ENVIRONMENTAL AND GENETIC VARIATION OF THE ANTI-CANCER PEPTIDES LUNASIN AND BOWMAN-BIRK PROTEASE INHIBITOR IN *GLYCINE MAX*

BY

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THESIS

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ABSTRACT

The economic importance of soybean (*Glycine max*) as a foodstuff has, in recent years, been supplemented by its importance as a source of bioactive compounds. Two of these compounds, the Bowman-Birk protease inhibitor (BBI) and the peptide lunasin, exhibit anti-cancer properties, and clinical studies suggest that the two may act in a complementary fashion. The present work surveys the levels of these two compounds across a time course of soybean development, a number of diverse soybean genotypes from the USDA Soybean Germplasm Collection, and a collection of soybean lines grown at Urbana and Bellflower, IL in the summer of 2012. Concentrations of BBI measured across mature lines in the study ranged from .101 to .38 mg/g defatted seed, while concentrations of lunasin ranged from 1.06 to 6.12 mg/g defatted seed. BBI and lunasin were significantly correlated through seed development ($p = .0097$), but concentrations of the compounds were not related in mature seeds. Genotype and the interaction of genotype by environment were found to significantly impact BBI ($p = .03$; $p = .01$), and this interaction effect was found to vary in direction depending on genotype. Environment ($p = .03$) and genotype had significant effects ($p = .03$; $p < .01$) on lunasin. Soil moisture may be the most important environmental factor in determining lunasin concentrations.

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Literature Review

The domesticated soybean (*Glycine max* (L.) Merr) of the family Leguminosae, subfamily Papilionoideae, and tribe Phaseoleae (Doyle & Doyle, 1993), is among the most important agricultural crops in the United States. In 2011, approximately 74 million acres of soybeans were planted in the United States, yielding a total of over 3 billion bushels with an economic value of nearly 37 billion dollars (USDA ERS, 2012). This value is primarily derived from the protein and oil of the soybean seed; on a 13% seed moisture basis, the average protein content of US soybeans grown from 1986 to 2003 was 35.5%, while the average oil content was 18.6% (Brumm & Hurburgh, 2006). Soybean meal, a product with nearly 13 billion dollars in combined domestic and export sales in 2011 (USDA ERS, 2012), is important as a source of high-protein livestock feed, and soybean oil has a variety of industrial, fuel, and food applications (Clemente & Cahoon, 2009). Soybean has also been utilized as a high-protein vegetable food for human consumption since its original domestication in China in approximately the 11th century BCE (Hymowitz, 1970). Indeed, the first soybeans brought to the United States by Samuel Bowen in 1765 were grown for the production of soy sauce and soybean noodles (Hymowitz and Harlan, 1983). More recently, health studies on the components of the soybean seed, especially several of its bioactive compounds, have greatly increased the demand for soy products as functional human foodstuffs.

Soy fiber, for example, has been investigated as a functional component of soy foods. Soy hulls and soy flour have different fiber profiles: the fiber of hulls is primarily cellulose and hemicellulose (Erdman & Weingartner, 1981), while that of flour is primarily non-cellulosic (Lo, 1989). Up to 93% of cotyledon-derived soy fiber is digestible in humans (McNamara *et al.*, 1986), which suggests that the fiber may be readily metabolized. Indeed, soy fiber has been

demonstrated in several studies to have superior functional properties for human health, reducing LDL levels and improving glucose tolerance to a greater degree than comparable fiber sources (Erdman & Weingartner, 1981; Tsai *et al.*, 1983; Lo *et al.*, 1986). The reduction in LDL is especially vital to the cardiovascular system, as it prevents the buildup of arterial plaque that may lead to blockage and myocardial infarction (de Lemos *et al.*, 2010). Bowel function in humans is also improved by soy fiber, as indicated by increases in stool weight and decreases in gastrointestinal transit time (Slavin, 1991) after soy fiber supplementation.

Soybean and soy foods have also been recognized as significant sources of isoflavones (Wang & Murphy, 1994), plant-derived compounds that can function as estrogen analogues in mammals like humans (Setchell, 1998). The first soy isoflavone to be discovered was genistin (Walter, 1941), and further work identified two others, daidzin and glycitin (Naim *et al.*, 1974), along with different naturally-occurring forms for each. These compounds can be processed into bioactive forms by human gut flora (Setchell *et al.*, 2002), and both the raw compounds and the resulting metabolites have been implicated as beneficial against a number of diseases (Tham *et al.*, 1998). For example, soy isoflavones have been shown to bind with osteoblast estrogen receptors and stimulate insulin-like growth factor production (Arjmandi & Smith, 2002), providing protection against osteoporosis. However, individual metabolic differences may mediate the effectiveness of isoflavones against bone resorption: a recent study indicated that postmenopausal women capable of metabolizing dietary isoflavones into equol are at significantly less risk for osteoporosis than women who are unable to process the compounds (Weaver & Legette, 2010).

Soy isoflavones have attracted additional interest for their impacts on cardiovascular health, particularly atherosclerotic risk. Three months of a high-isoflavone diet were shown to

reduce low-density lipoprotein (LDL) and the LDL/HDL ratio in the plasma of postmenopausal women (Wangen *et al*., 2001), and two months of a diet containing isoflavone-enriched pasta improved both serum lipid characteristics and arterial stiffness (Clerici *et al.*, 2007). It is worth noting that the previous study also considered equol metabolic ability; the benefits of isoflavones were evident in both equol-producing and equol-deficient subjects, although they were greatest among equol producers. Primate studies have suggested other mechanisms for the cardiovascular benefits of isoflavones, including antioxidative activity and antiproliferative impacts on smooth muscle cells (Anthony *et al.*, 1998). Evidence linking soy isoflavones to a reduced risk of breast cancer in human populations has been reported (Yamamoto *et al.*, 2003), although these effects are less established than the other benefits of soy. Laboratory work to confirm the anticancer effects of isoflavones has yielded conflicting results, with studies that suggest isoflavones both stimulate tumor growth (Ju *et al*., 2001) and prevent immunosuppression of cancer cells (Jiang *et al.*, 2008). Whether soy isoflavones are beneficial or harmful to cancer may be dose-dependent, as high levels of these compounds are generally observed to inhibit tumor formation and growth (Wood *et al.*, 2006; Zhang *et al.*, 2012).

Diets high in soy protein also have demonstrated benefits for a number of health markers. A meta-analysis of 38 clinical soy protein trials detected significant decreases in total cholesterol, triglycerides, and LDL levels in comparison with control diets (Anderson *et al*., 1995). The 7S-globulin storage protein is thought to be at least partially responsible for these changes, as demonstrated by a mouse study in which the protein reduced triglyceride levels in comparison with casein or glycinin diets (Moriyama *et al*., 2004). This reduction was associated with the upregulation of beta-oxidation and downregulation of fatty acid synthase, as well as with reductions in serum glucose and insulin. Soy protein may positively impact bone retention,

but this effect is most likely due to the isoflavones present in a soy diet, as previously discussed. In one clinical study, a year of soy protein supplementation improved a number of bone health markers in postmenopausal women, but it did not lead to a reduction in actual bone loss (Arjmandi *et al.*, 2005). Similarly, a six-month clinical study in postmenopausal women only detected the protective effects of a soy protein diet against spinal bone loss when the diet was supplemented with higher isoflavone levels (Potter *et al.*, 1998). Finally, a recent meta-analysis associated soy protein intake with reduced risk for a number of cancers, including prostate, breast, and colon cancer (Badger *et al.*, 2005). The same authors confirmed these effects for breast and colon cancers in a rat model, and microarray analysis revealed that the soy diet downregulated a number of genes involved in the activation pathway for a common carcinogen.

From a nutritional standpoint, soybean protein is generally considered to be a "complete" protein, as it contains adequate levels of all essential amino acids (Young, 1991). Human feeding studies have demonstrated the sufficiency of soy as the lone protein source (Beer *et al.*, 1989), alleviating early concerns about the plant's relatively low levels of methionine. Levels of this essential sulfur-containing amino acid have been shown to vary across the US soybean production region, with higher levels detected in the center of the region than in the northern or southern edges (Karr-Lilienthal *et al*., 2005). Methionine variation is thought to be due primarily to differences in the accumulation of the 11S glycinin storage proteins (Mahmoud *et al.*, 2006), which are associated with both genetic and environmental factors (Fehr *et al.*, 2003).

The majority of the protein present in soybean is storage protein. Approximately 34% of the protein is in the 11S fraction as glycinin, while approximately 27% of the protein is present in the 7S fraction as β-conglycinin (Petruccelli & Anon, 1995). The relative amounts of these storage proteins vary among genotypes, as do the proportions of the subunits that compose each

storage protein (Riblett *et al.*, 2001). Interestingly, the proportion of the *β* subunit of *β*-conglycinin is lower in modern soybean cultivars than in ancestral varieties, and as this subunit contains no sulfur-containing amino acids, the protein quality of soybean has been improved over time (Mahmoud *et al.*, 2006). A number of other storage globulins, such as γ-conglycinin and the basic 7S globulin, are present in less abundant levels in the 11S and 7S protein fractions (Thanh *et al.*, 1975). The most abundant functional proteins in the seed include cytochrome *c*, lipoxygenase, *β* –amylase, hemagglutinin, and the soybean trypsin inhibitors (Wolf, 1970): the Kunitz trypsin inhibitor (KTI) (Kunitz, 1945) and the Bowman-Birk protease inhibitor (BBI) (Bowman, 1946; Birk, 1985).

Soybean protease inhibitors

The Kunitz trypsin inhibitor was first described by Kunitz in 1945 using X-ray crystallography. Subsequent work showed the protein to be composed of 181 amino acids (Koide *et al.*, 1972), with a single active site that inhibits both trypsin and α-chymotrypsin (Bidlingmeyer *et al.*, 1972). The genetics of KTI inheritance are somewhat complex; early classical genetic work revealed the presence of four distinct alleles at a single locus (Orf & Hymowitz, 1979). Three of these alleles are codominant and encode minor variations of the inhibitor, designated Ti^a, Ti^b, and Ti^c, which are distinguishable through electrophoresis (Orf & Hymowitz, 1977). The amino acid sequences of these variants are all of the same length, and Ti^b and Ti^c both differ from the more prevalent Ti^a only by the order of a single amino acid (Kim *et al.*, 1985). These small differences do appear to have functional relevance: Ti^b is significantly less effective at inhibiting trypsin than the other variants, and while Ti^c is slightly more effective than Ti^a, it is also less thermally stable (Freed & Ryan, 1980), providing a possible selective pressure for the Ti^a allele among soybean accessions. Shortly after the discovery of these

variants, near-isogenic lines (NILs) derived from the cultivar "Williams" that were homozygous for each variant were developed and released as L82-2024 (Ti^b) and L82-2051 (Ti^c) (Bernard & Hymowitz, 1986a).

The fourth allele, designated *ti*, codes for an absence of KTI and is recessive to each of the three variant alleles (Orf & Hymowitz, 1979). This allele was quite uncommon among the accessions screened in this early work, with only two Korean plant introductions (PIs) possessing the null (Hymowitz, 1986). One of these accessions, PI 157440, was used to develop three NILs with the cultivars "Williams 82" (L81-4590), "Clark 63" (L81-4871), and "Amsoy 71" (L83-4387) that were homozygous for the KTI null (Bernard & Hymowitz, 1986b). After further testing and development, the NIL derived from Williams 82 was released commercially as the cultivar "Kunitz" (Bernard *et al.*, 1991). Although no further cultivars containing the KTI null have been released commercially, a number of experimental lines with double nulls were developed that combined the KTI null with nulls for lectin, lipoxygenase-1, μ-amylase, and urease (Prischmann & Hymowitz, 1988).

Further genetic study has revealed at least 10 genes in the soybean genome that bear close similarity to the primary Kunitz gene (Jofuku & Goldberg, 1989). However, most of the genes appear to code for proteins that lack inhibitory activity, suggesting that a single gene, KTI-3, accounts for most of the observed protein and activity. Agricultural species besides soybean that have been shown to contain members of the Kunitz family include *Populus tremuloides* (quaking aspen) (Haruta *et al.*, 2001), *Cicer arietinum* (chickpea) (Srinivasan *et al.*, 2005), and *Populus trichocarpa* × *Populus deltoides* (hybrid poplar) (Major & Constabel, 2008).

BBI was first detected by Bowman in 1946, who distinguished it from the previously known KTI, but the compound was largely ignored until the 1960s. Much of the early

purification and characterization work was conducted by Birk and colleagues (Birk, 1961; Birk *et al.*, 1963), and in recognition of the efforts of both scientists, the name "Bowman-Birk inhibitor" was given to the protein (Steiner & Frattali, 1969). BBI consists of 71 amino acids, including 14 half-cystine residues (Odani and Ikenaka, 1972), and contains two active sites, one inhibiting trypsin and the other inhibiting chymotrypsin (Odani and Ikenaka, 1973). These two sites are present in almost symmetrical halves of the molecule, yielding a characteristic "double headed" molecular structure (Qi *et al.*, 2005). Further work revealed that the initial BBI discovered by Bowman and Birk was only the most prevalent component of an entire class of protease inhibitors present in soybean and other flowering plants. Soybean contains this "major" or "classical" BBI along with 9 similar proteins (isoinhibitors) (Tan-Wilson *et al.*, 1985), although only 4-7 isoinhibitor forms are found in any particular accession. Despite the similar targets of BBI and KTI, there is little to no amino acid sequence similarity observed between the two proteins. There have also been no nulls detected for the major BBI in either domesticated soybean or its wild ancestor, *Glycine soja* (Domagalski *et al.*, 1992). Significant variation for the compound nonetheless exists across soybean accessions (Gladysheva *et al*., 2000; Pesic *et al.*, 2007), and growing conditions have been shown to impact BBI levels (Krishnan *et al*., 2012).

BBI nulls have been detected in many of the perennial *Glycine* species. An initial survey of *Glycine* diversity using ELISA found BBI nulls in *G. curvata* Tind., *G. cyrtoloba* Tind., *G. latifolia* (Benth.) Newell & Hymowitz, *G. microphylla* (Benth.) Tind., *G. tabacina* (Labill.) Benth., and *G. tomentella* Hayata (Domagalski *et al.*, 1992). Kollipara *et al.* (1995) confirmed these previously reported nulls and also found BBI nulls in the recently discovered species *G. albicans* Tind. and Craven. Interestingly, the presence or absence of BBI appeared to be linked with the intergenomic relationships of the genus: species with B- or C-genomes were BBI null,

while species with A-, E-, and F-genomes were BBI positive. Both BBI null and BBI positive accessions were present in 2n = 40 *G. tomentella* accessions and 2n = 80 *G. tabacina*. Very little work has been conducted to determine the causes or inheritance patterns of these BBI nulls. A genetic study in *G. tomentella* demonstrated that the BBI null allele was recessive and monogenic; the locus was weakly linked to another protease inhibitor (Kollipara *et al.*, 1996). Inheritance was not studied in *G. microphylla*, but cloning of the BBI null allele revealed a four-nucleotide deletion that caused a frameshift in the coding region, presumably preventing a buildup of the product in the seed (Krishnan & Kim, 2003). None of the other perennial *Glycine* BBI nulls have been characterized.

The DNA sequence for the major BBI in domesticated soybean has been cloned, along with those for several of its isoinhibitors (Baek *et al.*, 1994), and allelic variation has been observed within the family (Wang *et al.*, 2008). *In silico* analysis has identified putative sequences for the remaining soybean BBI isoinhibitors (de Almeida Barros *et al.*, 2012). Previous work shows that BBI appears in soybean seeds 7 weeks after flowering, increases through maturity, and remains in the mature seed (Park *et al.*, 2005). Members of the BBI family have been found in *Triticum aestivum* (common wheat) (Odani *et al.*, 1986), *Coix lacryma-jobi* (Job's tears) (Ary *et al.*, 1988), *Setaria italica* (foxtail millet) (Tashiro *et al.*, 1990), *Vicia faba* (broad beans) (Ye *et al.*, 2001), *Oryza sativa* (rice) (Qu *et al.*, 2003), *Hordeum vulgare* (barley) (Park *et al.*, 2004), *Lens culinaris* (lentils) (Caccialupi *et al.*, 2010), and *Lathyrus sativus* (grass pea) (De Paola *et al.*, 2012), among other species.

The soybean protease inhibitors have been shown to have antinutritional effects in several animal species (Liener, 1994). Early studies on rats (Klose *et al.*, 1946) and chicks (Ham *et al.*, 1945) demonstrated that animals raised on diets containing raw soybean meal performed more

poorly than those fed diets without raw soybeans. The inhibitors were shown to cause hypertrophy of the pancreas and a proliferation or hyperplasia of the acinar cells in the pancreas (Chernick *et al.*, 1948) in response to reduced digestive enzyme activity. An enlarged and overactive pancreas diverts resources from the rest of the organism to produce more digestive enzymes (Lyman and Lepkovsky, 1957), retarding its growth. Additionally, the overactivity of the acinar cells causes them to become susceptible to certain carcinogenic agents, including di(2-hydroxypropyl)nitrosamine (McGuinness and Wormsley, 1986). Nearly all soybean products are processed using heat to denature these inhibitors (Anderson and Wolf, 1995). However, this process is not completely effective at inactivating the proteins, and anywhere from 5-20% of the inhibitory action of raw soybean flour is present in heated or toasted flours (Friedman & Brandon, 2001). The human pancreas has been shown to increase its output of digestive enzymes in response to the reduction of trypsin and chymotrypsin activity caused by the Bowman-Birk inhibitor (Liener *et al.*, 1988). Such feedback effects may have detrimental impacts in humans, as they do in rats, but the clinical evidence on this point is rather sparse.

More recent research has suggested that these protease inhibitors may actually cause beneficial effects in humans. The KTI from soybeans has been shown to suppress the metastasis of human ovarian cancer by preventing the expression of a signaling protein in tumor cells (Kobayashi *et al.*, 2004). This inhibitor also downregulates the production of a tumor necrosis factor in human keratinocytes, possibly offering protection against skin cancers caused by ultraviolet radiation (Kobayashi *et al.*, 2005). Mice fed a diet supplemented with KTI showed significantly less inflammation due to lipopolysaccharides than mice on a control diet (Kobayashi *et al.*, 2005), and similar effects have been observed for a number of proteins with Kunitz-type active sites (Shigetomi *et al.,* 2010), suggesting that KTI may be valuable for the

treatment of arthritis, heart disease, asthma, and other inflammation-related diseases. BBI suppresses radiation-induced carcinogenesis in a number of different cell types (Yavelow *et al.*, 1985; Kennedy *et al.*, 2006), prevents the growth and survival of prostate cancer cells (Malkowicz *et al.,* 2001; Kennedy and Wan, 2002), and inhibits the esophageal carcinogenic effects of *N*-nitrosos-methylbenzylamine (von Hofe *et al.*, 1991). Interestingly, BBI has also been found to reduce the negative effects of muscle atrophy by buffering against oxidant activity (Arbogast *et al.*, 2007), a property that may be useful to astronauts on lengthy space journeys. BBI may be important not only due to its own beneficial effects but also due to the role it plays in protecting another soybean compound, lunasin, from digestion.

Lunasin peptide

Lunasin is a bioactive peptide, originally discovered in soybean, with cancer-preventative properties (de Lumen, 2005). The peptide was first detected as part of a larger hydrophobic protein and was noted for its poly (L-aspartic acid) tail (Odani *et al.*, 1987; Odani *et al.*, 1987). The gene with which this peptide was associated was not determined for another decade, until a team at the University of California, Berkeley purified a large methionine-rich protein from soybean (Revilleza *et al.*, 1996) and used its amino acid sequence to clone a cDNA for a posttranslationally processed 2S albumin (Gm2s-1) (Galvez *et al.*, 1997). This cDNA not only encoded the methionine-rich protein, but also a signal peptide, a small subunit (lunasin), and a linker peptide. As the experimenters attempted to isolate lunasin from *Escherichia coli* cells transformed with the small subunit coding region of the Gm2s-1 cDNA, they noticed that the transformed bacteria exhibited reduced growth and elongated filaments, signs of disrupted mitosis (Galvez and de Lumen, 1999). *E. coli* transformed with cDNAs in which the polyaspartyl end of the lunasin had been deleted, in contrast, showed normal growth patterns. Further work

demonstrated that mammalian cancer cells transfected with the lunasin-coding cDNA also showed disrupted mitotic patterns; again, cDNAs with the deletion generated no effect. This anti-mitotic effect was cytotoxic in many cases, an effect hypothesized to be caused by the inability of the cell lines to accommodate high genome dosages and the disruption of spindle formation (Galvez and de Lumen, 1999). These modes of action are similar to those of the taxanes, an established class of anti-cancer drugs (Rowinsky *et al*., 1992), and the promise of the compound as a possible cancer treatment led de Lumen and Galvez to patent the lunasin peptide from soybean in 2000.

The primary structure of lunasin consists of 43 amino acids: it contains a polyaspartyl tail of 9 aspartic acid residues at the carboxyl end, an Arg-Gly-Asp cell adhesion motif, and a helical region with structural homology to a conserved region present across chromatin-binding proteins (Galvez *et al.*, 2001). As previously noted, the lunasin cDNA has been cloned, and this sequence maps onto the Williams 82 genome chromosome 13 between the bases 37700716 and 37701192 (Grant *et al.*, 2010). This sequence is within 0.2 Mbp of the molecular marker Satt254. Lunasin RNA is expressed in the soybean from 3 weeks after flowering onward, and this expression only occurs in the cotyledon (de Lumen *et al.*, 1999); no lunasin RNA is found in leaf, stem, or root tissue. The compound itself, however, has not been shown to appear in detectable quantities until 5 weeks after flowering (Jeong *et al.*, 2003). Levels of lunasin have been observed to increase as the seed approaches maturity, and some lunasin is maintained in the mature seed (Park *et al.*, 2005).

To date, there have been only two studies examining variation among soybean genotypes for lunasin content. An initial survey quantified the amount of lunasin present in 144 soybean lines, covering all 13 maturity groups (MG) and a number of geographical origins (de Mejia *et*

al., 2004). A range of 1 to 13 mg lunasin/g defatted flour was detected, with the highest lunasin levels recorded in accessions from MG IX and X. However, the samples analyzed in this study were taken directly from the USDA Soybean Germplasm Collection, which is maintained by growing out a portion of the accessions every year. Therefore, the environmental conditions under which the lines of the study had been grown were not consistent across entries, and year and location effects were confounded with the effect of the genotype. A subsequent experiment grew 5 soybean accessions under a number of controlled temperature and moisture conditions in an attempt to separate out environmental variability (Wang *et al.*, 2008). Significant genotypic differences did exist between the cultivars, but temperature also had a significant effect, with soybeans grown under low (13/23°C night/day) or intermediate (18/28°C night/day) temperatures during the R6 and subsequent growth stages containing more lunasin than soybeans grown at high (23/33°C night/day) temperatures. Soil moisture had no significant main effect but was implicated in a number of interactions with cultivar and temperature.

Although first isolated in soybean, lunasin has subsequently been isolated in barley (Jeong *et al.*, 2002), common wheat (Jeong *et al.*, 2007), *Solanum nigrum* (black nightshade) (Jeong *et al.*, 2007), *Amaranthus hypochondriacus* (amaranth) (Silva-Sanchez *et al.*, 2008), *Secale cereale* (rye) (Jeong *et al.*, 2009), *Panicum miliaceum* (common millet) (Park *et al.*, 2009), and × *Triticosecale* (triticale) (Nakurte *et al.*, 2012). Of these sources, soybean appears to have the highest lunasin quantities, although triticale contains comparable amounts (Muceniece *et al*., 2012). The majority of medical studies involving the peptide, however, have been conducted with synthetic lunasin, due to the availability of its primary sequence. The large-scale production and isolation of natural lunasin from soybeans, while suggested by Jeong *et al.* (2003), has only recently been made feasible. A commercial-scale chromatography and

ultrafiltration method is now available to isolate lunasin from soy flour (Seber *et al.*, 2012), and the production of the peptide in recombinant *E. coli*, while still somewhat hindered by the mitotic abnormalities observed in early experiments, has significantly improved (Liu and Pan, 2010; Kyle *et al.*, 2012). Another recent study presents an unusual method of lunasin enrichment for a variety of flours through the use of sourdough lactic acid bacteria fermentation (Rizzello *et al.*, 2012), although this strategy might be more suited for the nutraceutical market than for scientific or medicinal production.

The first *in vivo* demonstration of lunasin's cancer-preventative properties was conducted in the SENCAR (SENsitive to CARcinogenesis) mouse skin tumor model (Galvez *et al.*, 2001). This study used topically-applied lunasin to achieve significant reductions in tumor formation in comparison with a control group. Lunasin prevented the formation of foci in the fibroblast cells (Lam *et al.*, 2003). Both cells treated with chemical carcinogens and those transformed with the E1A oncogene were effectively suppressed by the peptide, while control cells were unaffected. Established cancer cell lines, however, were also unaffected by lunasin. Subsequent studies suggested that lunasin may prevent carcinogenesis through binding to the deacetylated histones of cancerous cells, inhibiting reacetylation and causing the cell to undergo apoptosis (Jeong *et al.*, 2002; Jeong *et al.*, 2007). Several more detailed apoptotic mechanisms have been observed in more recent studies. In leukemia cells, for example, lunasin promotes apoptosis through the activation of the caspase-3 (Cysteine-ASPartic acid proteASE) protein (de Mejia *et al.*, 2010), and in colon cancer cells, lunasin induces the expression of a pro-apopotic clusterin isoform (Dia and de Mejia, 2010). Lunasin has been found to prevent the adhesion of metastatic colon cancer cells to the extracellular matrix and to healthy cells through regulatory changes in a number of relevant genes, such as those coding for integrins and the endothelial-leukocytre adhesion

molecule 1 (SELE) (Dia and de Mejia, 2011). Additionally, lunasin appears to have a synergistic effect with aspirin, inducing highly significant levels of apoptosis in breast cancer (Hsieh *et al.*, 2010) and fibroblast (Hsieh *et al.*, 2011) cells by arresting them at complementary phases in the cell cycle. Although no *in vivo* clinical trials of the peptide have been conducted in humans, it has been demonstrated that the consumption of soy protein does provide bioavailable lunasin to humans (Dia *et al.*, 2009).

These promising medicinal qualities, combined with the availability of commercial-scale purification techniques, make high-lunasin soybean lines greatly desirable to the health market. The peptide is already being marketed as a fortifying ingredient in a number of soy protein formulations, such as LunaRich® and LunaRich X™ by Reliv and Lunasin XP® by the Scoular Company, which provide lunasin at levels ranging from 5 to 200 times those observed in regular soy protein.

BBI-Lunasin interactions

Due to its protease inhibitory activity, BBI is stable in the human digestive system, while lunasin, a small peptide with no protease inhibition, is easily digested (Park *et al*., 2007). When lunasin and BBI are consumed together, as they are in naturally occurring soy protein, the lunasin is protected from degradation (Park *et al.*, 2007). This observation has clinical significance for anti-cancer trials of Bowman-Birk Inhibitor Concentrate (BBIC). BBIC has been shown to reduce the incidence of breast cancer tumors in a mouse model (Hsieh *et al.*, 2010), an effect originally believed to be caused by the BBI in this concentrate. However, injection with BBI alone generated no reduction of tumor incidence, while injection with lunasin alone caused significant reductions (Hsieh *et al.*, 2010). This result suggests that BBIC is perhaps misnamed, as lunasin provides the bioactive anti-cancer effect in the product.

Aside from the previously mentioned protective interaction of BBI with lunasin in the process of digestion, the two compounds may be linked at a more basic level in the soybean plant itself. A survey of lunasin concentrations detected no lunasin in a major BBI null accession of the perennial *Glycine latifolia* (de Mejia *et al.*, 2004). As previously discussed, domesticated soybean lacks BBI null accessions, but significant variation in the compound has been observed, and other perennial *Glycine* species possess BBI nulls. A transgenic line has also been recently developed from the soybean cultivar "Asgrow 3237" that exhibits half as much chymotrypsin inhibition activity (Livingston *et al.*, 2007). Lunasin levels in these BBI variants are completely unknown; if lunasin is found to be less prevalent or totally absent in plants with reduced levels of BBI, further study may reveal a common genetic or regulatory basis for the two compounds.

The associations of lunasin and BBI uncovered in these studies are of great interest to both medical and agricultural science. If these two compounds are associated in their beneficial health effects, it would be useful to select for soybean lines with appropriate amounts of both. Little is known about the relative levels of lunasin and BBI in soybean accessions, the genetic or environmental basis of these levels, or the association of the two compounds in the seed. Through quantifying lunasin in a number of accessions, determining the factors underlying its variation, and analyzing its correlation with BBI, the present study aims to offer breeders the information needed to develop lines for future pharmaceutical and nutraceutical enterprises. The objectives of this research are 1) to examine the correlation of lunasin and BBI in a number of soybean accessions; 2) to track the levels of lunasin and BBI over the course of seed development; and 3) to determine the relative importance of genotype and environment to the levels of lunasin and BBI in these accessions.

Materials and Methods

Plant Information and Field Techniques

Ten soybean accessions were obtained from the USDA Soybean Germplasm Collection at the University of Illinois at Urbana-Champaign. These lines represented a subsample of the 144 lines analyzed for lunasin content by de Mejia *et al.* (2004). Accessions were chosen from the lines with the highest and lowest previously observed lunasin levels for a range of maturity groups (MGs) to represent a high amount of diversity.

Out of the previously studied 144 lines, 10 accessions from MG III were selected to be grown at two locations in the summer of 2012 at the Crop Sciences Research and Education Center at the University of Illinois at Urbana-Champaign in Urbana, IL and a rented field near Bellflower, IL. These lines were chosen to represent the highest and lowest previously observed levels of lunasin in the maturity group and included exotic accessions from a range of geographical origins, including four Chinese provinces, North Korea, and South Korea, along with two U.S. cultivars. At the Bellflower location, two replicates of 30 seeds each from each accession were hand planted in rows 1.2 m long and 76 cm apart on May 16, 2012. The same experiment with the same plot size was planted at the Urbana location on May 19, 2012. Fields were maintained with standard agronomic practices.

One MG III line, NE3399, was chosen to be studied in a time course experiment. This accession was selected for its relatively high yields and adaptation to central Illinois to ensure reliable seed production for the experiment. Seven plots of 30 seeds of NE3399 were planted in rows 1.2 m long and 76 cm apart at the Urbana location on May 19, 2012 and divided into two replications of three and a half rows each. Starting on August 9, 2012 and ending on October 8, 2012, approximately 30 pods were harvested weekly from between the 5th and 15th nodes of

each of replication, with pods taken evenly from across each replication. These pods were dried in an oven at 40 °C for at least 72 hours, then removed and stored at room temperature until further use. Previous work has demonstrated the sufficiency of room temperature storage for the long-term stability of both BBI and lunasin (E. de Mejia, personal communication).

BBI and Lunasin Quantification by ELISA

Approximately 1 g of seed from each accession was ground into flour using a Wiley Intermediate laboratory mill (Thomas Scientific, NJ). Homogeneity of particle size was ensured by passing the flour through a 40 mesh filter, and the resulting product was stored at 4 °C until further use. The flour was defatted for approximately 6 hours in a Soxhlet extractor using a mixture of nonspectro-grade, > 85% pure hexanes (Tvap = 67.5 °C) (American Burdick & Jackson, Muskegon, MO) as the solvent. After defatting, the flour was allowed to air-dry in a fume hood overnight (14-16 h) and stored at 4 °C until used.

Extractions were performed in triplicate on 50 mg samples of the defatted flour suspended in 1 mL aliquots of an extracting buffer (1% polyvinylpolypyrrolidone (PVPP) in 0.05 M Tris-buffered saline (TBS)) (Sigma Chemical Company, St. Louis, MO) in Eppendorf centrifuge tubes. The Tris[hydroxymethyl]aminomethane hydrochloride (Tris-HCl) used for the TBS buffer solution was biotechnology performance certified, with a minimum of 99% purity. The samples were placed in an ultrasonic bath (Bransonic model 2510, Branson Ultrasonic Corporation, Danbury, CT) for 70 minutes at 40 °C; the water temperature was maintained using a recirculating bath (Endocal model RTE-9, Neslab Instruments, Portsmouth, NH). The samples were vortexed every 10 minutes during this process to ensure adequate suspension. The sonicated samples were then centrifuged at 20000 g for 30 min at 4 °C in an Eppendorf

centrifuge (model 5417R, Brinkmann Instruments, Westbury, NY), and the resulting supernatant was transferred to a new set of Eppendorf tubes for storage at -20 °C.

Soluble protein concentration for each sample was determined using the Protein DC-Microplate Assay (Reagents Package 500-0116, Bio-Rad Laboratories, Hercules, CA). Samples were diluted 1:20 in a buffer solution (0.05 M TBS). A standard curve was prepared using concentrations of 1500, 1200, 900, 600, 300, 100, 30, and 10 µg/mL buffer solution of > 98% pure bovine serum albumin (BSA) (Sigma Chemical Company, St. Louis, MO) and measured along with the samples for each run. Each standard, sample, and a buffer blank was plated (5 µl/well) in duplicate on a 96 well microtiter plate. To each well, 25 µl of reagent A (alkaline copper tartrate) and 200 µl of reagent B (dilute Folin reagent) were added, and the plate was gently agitated. After 15 minutes of incubation at room temperature, the absorbances of the plate were read using a photospectrometer (ELx 808 IU, Bio-Tek Instruments, Winooski, VT) at 690 nm. Protein concentrations of the samples were determined from the values of the standard curve derived from the protein standards.

Aliquots (100 µL) of each sample, diluted 1:50 in a buffer solution (1% Tween in 0.05 M TBS), were coated on the wells of a Nunc Maxisorp 96 well plate specifically designed for ELISA use. A standard curve of pure synthetic BBI or pure synthetic lunasin (FilGen BioSciences, Fairfield, CA), consisting of at least 6 concentrations from 8 to 100 ng/mL and prepared in the same buffer solution, was plated along with the samples for each run. These standards were plated in triplicate, samples were plated in duplicate, and blanks were plated in at least duplicate. All three extractions for each sample were run on the same plate to provide duplication.

Coated plates were incubated at 4 °C overnight (14-16 h), then washed 6 times using 300 µL of washing solution (0.05% Tween-20 in 0.01 M PBS) (Sigma Chemical Company, St. Louis, MO) at a dispensing rate of 150 µL/well/s and an aspiration rate of 5 mm/s. This phosphate buffered saline (PBS) with polyoxyethylene sorbitan monolaurate (Tween 20) buffer was mixed from packets with the proper concentrations and pH (0.01 M PBS, 0.05% Tween 20, pH 7.4) pre-measured. The plates were then blocked with 300 µL/well of a BSA blocking solution (5% BSA and 1% Tween in 0.05M TBS) and allowed to incubate at room temperature for 1 h. After blocking, the plates were washed again using the same protocol.

For BBI quantification, 50 µL of a 1:1000 dilution of mouse-derived monoclonal BBI antibody (Agdia, Inc., Elkhart, IN) in a BSA buffer solution (3% BSA and 1% Tween in 0.05 M TBS) were then added to each well, and the plates were incubated for another hour. Following another wash, 50 µL of a 1:2000 dilution of alkaline phosphatase conjugate anti-mouse IgG secondary antibody in the BSA buffer solution were added to each well. For lunasin quantification, 50 µL of a 1:100 dilution of rabbit-derived monoclonal lunasin antibody (provided by Dr. Benito de Lumen, University of California, Berkley) in a BSA buffer solution (3% BSA and 1% Tween in 0.05 M TBS) were added, and after incubation and washing, 50 µL of a 1:1000 dilution of alkaline phosphatase conjugate anti-rabbit IgG secondary antibody in the BSA buffer solution were added to each well.

For both BBI and lunasin quantification, the plates were again incubated for an hour at room temperature and washed according to the described protocol. Development of the plates was conducted using 100 µL/well of p-nitrophenyl phosphate (pNPP, Sigma N-7653), the color substrate for alkaline phosphatase. After 20 minutes of incubation at room temperature, the plates were read in an ELISA plate reader (ELx 808 IU, Bio-Tek Instruments, Winooski, VT) at

405 nm. The development reaction was stopped at 30 minutes using 100 μL/well of 3 N NaOH, and the plates were read again at 35 minutes.

Standard curves were obtained from the average of at least two ELISA replications. Absorbances for BBI were converted into mg/g defatted flour using the standard curve $y = .0119x + .0613$, and absorbances for lunasin were converted using the standard curve $y = .0011x + .1428$. The data were analyzed using analysis of variance (ANOVA), Tukey's range test, correlation, and linear regression in SAS 9.1 (SAS Institute Inc., 2003).

Results and Discussion

Significant differences (Table 1; $F = 3.75$, $p = .03$) did exist among the entries grown in the field for BBI concentrations. Tukey's range test was used to separate the entries into classes based on BBI concentration (Table 2). It was determined that the highest BBI levels were observed in the cultivar Lincoln, followed by Jogun and A.K. (Harrow). The lowest BBI levels were seen in PI 567362A (Ningxia, China) and PI 567775A (Jiangsu, China). The observation of significant BBI variation among the entries supports the conclusions of the previous literature. Gladysheva *et al.* (2000) found a threefold difference in BBI between the highest and lowest levels among 9 entries, similar to the range reported here, and Pesic *et al.* (2007) also detected significant differences in BBI levels among a set of 12 Serbian soybean lines.

Table 1. ANOVA table for BBI concentrations among diverse soybean entries grown at two locations in Illinois in 2012.

Source	Sum of Squares	DF	Mean Squares	*F* value	*p* value
Environment	.000847	1	.000847	0.15	0.71
Entry	.195781	9	.021753	3.75	0.03
Environment x Entry	.052153	9	.005795	3.42	0.01
Residual	.032209	19	.001695	.	.

Table 2. Average BBI and lunasin concentrations among diverse soybean entries grown at two locations in Illinois in 2012. Different letters in the same column indicate significantly different means as determined by Tukey's range test ($p < .05$).

Entry	Geographical Origin	BBI (mg/g defatted flour)	Lunasin (mg/g defatted flour)
PI 567775 A	China	.101 a	2.64 a
PI 567362 A	China	.119 a	2.39 a
PI 398261	Korea	.148 ab	4.06 b
PI 594871	China	.149 ab	4.17 b
NE3399	USA	.155 ab	3.26 ab
PI 594444 B	China	.165 ab	2.93 ab
PI 603167	China	.203 abc	3.99 b
A.K. (Harrow)	China	.251 bcd	3.67 ab
Jogun	Korea	.295 cd	3.18 ab
Lincoln	USA	.319 d	3.51 ab

There was also a significant interaction effect of entry and environment on BBI concentration (Table 1; $F = 3.42$, $p = .01$). Using contrasts to separate the interaction effect, it was determined that Lincoln and A.K. (Harrow) contained significantly more BBI (Table 3; $F = 5.58$, $p = .03$; $F = 8.04$, $p = .01$) at the Urbana location than at the Bellflower location, while Jogun contained significantly more BBI (Table 3; $F = 11.62$, $p < .01$) at Bellflower than at Urbana. Krishnan *et al.* (2012), however, found no interaction between location and entry for BBI levels. Interestingly, the three entries with the highest BBI concentrations in this study were also the only entries with significant interaction effects, but these interactions, as previously

noted, were not same for all three entries. However, none of the genotypes used in the present study were tested by Krishnan *et al*. (2012), so a direct comparison cannot be made. Further research with more genotypes and better environmental data might more accurately determine the presence or absence of a genotype by environment interaction effect on BBI variation.

Table 3. Significance of environment x entry interaction for BBI concentration among diverse soybean entries grown at two locations in Illinois in 2012.

Entry	Numerator DF	Denominator DF	*F* value	*p* value
PI 603167	1	19	0.74	0.40
Lincoln	1	19	5.58	0.03
PI 594444 B	1	19	0.50	0.49
Jogun	1	19	11.62	< 0.01
PI 567362 A	1	19	0.02	0.88
PI 594871	1	19	1.95	0.18
PI 567775 A	1	19	0.08	0.78
NE3399	1	19	2.39	0.14
AK(Harrow)	1	19	8.04	0.01
PI 398261	1	19	0.12	0.73

Although there were significant entry x environment interactions, no significant main effect for environment on BBI (Table 1; $F = .15$, $p = .71$) was observed in the field experiment, a result in contrast with that of Krishnan *et al.* (2012). These authors grew 8 soybean lines in 3 locations and detected a consistent environmental effect on BBI, as measured by immunoblot

analysis. The previous paper was, however, unable to determine what environmental differences generated the significant effect; although soil analyses eliminated differences in available sulfur or nitrogen as causative factors, temperatures and moisture levels at the different locations were not reported. It is therefore difficult to conclude to what factor the significant environmental main effect may have been attributed, and conversely, to conclude if the environments of the present study did not differ for that factor.

The BBI concentrations observed in the present study are within the range of previously reported values. Yeboah *et al.* (1996) reported a yield of .185 mg BBI/g defatted flour using a rapid purification method, while Gladysheva *et al.* (2000) found a value of .23 mg BBI/g defatted flour; both values are relatively close to the average BBI concentration of .191/g defatted flour observed across the field experiment.

Significant differences also existed among entries in the field experiment for levels of lunasin (Table 4; $F = 6.09$, $p = .01$). Tukey's range test was used to separate the entries into classes based on lunasin concentration (Table 2): PI 603167 (North Korea), PI 594871 (Yunnan, China), and PI 398261 (Kyonggi, South Korea) had the highest lunasin levels of the entries, while PI 567362A and PI 567775A had the lowest levels. Interestingly, these two entries also had the lowest observed BBI levels in the present study. The remaining entries were not significantly different from either of these two classes. The observation of significant lunasin variation among entries corroborates the previous results of Vasconez (2004) and Wang *et al.* (2008). Vasconez (2004) indicated that genotypes of Chinese origin may contain more lunasin on average than genotypes from the Korean Peninsula; this effect did not appear to be supported in the present work, as both of the lowest-lunasin entries came from China and two of the highest-lunasin entries came from the Korean Peninsula. However, the previous work had also shown

greater lunasin variability within these regions than between them, so a failure to detect similar differences due to geographical origin with the present study's relatively small sample size should not be entirely unexpected.

Table 4. ANOVA for lunasin concentrations among diverse soybean entries grown at two locations in Illinois in 2012.

Source	Sum of Squares	DF	Mean Square	*F* value	*p* value
Environment	79.470132	1	79.470132	301.86	< 0.01
Genotype	14.440088	9	1.604454	6.09	0.01
Environment x Genotype	2.369423	9	.263269	1.00	0.47
Residual	4.997216	19	.263011	.	.

The main effect of environment on lunasin in the field experiment was highly significant (Table 4; $F = 301.86$, $p < .0001$), with concentrations at the Bellflower location averaging less than half (1.92 mg/g defatted flour) those at the Urbana location. The importance of environment to lunasin variation is further supported by the lack of similarity between the lunasin values of the present study and those of Vasconez (2004) for the same genotypes. The seeds in that previous work were obtained directly from the USDA Soybean Germplasm Collection, which, as previously discussed, is maintained by growing out a portion of its accessions every year. This practice means that the samples for each genotype used in the previous study were grown in different environmental conditions from each other and from the seeds analyzed in the field

experiment. The rankings of the entries for lunasin were different between the Vasconez study (2004) and the present study, suggesting that environment is more important than genetic differences in determining the amount of lunasin present in soybean.

The lunasin concentrations of the field experiment are within the range reported in the existing literature, but the concentrations at Bellflower fall far below the average of the 19 previously reported MG III genotypes (Vasconez, 2004), and some entries had lunasin concentrations below the previous minimum value (1.9 mg/g) reported for MG III. As previously discussed, de Mejia *et al.* (2004) reported a range of 1 to 13.3 mg lunasin/g defatted flour across a diverse array of soybean genotypes, and the cultivars grown in greenhouse conditions by Wang *et al.* (2008) ranged from 5.7 to 11.4 mg lunasin/g defatted flour. The average lunasin concentrations for the entries in this study range from 2.39 to 4.17 mg/g defatted flour, and the MG III plants grown at the Urbana location had an average lunasin concentration of 4.77 mg/g defatted flour, nearly identical to the value reported for the MG III genotypes (4.7 mg/g defatted flour) by de Mejia *et al.* (2004), which had also been grown in Urbana.

However, environmental conditions have previously been associated with differences in lunasin concentration of similar magnitude to those observed between Bellflower and Urbana, as reported by Wang *et al.* (2008). For example, when grown under high temperature and low moisture, the cultivar Imari yielded only 5.36 mg lunasin/g defatted flour, but when grown at an intermediate temperature with high moisture, the same cultivar yielded 9.36 mg/g, a difference of 4 mg/g. This proportional difference (~43%) due to environment is similar to that observed in the present work. Of particular interest to the present study are the differences recorded for the cultivar Jack. This cultivar yielded 11.40 mg lunasin/g defatted flour under intermediate temperature and low moisture but only 7.85 mg/g when grown at intermediate temperature and

high moisture. Over the growing season of the present study, rainfall at Urbana was approximately 33% less than that at Bellflower (Table 5) (wunderground.com); although this difference is less than that between the low and high moisture conditions (70%) employed in the previous work, it is a significant reduction in available moisture, and plants grown at Urbana yielded significantly more lunasin. Both environments had similar average temperatures over the growing season, suggesting that soil moisture was the driving factor behind the observed differences.

Table 5. Average monthly weather conditions for Bellflower and Urbana, IL.

	Bellflower		Urbana	
Month	Average Temperature (°C)	Total Precipitation (mm.)	Average Temperature (°C)	Total Precipitation (mm.)
May	20	63	21	62
June	23	49	21	7
July	27	34	27	1
August	22	173	23	112
September	17	202	18	148
October	10	163	11	123

The consistency of the lower lunasin values at Bellflower is perhaps of the greatest interest, as previous work has indicated that different genotypes respond in different directions to the same change in environmental conditions. According to Wang *et al.* (2008), both the genotype by temperature and genotype by soil moisture interactions for lunasin concentration were significant, but the genotype by environment interaction in the present study was not

significant (Table 3; $F = 1.00$, $p = .4719$). Without exception, every entry contained less lunasin at Bellflower than at Urbana. However, it is possible that the variability of external factors (*e.g.*, disease pressure, microenvironmental differences) introduced by a field experiment may have influenced these interactions, and caution must be exercised in the comparison of greenhouse and field data. Further experimentation using greenhouse controls and a greater number of genotypes will be necessary to determine the most common pattern of lunasin's response to environmental factors and which of these factors are most important.

Despite the existing information about lunasin's properties and presence in soybean, its biological role in the plant remains unknown. Some of the data obtained in this study may offer a hypothesis for its function. Lunasin was observed at higher concentrations in the more water-stressed environment of Urbana; water stress is known to generate an excess of reactive oxygen species (ROS) in plants (Iturbe-Ormaetxe *et al.*, 1998; Lima *et al.*, 2002), which can lead to significant oxidative damage of key cellular components. Nuclear DNA can be harmed by ROS, and although plants have a number of mechanisms to repair (Britt, 1996) or resist (Badawi *et al.*, 2004) this damage, it may reach unsustainable levels in some cells. In this case, the plant often resorts to programmed cell death in the damaged cells (Foyer & Noctor 2005). Lunasin has been shown to encourage apoptosis in cancerous cells, many of which also are derived through DNA damage from oxidative stress (Valko *et al.*, 2006). In the developing soybean seed, lunasin may serve to target the elimination of defective cells under drought conditions, ensuring the normal development of these vital propagules. It is reasonable that regulatory mechanisms would exist in some soybean cultivars to increase lunasin under drought conditions if the peptide does indeed provide this function. This line of reasoning is highly speculative, but would serve to explain some of the results of the present experiments. Future work might examine metabolic variables

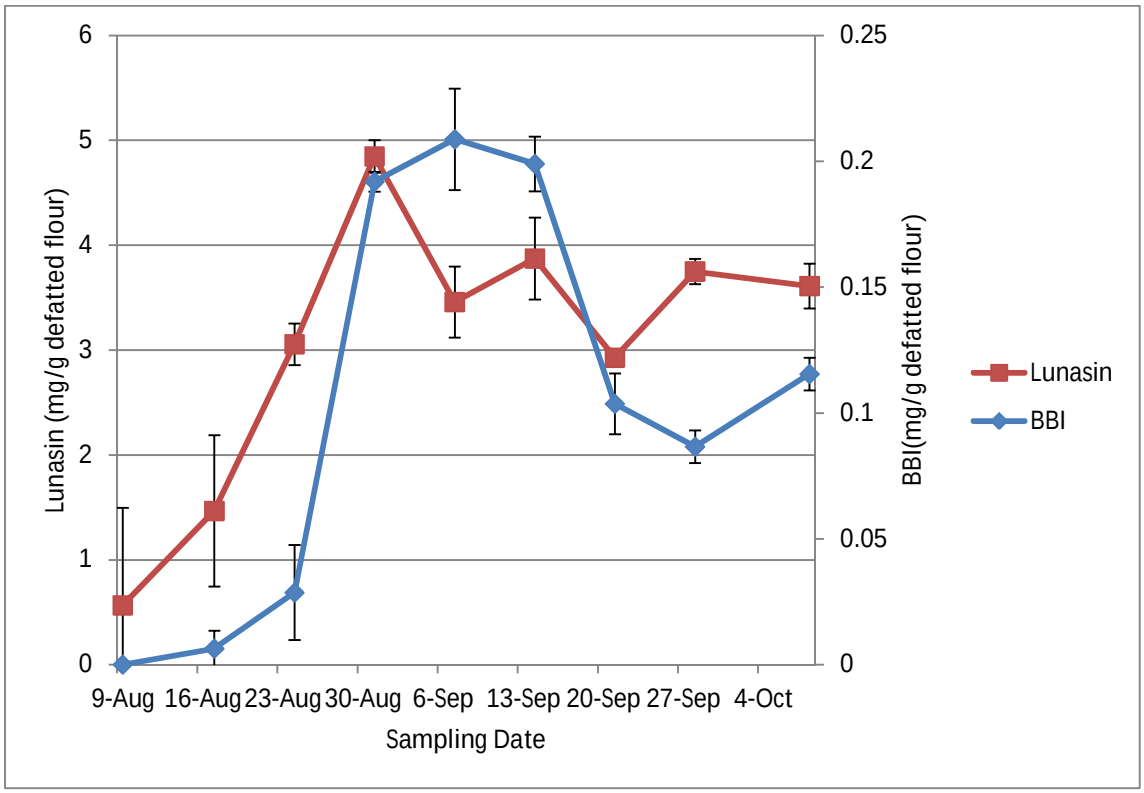
in soybean genotypes with diverse lunasin contents or apply pure lunasin to drought-stressed cells to determine if this hypothesis has merit.

The results for BBI and lunasin concentration at each of the harvest dates in the time course experiment are presented in Table 6, organized by sampling date. The highest level of BBI (.21 mg/g defatted flour) was observed at the 5th week of sampling, and the highest level of lunasin (4.85 mg/g defatted flour) was observed at the 4th week of sampling. A visual representation of the levels of the two compounds over the experiment is given in Figure 1. A significant correlation was detected between BBI and lunasin concentrations (Figure 2; R^2 = .6396, F = 12.42, p = .0097) over the time course, hinting at a similar regulatory mechanism or set of mechanisms governing their levels in the growing soybean. The lack of a significant correlation between either BBI (R^2 = .1763, F = 1.5, p = .2605) or lunasin (R^2 = .2793, F = 2.71, p = .1436) with overall protein is of particular interest, as this result suggests that BBI and lunasin are governed by factors beyond the general increase in protein associated with seed fill.

Table 6. Average BBI, lunasin, and soluble protein concentrations over 9 weeks for NE3399 grown at one location in Illinois in 2012.

Calendar Date	BBI (mg/g defatted flour)	Lunasin (mg/g defatted flour)	Soluble Protein (mg/g defatted flour)
8-9	0.00	0.57	76
8-17	0.01	1.47	101
8-24	0.03	3.05	107
8-31	0.19	4.85	148
9-7	0.21	3.46	185
9-14	0.20	3.87	136
9-21	0.10	2.93	246
9-28	0.09	3.75	223
10-8	0.12	3.61	240

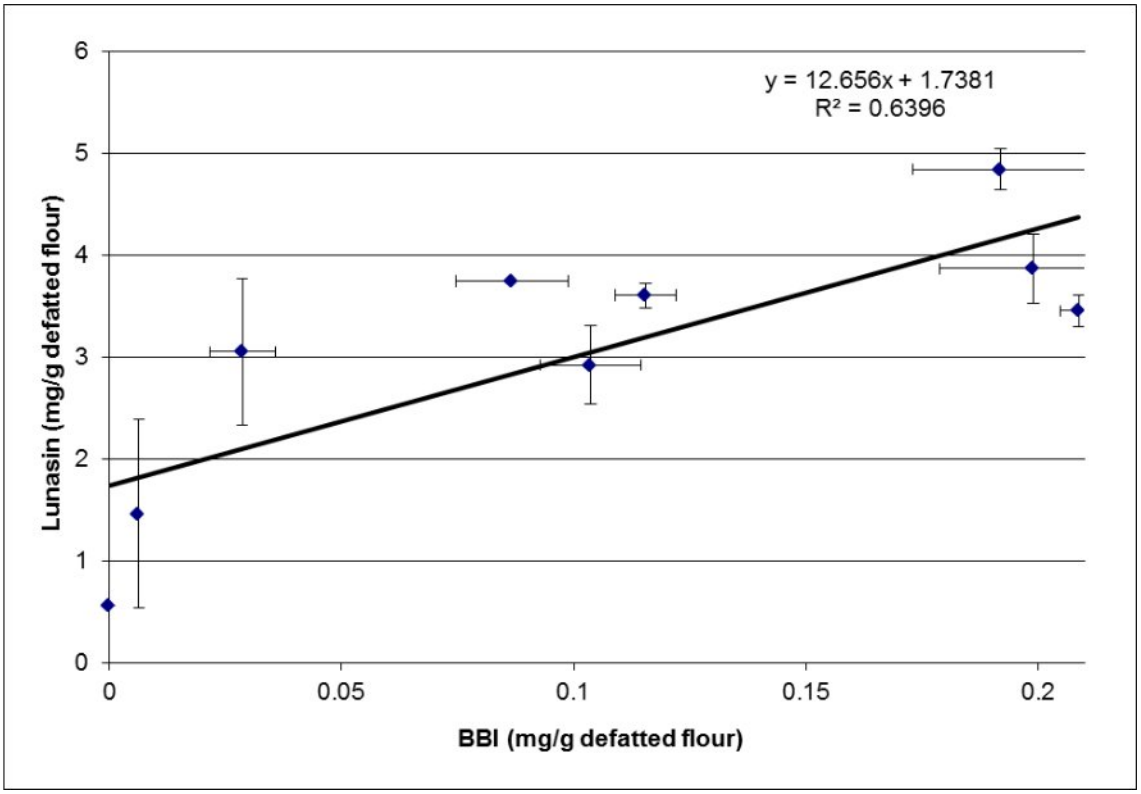
Figure 1. Average BBI and lunasin concentrations over 9 weeks for NE3399 grown at one location in Illinois in 2012. Error bars indicate the standard error of the means for lunasin and for BBI.



Both BBI and lunasin peak around the 4th or 5th week of the time course, approximately the R6 stage of seed development, then decrease and maintain a lower concentration while overall protein continues to increase. The Western blot method used by Park *et al.* (2005) was not as sensitive as the ELISA method employed in the present study, but these authors also detected peak concentrations of BBI and lunasin sometime before the end of seed development and a subsequent decline. BBI associates with the 2S soybean protein fraction (Wolf, 1970), and lunasin has also been shown to belong to this fraction (Seber *et al.*, 2012). In previous work, the 2S fraction has been shown to increase early in seed development before reaching a plateau (Hill & Briedenbach, 1974). Although many other proteins and peptides besides BBI and lunasin are

present in the 2S fraction, this result is consistent with the finding that the two compounds do not increase linearly throughout the entirety of soybean seed development.

Figure 2. Significant correlation of average BBI and lunasin over 9 weeks among diverse soybean entries grown at one location in Illinois in 2012. Vertical error bars indicate standard error of the mean for lunasin at each harvest date. Horizontal error bars indicate standard error of the mean for BBI at each harvest date.



As previously discussed, lunasin may serve as a drought protective mechanism in actively developing seeds. It may be supposed that once the soybean seed is no longer actively developing, the need for protection from DNA damage would be reduced, and lunasin levels might decrease, as was observed in the time course experiment. Similarly, BBI may also serve a protective role in soybean as an insect deterring (Ryan, 1990) or even insecticidal (Prasad *et al.*,

2010) agent. Although BBI has not been shown to be induced by insect wounding in soybean, members of the BBI family have shown this response in other species. The first wound-inducible BBI was observed in alfalfa (Brown *et al.*, 1985), and subsequent molecular work has shown that transcripts for BBI family proteins accumulate after wounding in both maize (Eckelcamp *et al.*, 1993) and rice (Qu *et al.*, 2003). As with drought protection, insect protection is likely more important in plants undergoing the metabolically intense process of seed fill. Therefore, it would be advantageous to accumulate BBI and lunasin during active seed fill, but the plant might make better use of the nutrients (especially those of the sulfur-rich BBI) elsewhere later in the season and break down the compounds for remobilization. Again, the present study lacks the data to support or refute this hypothesis, but future work might examine the impacts of insect pressure on BBI induction and accumulation in soybean to illuminate this issue.

Although the relative levels of BBI and lunasin in the developing seed may be significantly correlated, the absolute levels of the two compounds seem to be independent across the studied genotypes. There appeared to be little relationship between the lunasin and BBI concentrations observed in the field experiment. To this researcher's knowledge, this is the first study to compare BBI and lunasin levels among soybeans grown in common environments. Additionally, no relationship between the BBI levels of the accessions taken from the USDA Soybean Germplasm Collection and the lunasin levels reported by Vasconez (2004) for the same accessions was observed (Table 7). The seed sources used in the previous work were all different from those used in the present study and were grown under different environmental conditions in different years (R. Nelson, personal communication). These results emphasize the importance of environment to BBI and lunasin and suggest that reliable testing for genotypic differences in

concentrations of the two compounds must be conducted under constant environmental conditions.

Lunasin's biological role is unknown, but it is not thought to be insecticidal like BBI. Therefore, if pest pressure is a significant selective force on BBI, and there is no linkage between the genes for BBI and lunasin (as is likely; none of the BBI family sequences reported by de Almedia Barros *et al.* (2012) are present on chromosome 13, the location of the lunasin sequence as reported by Grant *et al.* (2010)), BBI would be selected for independently of lunasin.

Table 7. Maturity group, geographical origin, and average BBI concentrations for entries taken from the USDA Soybean Germplasm Collection and average lunasin concentrations for the same entries as reported by Vasconez (2004).

Entry	Maturity Group	Geographical Origin	BBI (mg/g defatted flour)	Lunasin (mg/g defatted flour)
Cisne	IV	USA	0.12	7.56
PI 587670B	VII	China	0.26	8.51
PI 603309	I	China	0.27	2.19
PI 228056	VIII	Japan	0.28	2.22
PI 90243	V	Korea	0.32	1.87
PI 603334	0	China	0.34	2.22
PI 507298	VI	Japan	0.36	1.90
FC 33243	IV	Unknown	0.37	7.58
Council	0	USA	0.38	7.41
PI 424474-1	VII	Korea	0.38	2.41

The apparent independence of BBI and lunasin is useful information for breeders who may wish to improve soybeans for the nutraceutical market. The value of BBI in protecting lunasin from digestion has been established (Park, 2007), and breeding efforts would be necessary to increase the levels of each compound independently. However, the genetic variation observed for both compounds in the present study suggests that there exists sufficient diversity in the germplasm for breeders to make significant gains. Conversely, the environmental variation seen for lunasin concentrations is promising for companies that wish to extract the peptide alone. Sourcing soybeans from drought-stressed areas may allow for more lunasin to be derived from the same amount of seed, making the prospects of large-scale lunasin purification and marketing more economical.

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