

ISB NEWS REPORT

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Recent US Supreme Court Decisions Fortify Patents

Phillip Jones

Stanford University reportedly looked at Roche for more than \$200 million in damages for infringing patents on a polymerase chain reaction method for measuring human immunodeficiency virus RNA in human blood samples. On June 6, 2011, the US Supreme Court told Stanford to look elsewhere.

In 1988, around the time that Dr. Mark Holodniy joined Stanford as a research fellow in the Department of Infectious Diseases, scientists in the department started collaborating with Cetus scientists on methods to test the effectiveness of new drugs to treat AIDS. Holodniy worked on a PCR technique for quantifying HIV levels in patient blood samples. Since he had limited experience with PCR, Holodniy’s supervisor arranged for him to continue his research at Cetus. For nine months, Holodniy worked with Cetus researchers and devised a PCR assay to measure HIV RNA in human plasma. He published his findings with Cetus co-authors. After he returned to Stanford, Holodniy collaborated with Stanford researchers to test the PCR assay on samples from patients undergoing antiretroviral drug therapy. Their results showed that levels of HIV RNA in human blood provided a marker of antiviral drug efficacy, and they filed a patent application that eventually matured into three patents.

After Roche purchased Cetus’ PCR business in December 1991—a package deal that included Cetus’ agreements with Stanford researchers—the company began to manufacture PCR kits for detection of HIV RNA. In May 1992, Stanford filed the PCR-HIV RNA patent application. Holodniy and the other inventors assigned their patent rights to Stanford. In April 2000, Stanford asserted its ownership of the HIV RNA assay and offered Roche an exclusive license to all related patents. Roche declined the offer, responding that it owned the patent rights. Following years of failed negotiations, Stanford filed a lawsuit against Roche in the Northern District of California on October 14, 2005.

The university alleged that Roche’s HIV PCR detection kits infringe its patents. Among other things, Roche declared that the company owns the patents through the acquisition of Cetus’ PCR assets. Roche pointed out that Holodniy had assigned his rights in Cetus’ Visitor’s Confidentiality Agreement, which he had signed before collaborating with Cetus scientists. Stanford replied that Holodniy had no rights to assign to Cetus, because the University’s HIV research had been federally-funded, which gave the university superior rights in the invention under the Bayh-Dole Act. The district court agreed with Stanford; the Bayh-Dole Act enables an inventor to obtain title to a federally-funded invention *only if* the government and the contracting party (i.e., Stanford University) declined to do so. That was not the case here: In 1995, Stanford had formally notified the government that it elected to retain title to the PCR-HIV inventions under the Bayh-Dole Act. Roche appealed the decision to the Court of Appeals for the Federal Circuit.

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bution.

To determine whether Stanford owned the technology, the Federal Circuit reviewed the various agreements that Holodniy had executed. In 1988, Holodniy had signed Stanford's Copyright and Patent Agreement, which states "I agree to assign or confirm in writing to Stanford and/or Sponsors that right, title and interest in . . . such inventions as required by Contracts or Grants." The phrase "agree to assign" signals a simple promise to assign rights in the future, the court said. In 1989, Holodniy signed Cetus' Visitor's Confidentiality Agreement, which states "I will assign and do hereby assign to CETUS, my right, title, and interest in each of the ideas, inventions and improvements" that Holodniy may devise "as a consequence of" his work at Cetus. The Federal Circuit decided that Holodniy's invention of the PCR assay for HIV RNA arose from his collaboration with Cetus, and that the invention was a consequence of his work at the company. Turning to the wording of the two documents, the court stressed that, unlike the Stanford agreement, the Cetus document had created a present assignment of Holodniy's future inventions. Cetus had immediately gained equitable title to Holodniy's inventions. Holodniy had completed his invention by the time that he filed his first HIV PCR patent application that matured to a patent. No later than that filing date, the court explained, Cetus's equitable title converted to legal title. By the time that the inventor had assigned his rights in the patent application to Stanford, the court said, Cetus's legal title had vested, and Holodniy no longer retained rights in the patent application that he could assign to Stanford. Moreover, the Federal Circuit held that the Bayh-Dole statutory scheme did not automatically void the patent rights that Cetus received from Holodniy.

Stanford appealed the decision to the Supreme Court, focusing on one issue: Whether a federal contractor university's right under the Bayh-Dole Act in inventions arising from federally-funded research can be terminated unilaterally by an inventor through a separate agreement intending to assign the inventor's rights to a third party. Universities and organizations representing universities filed amicus briefs in support of Stanford. The Wisconsin Alumni Research Foundation argued that the Federal Circuit's decision negates the Bayh-Dole Act, jeopardizing decades of public benefits from transfer of university-developed research. In its brief, the US government declared that title to the HIV-PCR invention initially vested in Stanford as contractor, and Stanford exercised its rights under the Bayh-Dole Act to retain title. Holodniy could not have assigned his rights in the invention to Cetus; he never had the rights.

Writing for the Supreme Court, Chief Justice Roberts summarized the majority's view in the first paragraph of the opinion. "Since 1790, the patent law has operated on the premise that rights in an invention belong to the inventor," he wrote. "The question here is whether the University and Small Business Patent Procedures Act of 1980—commonly referred to as the Bayh-Dole Act—displaces that norm and automatically vests title to federally funded inventions in federal contractors. We hold that it does not."

The Chief Justice explained that the general rule is that rights in an invention belong to the inventor. An inventor can assign rights in an invention to a third party. However, unless there is an agreement to the contrary, an employer does

not have rights in an invention that was conceived by the employee. Roberts wrote that the Bayh-Dole Act does not change these rules. “Stanford and the United States as amicus curiae contend that the Bayh-Dole Act reorders the normal priority of rights in an invention when the invention is conceived or first reduced to practice with the support of federal funds,” he wrote. “In their view, the Act moves inventors from the front of the line to the back by vesting title to federally funded inventions in the inventor’s employer—the federal contractor.” But the Bayh-Dole Act does not expressly vest title in contractors or anyone else. Nor does the Act expressly deprive inventors of their interest in federally funded inventions.

Howard Bremer, emeritus patent counsel for the Wisconsin Alumni Research Foundation, summed up the lesson of the *Stanford* case for *Genetic Engineering & Biotechnology News*. “The ultimate effect on the universities might be,” he said, “number one, they have to do a better job, or try to do a better job, of educating faculty on what they should and should not sign.” That’s advice worth at least \$200 million.

Destroying a Patent is an Arduous Task, Supremes Say.

According to §282 of the Patent Act of 1952, a patent is presumed valid, and the burden of establishing invalidity rests upon the party who asserts that patent claims are invalid. But what is the standard of proof? Convincing a trier of fact (a jury, or judge in a trial without a jury) beyond a reasonable doubt is an unreasonably high standard. In *Microsoft Corporation v. i4i Limited Partnership et al.*, Microsoft wanted to prove an i4i patent to be invalid using a preponderance of evidence standard. In this case, Microsoft would need to provide enough evidence to make it more likely than not that something is true. Basically, a person need only just tip the scales to make a point with a preponderance of evidence standard. On June 9, 2011, the Supreme Court decided that Microsoft has a much greater hurdle to overcome if the company wants to invalidate the i4i patent.

The case concerned i4i’s patent claims for an improved method for editing computer documents,

which stores document content separately from the metacodes associated with a document’s structure. In 2007, i4i sued Microsoft, claiming that Microsoft Word products infringe i4i’s patent. Among other things, Microsoft sought a declaration that the i4i patent is invalid and unenforceable, and introduced alleged prior art that Microsoft said rendered the i4i patent invalid. Microsoft wanted the judge to instruct the jury that the company’s burden of proof with regard to its invalidity defense is by preponderance of the evidence. The judge, however, instructed the jury that Microsoft has the burden of proving invalidity by clear and convincing evidence. This meant that the jury had to decide that invalidity is more highly probable to be true than not. It’s not quite a “beyond a reasonable doubt” standard, but much tougher than a “preponderance of the evidence” standard. The jury decided that Microsoft had failed to prove invalidity of the i4i patent. Microsoft appealed, but the Federal Circuit confirmed the lower court. So, Microsoft appealed to the Supreme Court.

The Court disagreed with Microsoft. By the time that Congress enacted §282 and declared that a patent is presumed valid, Justice Sotomayor wrote, the presumption of patent validity had been a part of US common law for more than a century. According to common law, a defendant who raised an invalidity defense bore a heavy burden of persuasion, one that required proof by clear and convincing evidence. Microsoft argued that judges had not applied a clear and convincing standard of proof in every case involving a patent invalidity defense. “Squint as we may,” Sotomayor said, “we fail to see the qualifications that Microsoft purports to identify in our cases.” The clear and convincing evidence standard remains, the Court said, unless Congress decides to alter the law.

“This decision,” Stephanie Fischer wrote for BIOtechNOW, “is a huge relief for the biotechnology industry, which relies heavily on the presumed validity of patents to generate investment and a reasonable return thereon.” BIOtechNOW is a publication of the Biotechnology Industry Organization, which filed an amicus brief with CropLife International and the Association of University Technology Managers in support of the clear and convincing evidence standard.

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USDA Seeks Public Comment on Draft Environmental Assessment for Genetically Engineered Soybean

WASHINGTON, June 28, 2011

The U.S. Department of Agriculture's Animal and Plant Health Inspection Service (APHIS) has prepared a plant pest risk assessment and a draft environmental assessment (EA) to address a request from the Monsanto Company seeking a determination of nonregulated status. The request is for soybeans that have been genetically engineered (GE) to have a modified fatty acid profile and a tolerance to the herbicide glyphosate.

Monsanto's GE soybean, event MON 87705, produces soybean seeds with lower levels of saturated and polyunsaturated fatty acids and higher levels of monounsaturated fatty acid than those found in non-modified soybean seeds. MON 87705 also expresses tolerance to glyphosate, which is the active ingredient in Roundup®.

APHIS' risk assessment indicates that MON 87705 is unlikely to pose a plant pest risk. APHIS is making available for public comment the Monsanto petition, the agency's risk assessment and the draft EA for the proposed determination of nonregulated status.

APHIS has prepared its draft EA to consider the potential environmental effects of a determination of nonregulated status consistent with the Council on Environmental Quality's regulations implementing the National Environmental Policy Act regulations and procedures.

This EA has been prepared in order to specifically evaluate the effects on the quality of the human environment that may result from a nonregulated status determination of MON 87705. MON 87705 is currently under APHIS regulation. Interstate movements, importations and field testing have been conducted under notifications acknowledged by APHIS.

The notice of availability of the risk assessment, draft EA and the petition is published in the June 28 Federal Register. **Consideration will be given to comments received on or before Aug. 29.** You may submit comments by either of the following methods:

- Federal eRulemaking Portal: Go to www.regulations.gov/#!documentDetail;D=APHIS-2011-0046 to submit or view comments and to view supporting and related materials available electronically.
- Postal Mail/Commercial Delivery: Please send your comment to Docket No. APHIS-2011-0046, Regulatory Analysis and Development, PPD, APHIS, Station 3A-03.8, 4700 River Road Unit 118, Riverdale, MD 20737-1238.

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Biotechnologies for Agricultural Development: FAO Publishes the ABDC-10 Proceedings

John Ruane

FAO has just published “Biotechnologies for Agricultural Development,” a 592-page book that represents the proceedings of the FAO international technical conference on “Agricultural Biotechnologies in Developing Countries: Options and Opportunities in Crops, Forestry, Livestock, Fisheries and Agro-industry to Face the Challenges of Food Insecurity and Climate Change” (ABDC-10). The book can be downloaded at <http://www.fao.org/docrep/014/i2300e/i2300e00.htm>.

ABDC-10 took place in Guadalajara, Mexico, 1 – 4 March 2010, and was hosted by the Government of Mexico and co-sponsored by the International Fund for Agricultural Development (IFAD). The Consultative Group on International Agricultural Research (CGIAR), the Global Forum on Agricultural Research (GFAR), the International Centre for Genetic Engineering and Biotechnology (ICGEB), and the World Bank were also major partners in this initiative.

The Conference was dedicated to “agricultural biotechnologies,” a term covering a broad range of biotechnologies used in food and agriculture for a variety of different purposes, such as the improvement of plant varieties and animal populations to increase their yields or efficiency; characterization and conservation of genetic resources; plant or animal disease diagnosis; vaccine development; and improvement of feeds and the safety of foods.

The Proceedings contain a 2-page foreword signed by Jacques Diouf, the FAO Director-General, and is organized in two main sections. The first section contains ten chapters with an extensive series of FAO background documents, prepared before ABDC-10 took place. The second section contains five chapters dedicated to the outcomes of ABDC-10.

Background to ABDC-10

The first five chapters are sector-specific, covering the current status and options for biotechnologies in developing countries in crops, forestry, livestock, fisheries/aquaculture and, finally, in food processing and food safety. Each of the sector-specific documents

is organized into two parts.

The first part, entitled “Stocktaking: Learning from the past,” provides a brief overview of the history and status of application of conventional technologies in the sector; describes the biotechnologies that are covered in the chapter; documents the current status of application of these biotechnologies in developing countries; analyses the reasons for successes or failures of using biotechnologies in developing countries in the past; and presents a small number of relevant case studies for illustration purposes. The second part, entitled “Looking forward: Preparing for the future.” begins by identifying some key, unsolved problems in the sector where the use of biotechnologies could be useful. Then, based on the overview and analyses contained throughout the chapter, it identifies a number of specific options to assist developing countries in making informed decisions regarding adoption of biotechnologies as well as a set of priorities for action for the international community (FAO, UN organizations, non-governmental organizations, donors and development agencies).

Chapter 6 is dedicated to the moderated e-mail conference that was held in 2009 to complement the five sector-specific documents entitled “Learning from the past: Successes and failures with agricultural biotechnologies in developing countries over the last 20 years” (<http://www.fao.org/biotech/conf16.htm>). About 850 people subscribed to the e-mail conference. The chapter contains the Background Document prepared before the conference took place as well as the Summary Document, which summarizes the major issues discussed by participants. During the conference, 121 messages were posted by 83 people living in 36 different countries, the greatest number coming from people in India, Nigeria, Argentina, the United States, and Cameroon. The majority of messages (74%) were posted by participants living in developing countries. Most contributions focused on whether applications of one or more biotechnologies had been a success or a failure in the crop, livestock, forestry or food processing sectors, as well as the factors that determined their success or failure.

Chapters 7–9 are closely inter-connecting chapters that deal with policy options for strengthening national capacities to make informed choices about using biotechnologies for food and agriculture. All three chapters make extensive use of concrete examples from national biotechnology policy documents approved by 15 selected developing countries.

Chapter 7 attempts to “paint the broad picture,” covering some of the foundations and principles for countries to consider when targeting biotechnologies to the poor. It first describes the broad context (national and international) within which agricultural policies operate and then deals with the “why, what and how” of developing, approving, and implementing a national biotechnology strategy framework, including a list of the key policy issues that should be addressed at the governmental level. Options for the governance of agricultural biotechnologies are then provided, dealing with both structural and organizational aspects (such as leadership, coordination, and options for independent advice). The chapter concludes with the all-important issue of research and development (R&D) priority-setting at government, ministerial, and research institution levels, including the “division of labor” between the public and private sectors.

Chapter 8 deals with enabling R&D for agricultural biotechnologies. It provides an initial general overview of the global picture with respect to human and financial investments in agricultural science and technology, including biotechnology, and then describes funding instruments and options to be considered by countries. Numerous country-specific examples about capacity building and funding for agricultural biotechnologies are provided. The chapter also covers regulation, including, inter alia, options for establishing national biotechnology regulatory frameworks. Emphasis is also given to the international dimensions of biotechnology regulation, including international harmonization.

Chapter 9 is dedicated to ensuring access to the benefits of R&D. It covers three main subjects. The first,

intellectual property rights (IPR) and genetic resources, includes issues such as the establishment of laws and institutions, and IP policy options and mechanisms for accessing biotechnology tools and products by research institutes and national and international research funding and development agencies. The second, public awareness and participation, is covered from the standpoints of engaging the wider society in planning, implementing, and assessing biotechnology R&D and extension and in the regulation of GMOs. The third is agricultural extension.

The final background document (Chapter 10) builds on the previous documents and aims to synthesize the lessons learned and options available to developing countries for making informed decisions regarding adoption of agricultural biotechnologies within their national food security and rural development plans and policies. It also presents a set of priorities for action for the international community regarding agricultural biotechnologies for food security in developing countries, organized in three categories covering policy, capacity development, and coordination.

“Numerous country-specific examples about capacity building and funding for agricultural biotechnologies are provided.”

Outcomes of ABDC-10

During the ABDC-10 conference, 27 parallel sessions were held over the first three days. Short summary reports were prepared after the sessions were terminated and were presented to the Plenary Session by a Rapporteur the following morning.

Ten of the sessions were dedicated to sector-specific issues and were organized by FAO. Chapter 11 presents the summary reports of the 10 sector-specific parallel sessions, 5 of which were dedicated to the background documents (presented in Chapters 1 to 5) and 5 to case studies of successful applications of biotechnologies in crops, forestry, livestock, fisheries/aquaculture, or agro-industry in developing countries.

Twelve parallel sessions were dedicated to cross-sectoral issues, such as genomics, genetic resources, public-private partnerships, intellectual property rights

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and enhancing human capacities. Most were organized by different inter-governmental and non-governmental organizations. Chapter 12 presents the summary reports of these sessions.

Five parallel sessions were region-specific and were organized by relevant regional organizations. The regions covered were Latin America and the Caribbean; West Asia and North Africa; Sub-Saharan Africa; Asia-Pacific; and Europe and Central Asia. Chapter 13 presents these summary reports.

Chapter 14 contains four keynote presentations from the representatives of FAO, the Government of Mexico, and IFAD, as well as by M.S. Swaminathan, Honorary Chair of the ABDC-10 Conference Steering Committee.

Participation at the ABDC-10 conference was

by invitation and it brought together about 300 policy-makers, scientists, and representatives of intergovernmental and international non-governmental organizations. This included delegations from 42 FAO Member Countries, namely Algeria, Argentina, Bhutan, Brazil, Cameroon, Canada, Cape Verde, the Cook Islands, Cuba, the Dominican Republic, Egypt, El Salvador, Gabon, the Gambia, Grenada, Guatemala, Haiti, India, Indonesia, Kenya, Lesotho, Malawi, Malaysia, Mexico, Morocco, the Netherlands, Nigeria, Pakistan, Panama, Peru, Qatar, Senegal, Sri Lanka, Suriname, United Republic of Tanzania, Thailand, Trinidad and Tobago, Turkey, United States of America, Uruguay, Zambia and Zimbabwe. On the afternoon of the final day, the member countries adopted the conference report, which is provided in Chapter 15.

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Genetic Engineering for High Threonine Soybean

Qungang Qi and James Crowley

Introduction

World population is predicted to reach 8.3 billion in 2030 and 9.1 billion in 2050, representing a 34 percent increase compared to current levels. In order to feed this large population, annual cereal production would need to be about 3 billion tons in 2050, up from 2.1 billion today, and annual meat production will need to rise by over 200 million tons to 470 million tons¹. As we face pressure from this continuous population growth and limiting agricultural resources, we recognize that the challenge is not only to meet the future requirements of human food and animal feed, but also to address the need for nutritionally balanced crops and foods.

Protein quality has always been an important consideration in both human and animal nutrition. Because humans and many farm animals lack the enzymatic machinery for *de novo* synthesis of nine essential amino acids (EAAs) that are constituents of proteins necessary for their own growth and function,

these nine EAAs (lysine, threonine, methionine, phenylalanine, tryptophan, isoleucine, leucine, valine, and histidine) must be acquired through diet. The deleterious effects of diets that are sufficient in protein quantity (amount) but deficient in quality (the content of EAAs) are well documented². Enhancing EAAs or nutritional protein content in major grain crops will have a significant social and economical impact worldwide, especially in developing countries where caloric demand is more likely satisfied at the expense of balanced amino acid nutrition.

Major crops are deficient in one or more of EAAs for human food and animal feed. These deficiencies include Lys and Trp in maize³, Met and Thr in soybean⁴, Met, Cys, and Ile in potatoes⁵, and Lys and Thr in rice⁶. Therefore, chemically- or microbially-synthesized EAAs are routinely used as supplements to grain-based and other livestock diets, resulting in increased cost of these diets. An alternative approach—genetically developing

crops with enhanced/balanced EAAs—can offer a more efficient and sustainable system to nutritionally balanced animal diets.

Because Thr, Lys, Met, and Ile are the major limiting EAAs in crops, and all are produced from aspartate in plants via a branched pathway (Fig. 1), the aspartate family pathway has received increasing interest in plant molecular biology and biotechnology. An improved understanding of plant essential amino acid biosynthesis now makes it possible to metabolically engineer increased EAA content using plant breeding and biotechnology approaches. Here we describe one successful approach to genetically engineer high free threonine soybean seed with normal germination via expression of feedback-resistant *Xenorhabdus bovienii* aspartate kinase variants in developing seed.

Strategies to genetically enhance essential amino acids in grain crops

Among the different strategies used to date, three main ones have been used to enhance the levels of either free or storage protein-bound EAAs in crops: conventional plant breeding; genetic selection of high EAA mutants; and biotechnology. So far, the success of breeding and mutant selection approaches has been mostly restricted to development of the high-lysine corn through identification of natural variation/mutation³. This effort has been hampered by undesirable agronomic traits

associated with the mutation. A lack of natural genetic resources for plant breeding is mostly responsible for the relatively limited success in increased EAAs for other crop species.

In contrast, genetically engineering offers a more promising opportunity for improving the nutritional quality of seed. Recent advances in genomics and biotechnology provide an efficient and direct way of tagging the genes of interest and subsequently developing molecular markers to accelerate the incorporation of target genes into elite germplasm. In particular, biotechnology can use seed-specific promoters for directing specific expression of desired traits of interest, thus efficiently correcting undesirable traits associated with high amounts of a specific amino acid. Successful examples of biotech crops with improved quality traits include high-Lys LY038 corn³ (first commercial biotech crop with high nutritional value), high-Thr soybean⁷, and high-Trp soybean⁸.

With scientific advances in genomics, various biotechnology approaches have been attempted to enhance levels of EAAs in food crops^{8,9}. Three strategies stand out, namely 1) increasing the free EAA pool through the genetic engineering of EAA metabolism;

2) enhancing the nutritional quality of proteins with the appropriate EAA profile by changing the levels of natural EAA-rich proteins and/or expression of synthetic genes encoding EAA-rich proteins; and 3) increasing both free and protein-bound EAAs in crops. Increasing free EAAs and enhancing EAA-rich protein accumulation simultaneously would offer the advantage of reducing the potentially negative effects caused by high concentrations of a free amino acid or unbalanced flux in amino acid biosynthetic pathways.

Metabolically engineered soybean seed with enhanced threonine levels

Threonine (Thr) is one of several limiting essential amino acids, and its level in feed rations can impact the production of important meat sources, such as swine and poultry. As shown in Figure 1, aspartate forms the entire carbon skeleton for Thr through a primary path, where secondary

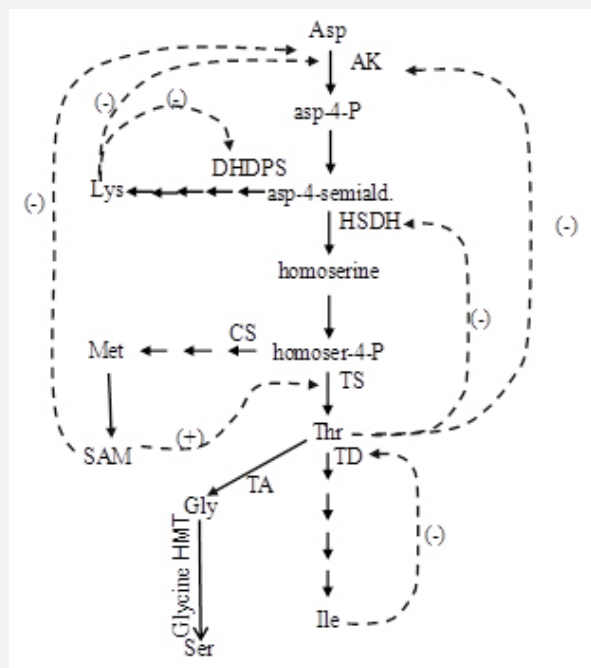


Figure 1. Schematic drawing of the aspartate family biosynthetic pathway and regulation
 AK= aspartate kinase; DHDPS=dihydrodipicolinate synthase; HSDH= homoserine dehydrogenase; CS=cystathione gamma-synthase; TS=threonine synthase; TA=threonine aldolase; TD=threonine deaminase; glycine HMT=glycine hydroxymethyltransferase

branches in the pathway allow for the synthesis of Lys and Met. The first enzyme in the pathway, aspartate kinase (AK, EC 2.7.2.4), catalyzes the ATP-dependent phosphorylation of aspartate to β -aspartyl phosphate. AK is the main regulatory step controlling metabolic flux through the pathway.

To elevate Thr levels in soybean seed while maintaining normal seed germination, we identified and tested feedback-resistant AK enzymes from various organisms that can be expressed in developing soybean seeds. We first purified and biochemically characterized AK from the enteric bacterium *Xenorhabdus bovienii* (*Xb*). Site-directed mutagenesis of XbAK identified two key regulatory residues, Glu-257 and Thr-359, involved in lysine inhibition. End-product feedback inhibition experiments demonstrated that the conservative replacement of XbAK Thr-359 with Ile increased the IC_{50} value for Lys by 104-fold, but no substantial differences were observed in other kinetic parameters, with the exception of a lower K_m^{ATP} value compared to the wild-type enzyme (**Table 1**). Mutation of the conserved Glu-257 to Lys also renders the enzyme insensitive to Lys, as shown by an 86-fold increase in the IC_{50} value compared to the wild-type enzyme. The results indicate an important role for both Thr-359 and Glu-257 residues in feedback inhibition of the enzymatic activity.

To test the ability of feedback-insensitive AK variants to enhance Thr accumulation in crop seeds, wild-type or Lys-resistant XbAK-T359I and XbAK-E257K alleles were expressed in soybean seed under the control of two different seed-specific promoters, *7S α '* or *USP99*⁷ (**Table 2**). A chloroplast targeting sequence, CTP1, was fused in-frame to AK coding sequences to target the protein to the plastids, where the aspartate pathway operates.

Free amino acids of mature soybean seed from transgenic events expressing the wild-type and Lys-insensitive XbAK genes were extracted and quantified using an HPLC method⁷. Levels of protein-bound Thr and other EAAs were not measured in this study but would be useful to be determined in future homozygous transgenic lines. For each independent transgenic event, 25 R_1 seeds (segregating 1:2:1, null: hemizygous: homozygous, with respect to transgene) were harvested at maturity and bulked for analysis of free amino acids. As shown in Table 2, all three constructs expressing feedback-resistant variants showed substantial increases

in free Thr levels compared to the non-transgenic wild-type control, with up to a 100-fold increase for seed from the highest event (3112 ppm in *USP99::CTP1::XbAK-T359I* vs. 31 ppm in WT). The mean free Thr level in the transgenic events from the three constructs expressing lysine-insensitive XbAK was 49- to 93-fold higher than the wild-type control. Expression of the Lys-sensitive wild-type XbAK did not result in any substantial change in Thr levels in the mature seed. The results suggest that allosteric feedback inhibition of AK enzyme may override the over-expression of these proteins and plays a critical role in controlling threonine production via the aspartate family pathway.

Further analysis of free amino acid profiling data reveals that in addition to Thr levels, similar increases were also observed in other aspartate-derived amino acids in XbAK transgenic seed (**Table 3**). For example, the changes in *USP99*-driven XbAK-T359I transgenic events included significant increases in methionine (3.9-fold), lysine (2.6-fold), isoleucine (3-fold), glycine (6-fold), and serine (31-fold). Substantial increases in accumulation of free Gly and Ser in the transgenic soybean seeds suggests that accumulation of free Thr levels are controlled not only by the rate of its synthesis, but also by the rate of its conversion to other amino acids.

High threonine soybean seed expressing XbAK-T359I or E257K exhibit normal seed morphology in appearance and germination and seedling growth under greenhouse conditions⁷. These results suggest that high Thr seed expressing Lys-insensitive XbAK has no deleterious pleiotropic effect on soybean agronomic performance.

Conclusions and future prospects

Here we report a successful strategy to produce high free threonine soybean seed with normal seed morphology and germination via expression of feedback-resistant XbAK variants in developing soybean seed. The strategy targets the allosterically regulated AK enzyme of the aspartate pathway that controls the metabolic flux into threonine, lysine, methionine, and isoleucine. High Thr soybean seed expressing Lys-insensitive XbAK variants also show a substantially increased accumulation of other essential amino acids including Lys, Met, and Ile. Thr availability for high Thr soybean-based meal could be

calculated after the total Thr content (free and protein-bound Thr) is determined in the transgenic seeds.

In addition to passing safety assessments, to be commercially useful crop varieties with improved seed quality traits must also possess good agronomic characteristics under field conditions. Therefore, for high threonine soybean seed, other agronomic characteristics such as pest susceptibility, environment adaptation, seed nutrient compositions, and heritability of the trait must be rigorously evaluated in field trials before the nutritionally improved soybean seed expressing XbAK variant can be commercialized.

There has been extensive research in developing commercial biotech crop varieties with enhanced nutritional quality of seed^{3,6,8,9}. There seems little doubt that we can envision the use of genetically engineered crops to help meet the ever-increasing nutritional needs of both livestock and humans. The success with high-Lys corn³ and high-Thr soybean⁷ provides an optimistic basis for development of a wide variety of efficient biotech crops with targeted EAA profiles. These highly nutritional crops will undoubtedly lead to further developments in the agricultural food and feed industries.

Table 1. Biochemical properties of the recombinant wild-type and Lys-insensitive XbAK enzymes purified from expressed E. coli BL21(DE3) cells. The data shown represent the mean of four replicates.

	K _m Asp mM	K _m ATP mM	IC ₅₀ Lys mM	V _{max} ¹ Asp μmoles •min ⁻¹ •mg ⁻¹	V _{max} ² ATP μmoles •min ⁻¹ •mg ⁻¹
X.bovienii AK	2.73 ab*	2.56 a	1.4 a	0.29 a	0.36 a
X.bovienii AK-T359I	2.31 a	1.82 b	145.4 b	0.26 ab	0.32 a
X.bovienii AK-E257K	3.06 b	1.89 b	121.5 c	0.20 b	0.22 b

* Significances were calculated using Tukey-Kramer HSD (JMP 6.0, SAS Institute) and means followed by different letters are significantly different from each other (α = 0.05).

Table 2. Up to 100-fold increase in free threonine levels in deregulated XbAK R1 soybean seed populations over wild-type seed. Abbreviations: 7Sa¹, Glycine max seed storage protein promoter; USP99, USP promoter derived from *Vicia faba* seed storage protein USP gene; CTP1, a modified chloroplast target peptide from the small subunit of the Arabidopsis ribulose biphosphate carboxylase.

Free threonine level, ppm*				
	No. of events	Highest Thr event**	Mean	Significance ^c
7Sa ¹ ::CTP1::XbAK	20	68	38 ± 1.6	a
7Sa ¹ ::CTP1::XbAK-E257K	14	2887	1139 ± 184	b
7Sa ¹ ::CTP1::XbAK-T359I	15	2999	1694 ± 177	c
USP99::CTP1::XbAK-T359I	14	3112	2142 ± 207	d
Non-transgenic (wild-type)	22 plants	31	23 ± 1.2	a

^a, parts per million, dry weight; ^b, Event with maximum seed Thr content analyzed for 25 R₁ segregating soybean seeds per event; ^c, Significance were calculated using Tukey-Kramer HSD (JMP 6.0, SAS Institute) and means followed by the same letters are not significantly different from each other (α = 0.05).

Table 3. Soybean seed over-expressing insensitive XbAK variants show substantially increased levels of free Lys, Met, Ile, Gly, and Ser. The data shown represent the mean of all events (see Table 2) from a given construct or the mean of 22 plants for non-transgenic seeds.

Amino acid, ppm ^a	Non-transgenic	7Sa':::CTP1::XbAK	7Sa':::CTP1::XbAK-E257K	USP99::CTP1::XbAK-T359I
Lys	99 ± 13 a	75 ± 16 a	168 ± 19 b	261 ± 21 c
Met	24 ± 7 a	32 ± 9 a	99 ± 10 b	146 ± 11 b
Ile	40 ± 5 a	81 ± 7 b	124 ± 8 c	117 ± 9 c
Gly	43 ± 14 a	39 ± 18 a	204 ± 21 b	269 ± 24 c
Ser	68 ± 17 a	48 ± 21 a	1407 ± 183 b	2116 ± 206 b

^a, parts per million, dry weight; ^b, means ± standard error, different letters on rows indicate statistically significant difference from each other (Tukey-Kramer HSD, $\alpha = 0.05$).

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