogenous Trx's and NADPH/TRX reductase's in ancient *China Antique* sacred lotus seeds

April: Using western blots to identify endogenous Trx's and NADPH/TRX reductase's in germinating modern sacred lotus seeds

May: Writing my Genetics and Plant Biology honors thesis
Presenting research and CNR Poster Session

Appendix

Fig 1: Activation of target enzyme by thioredoxin.[10]

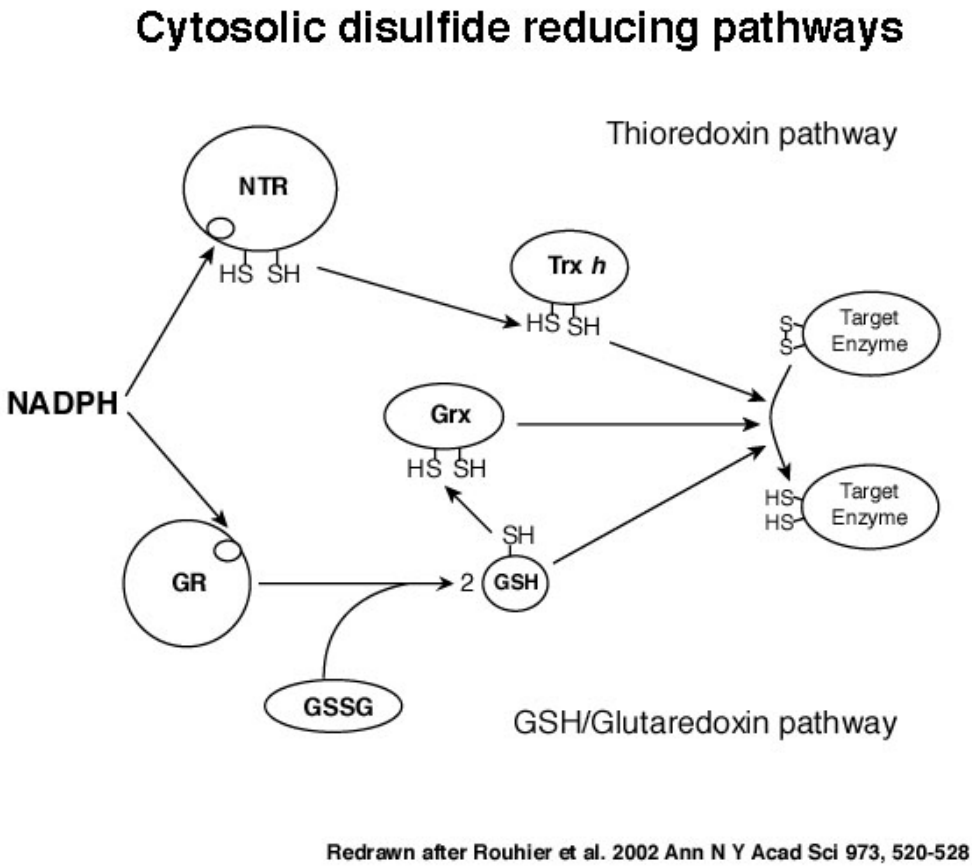

Fig.2: The thioredoxin and glutathione systems for reducing disulfide bonds
Thioredoxin (Trx) and glutathione (GSH) are both antioxidants that have the capacity to reduce disulfide bonds of oxidized proteins. The difference being that Trx is a protein and shows different reductant specificity than glutathione, which is a tripeptide. In the Trx system, NADPH reduces oxidized Trx via NADPH/Trx reductase. Similarly, NADPH can also reduce the oxidized form of glutathione (GSSG) via glutathione reductase (GR) yielding its reduced form (GSH). Both reduced Trx and GSH can reduce oxidized target proteins.

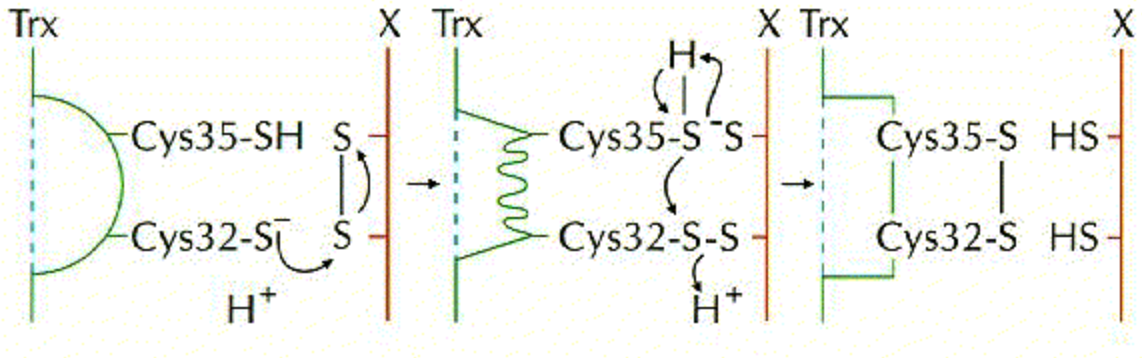

Fig.3: Mechanism of thioredoxin-catalyzed reduction of protein disulfide bonds.
Reduced thioredoxin (2(-SH)) binds to a target protein at its active site (left frame). Nucleophilic attack by the thiol/sulfhydryl group of one cysteine residue (Cys32) on the disulfide bond of another protein results in formation of a transient mixed disulfide (middle frame). Cys35 then becomes deprotonated and nucelophillic attack causes deprotonation of Cys 32 generating oxidized thioredoxin ($Trx_{S\text{-}S}$) and reduced protein. Note that conformational changes in thioredoxin (and the target protein) occur during the reaction. [9]

Fig. 4:

A) *Flowering ancient China Antique sacred lotus (Nelumbo nucifera)to reveal its yellow receptacle which houses reproductive organs.*[15]
B) *The maturing green, photosynthetic receptacle which assists in fruit—sacred lotus seed development.*[15]
C) *The brown receptacle protects matured sacred lotus seeds in its pockets.*

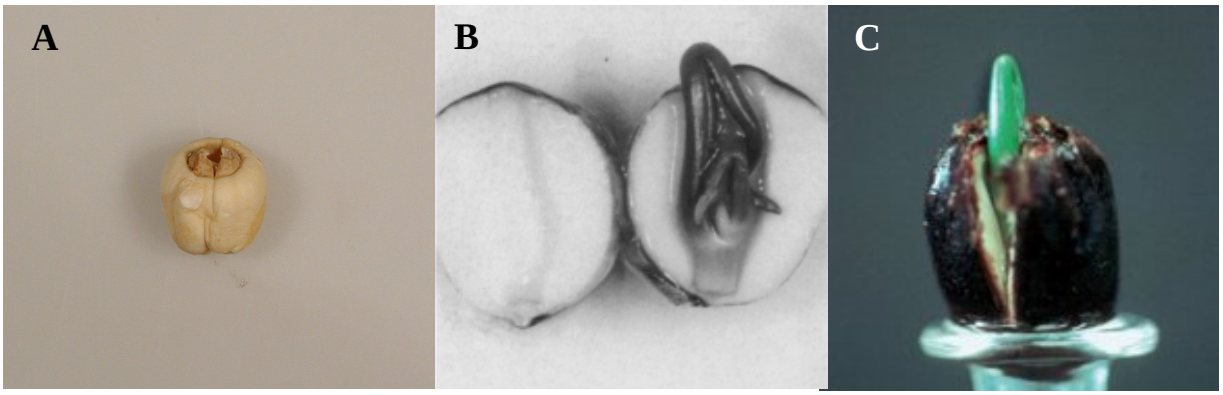

Fig. 5:
A) Dried sacred lotus seed with pericarp removed to reveal its beige cotyledon tissues.
B) Fresh sacred lotus seed with intact pericarp.[14] *A single seed was opened to reveal the fleshy embryonic axis flanked by two large cotyledons.*
C) A germinating 464 yr-old sacred lotus seed, which reveals the split brown pericarp, creamy cotyledons and emerging green embryonic axis.[15]

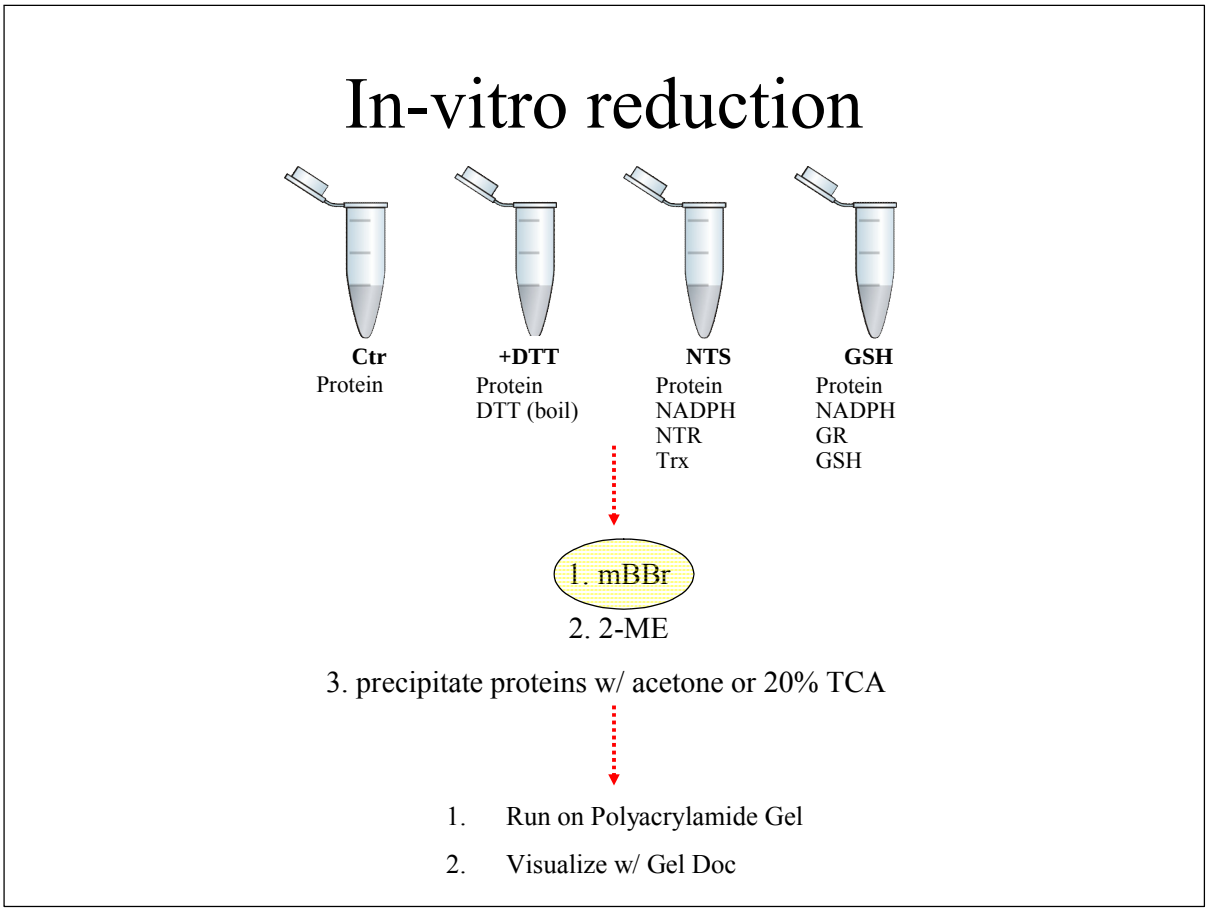

Fig. 6: Schematics for in-vitro reduction of proteins
The negative control has only sample proteins. The positive control is boiled and contains DTT, a reducing agent with two catalytically active sulfhydryl groups, is boiled to reduce the protein

fully. All sacred lotus seed proteins with disulfide bonds when treated with DTT and boiled will be reduced. NTS refers to the thioredoxin system in which NADPH is supplied to reduce Trx via NADPH/thioredoxin reductase (NTR). GSH is the glutathione system. Similarly, NADPH will reduce the oxidized form of glutathione via glutathione reductase (GR) yielding the reduced form. Both reduced thioredoxin and glutathione can reduce target proteins in the sacred lotus seeds, which are visualized on a polyacrylamide gel with a thiol-specific fluorescent probe, mBBr.

Selected Bibliography

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Budget Proposal

-Plant Materials

Ancient Chinese sacred lotus seeds	-
Modern viable sacred lotus seeds	$25

-Supplies

INVITROGEN mBBr (monobromobimane)	100
BIO-RAD Protein Assay for protein determination (QTY1@100.00/500mL)	100
XT Sample Buffer for 1-DE gel (QTY 2@12.00/10mL)	24
Rehydration Buffer for first dimension IEF of 2-DE gel (QTY 2@23.00/10mL)	46
Equilibration Buffer I with DTT for 2-DE gels (QTY 2@23.00/10mL)	46
Equilibration Buffer II with IAA for 2-DE gels (QTY 2@29.00/10mL)	58
Bio-Rad Criterion XT Bis-Tris gels for 1-DE analysis (QTY 30@13.50/gel)	405
Bio-Rad ReadyStrip IPG Strips for first dimension of 2-DE, 12/package (QTY 3@76.00/pk)	228
Bio-Rad Bio-Lyte 3/10 Ampholyte (QTY 1@32.00/mL)	32
Bio-Rad Criterion XT Bis-Tris gels, IPG+1 well for 2-DE analysis (QTY 24@13.50/gel)	405
FERMENTAS PageRuler Prestained Protein Ladder Plus (QTY 1@ 110.00/0.5mL)	110
FERMENTAS PageBlue Protein Staining Solution (QTY 1@80.00/Liter)	80
Transfer Buffer for Western blot	62
Nitrocellulose membrane for Western blot (QTY 1@159.00/10sht)	159
Bio-Rad Goat Anti-Rabbit IgG (H+L) Horseradish Peroxidase Conjugate (QTY 1@120.00/2mL)	120

-Services

Amino acid sequencing (of proteins)	-

Total Budget Request: $2,000

Approved by,

Bob B. Buchanan

Professor Bob B. Buchanan

Budget Narrative

Dr. Shen-Miller will kindly provide the ancient, 1996 and 2004 varieties of sacred lotus seeds for *in-vivo* reduction. Commercially available sacred lotus seeds are for germination and identifying Trx target proteins. Proteins extracts are collected from sacred lotus tissue via extraction buffers. Assessing protein redox status requires materials for separating proteins by running protein extracts in polyacrylamide gels (e.g. 1-DE, 2-DE) and reagents for visualizing protein (e.g. mBBr, protein staining solution). Amino acid sequencing services, provided by the USDA (Albany), are used to identify Trx targets and Trx activity in SL seeds can be detected via western blots. Upon completion of my research, I hope to present these results at the Joint Annual Meeting of the American Society of Plant Biologists following graduation.