ISB NEWS REPORT
AGRICULTURAL AND ENVIRONMENTAL BIOTECHNOLOGY

August 2017

FINAL ISSUE

CONTENTS

Farewell 1

Bt Eggplant: A Genetically Engineered 'Minor' Crop Comes of Age in Bangladesh and the Philippines 2

Using Biotechnology to Eliminate Mycotoxins 8

Addition of a New Nuclease Cpf1 in the Tool Box of CRISPR System 12

Agriculture in a Changing Climate 16

VirginiaTech
Invent the Future
Virginia Polytechnic Institute and State University

Farewell

Dear Readers,

Information Systems for Biotechnology will cease operations on September 14, 2017, as our 30-year association with USDA will be terminated. This is the final issue of the ISB News Report.

A bit of history seems in order. The Information Systems for Biotechnology (ISB) program was established at Virginia Tech in 1988 as part of the National Biological Impact Assessment Program (NBIAP), which was administered by USDA's Cooperative State Research Service for 20 years, and USDA APHIS for 10 years. ISB hosts the database for online searches of the USDA's Environment Releases database that catalogs all field tests and petitions for deregulation of genetically engineered organisms under USDA authority. Approximately 1,353,500 searches of the ISB database website are conducted annually, of which over 82,000 are unique visitors. Additionally, ISB has hosted many conferences, workshops and focus groups in our efforts to gather and distribute information about agricultural biotechnology research and product development, biotechnology and biosafety regulations, and environmental issues associated with small and large-scale releases of genetically modified organisms in agriculture.

Going forward, the USDA APHIS will take over hosting the environmental release database on their website.

You may continue to access and download our publications – the two workshop proceedings and the "Greenhouse Guide" – at our website until September 14th. After that, the publications will be available for download from the Virginia Tech Library website VTechWorks. We will be adding back issues of the News Report to VTechWorks as time allows.

VTechWorks: https://vtechworks.lib.vt.edu/handle/10919/78421

On behalf of the previous and current ISB staff, I want to express our gratitude for your interest in the ISB program, for reading the News Report, for participating in our workshops and conferences, and for all the countless authors who have contributed articles to the News Report over the years.

Kind regards,

Ruth Irwin
Editor and Principal Investigator

PUBLISHED BY

Information Systems for Biotechnology

Virginia Tech
1900 Kraft Drive, Suite 103
Blacksburg, VA 24061

Tel. 540-231-3747
Fax 540-231-4434

Subscribe to and access archived issues of the ISB News Report online:

www.isb.vt.edu

Editor:
Ruth Irwin
rirwin@vt.edu

ISB welcomes your comments and encourages article submissions. If you have a suitable article relevant to our coverage of agricultural and environmental applications of genetic engineering, please email it to the Editor for consideration.

Bt Eggplant: A Genetically Engineered 'Minor' Crop Comes of Age in Bangladesh and the Philippines

A. M. Shelton, K. E. Hokanson, D. M. Hautea, M. J. Hossain, M.A. Hossain, V. Paranjape, R.A. Hautea, L. McCandless, and S. H. Sarwer

It has been more than 20 years since the first genetically engineered (GE) crops were commercialized. GE crops are grown in 26 countries, and GE corn, cotton, and soybean now dominate their respective crops in the global commodity market[1]. Insect-resistant Bt crops have revolutionized integrated pest management (IPM) by providing an exceptional degree of host plant resistance, the foundation of IPM, through traits that make the crop effectively immune to the target pest[2,3]. Cumulatively on a global basis from 1996 to 2014, Bt corn and Bt cotton have provided $41.4 billion and $44.8 billion in economic benefit and have reduced the use of insecticides by 51.6 and 27.9%, respectively[4]. A reduced need for pesticides is very important to resource-poor farmers who often lack the training and protective equipment to use them properly. Bt crops have also been shown to conserve natural enemies and other valuable arthropods that contribute to ecosystem services[5,6].

While the advent of GE crops was a transformative success story in agriculture—indeed, the 2013 World Food Prize was awarded to pioneers in the field—the use of Bt crops has largely been limited to large acreage commodity crops. Biotechnology for use in the so-called 'minor' crops, sometimes referred to as 'orphan' or 'neglected' and 'underutilized' crops, has not been as forthcoming. Whatever term is used, minor crops are important for local and regional food security and historically lag behind large acreage crops in development of crop protection products. This is unfortunate since this group of crops includes fruits and vegetables that are critically needed for a balanced, nutritious diet and diversified farm income. Most people are surprised to learn that more insecticides are used on fruits and vegetables than on the large acreage crops of corn, cotton, and rice combined[7]. The pesticide application rate is driven by the higher value of fruits and vegetables and their higher cosmetic standards, as well as the diverse insect complexes that cause various maladies. Furthermore, biological control and other tactics rarely are sufficient to control insect pests of fruit and vegetables in open field conditions.

These factors should make fruits and vegetables suitable candidates for GE technologies that control insect pests or the pathogens they vector; however, there is only a small number of GE fruits and vegetables that have been commercialized. The poster child for the success of a GE minor crop is the GM papaya developed by Gonsalves and colleagues at USDA that controls the insect-transmitted virus causing papaya ringspot disease[8]. Although GM papaya is still opposed by some activist groups, all acknowledge that without the GE trait, economically profitable cultivation of papaya in Hawaii would not be possible[9]. A few more GE fruits and vegetables have proved to be

useful for pest management, e.g., GE virus-resistant squash and insect-resistant Bt sweet corn in North America[10,11], and virus resistant beans in Brazil[12]. But now there is a new crop that is playing a pivotal role in the future success of GE technology for 'minor' crops in developing countries—Bt eggplant.

Eggplant's Big Insect Problem

Solanum melongena L. (eggplant, also known as brinjal in India and Bangladesh, and talong in the Philippines) is one of the most important, inexpensive, and popular vegetable crops grown and consumed in Asia. Eggplant is low in calories and fat, rich in vitamins and minerals, and a good source of dietary fiber. It has abundant total water-soluble sugars, free reducing sugars, anthocyanin, phenols, and amide proteins, which provide medicinal benefits (http://www.medicalnewstoday.com/articles/279359.php). Eggplant production provides an important source of cash income, particularly for small, resource-poor farmers.

The biggest constraint to eggplant production throughout Asia is chronic and widespread infestation by the eggplant fruit and shoot borer (EFSB), *Leucinodes orbonalis* Guenée. The caterpillars damage eggplant by boring into the petiole and midrib of leaves and tender shoots, resulting in wilting and desiccation of stems (**Fig. 1**). Larvae also feed on flowers, which results in flower drop or misshapen fruits. The most serious economic damage caused by EFSB is to the fruit, because the holes, feeding tunnels, and frass (larval excrement) make the fruit unmarketable and unfit for human consumption (**Fig. 2**).

Figure 1. EFSB larva in an eggplant shoot, causing it to die and no fruit to be produced.

Figure 2. EFSB larva in the fruit, rendering it unfit for market.

To control this insect, farmers routinely spray broad-spectrum insecticides, often 2–3 times per week, and, in some cases, twice a day. Consequently, over 100 sprays per season may be applied, resulting in high residues on the marketable fruit. Such an insecticide-dependent strategy poses both environmental and health concerns. Environmental concerns include killing natural enemies that can help reduce pest populations, leaching of active pesticide ingredients into the soil and water, and harming pollinators. Health concerns include harm to the applicator and farm workers, as well as harm to the consumer from high pesticide residues on the crop. These problems have been well documented in Bangladesh and the Philippines (http://bteggplant.cornell.edu/content/facts; http://bic.searca.org).

Building a Better Eggplant

The development of Bt eggplant was initiated in 2000 by the India-based Maharashtra Hybrid Seed Company (Mahyco) under a partnership with the Monsanto Company. Mahyco used a *Bacillus thuringiensis cry1Ac* gene that had already been widely used in Bt cotton in India. The GE Bt eggplant (termed '*event*' EE-1) demonstrated control of EFSB in contained greenhouse trials. In late 2003, a partnership was formed between Mahyco, Cornell University, the United States Agency

for International Development (USAID), and public sector partners in India, Bangladesh, and the Philippines under the Agricultural Biotechnology Support Project II (ABSPII; http://absp2.cornell.edu). Bt eggplant was selected for the countries based on a priority setting process by representatives in each country. Each partner in ABSPII shared in the responsibility to get Bt eggplant into their respective markets.

This project had the unique vision to use two market channels to satisfy a diverse farming community[13,14]—a 'pro-poor' channel for the distribution of open pollinated (OP) lines, and a commercial channel through which the higher priced hybrid varieties would be sold. The belief at the time was that low resource farmers would not adopt hybrid eggplant. However, hybrid eggplant has proved to be immensely popular over time with resource-poor farmers in India, so future GE eggplant products may be incorporated straight into hybrid backgrounds in future.

ABSPII also operated in Bangladesh and the Philippines; however, different market channels created different requirements (see below). All three countries used the resistant EE-1 event created by Mahyco. When ABSPII ended in 2014, USAID re-competed the award, with emphasis shifting to the regulatory issues, scaling-up methods, and means of deployment/stewardship for farmers in Bangladesh and the Philippines. The Feed the Future South Asia Eggplant Improvement Partnership (http://bteggplant.cornell.edu) housed at Cornell University is the implementing partner working in partnership with the Bangladesh Agricultural Research Institute (BARI), the University of the Philippines Los Baños, the University of Minnesota, and Sathguru Management Consultants. The project has unfolded in different ways in the three countries.

The Indian Stalemate

After extensive field trials and safety evaluations conducted by the Indian biosafety body the Genetic Engineering Appraisal Committee (GEAC) recommended commercialization of Bt brinjal. However, responding to challenges from activist groups, the Indian Minister of Environment and Forests imposed a moratorium on release on February 9, 2010, until a political consensus was reached. That moratorium is still in place today[15].

Bangladesh Steps Forward

As in neighboring India, eggplant (brinjal) is an important vegetable crop in Bangladesh, where it is second only to potato in production and is grown on nearly 50,000 hectares. Similarly, EFSB is the main pest in Bangladesh, and the crop is intensively sprayed with insecticides that have limited efficacy against boring insects. The insecticides have negative impacts on humans and the environment, and are applied by often poorly trained farmers (http://bteggplant.cornell.edu/content/facts/pesticide-use-bangladesh). In addition, more than 40 years of conventional breeding has failed to produce highly resistant eggplant cultivars[16]. Consequently, the crop is a good candidate for genetically engineered host plant resistance. An ex-ante study indicated that the introduction of Bt eggplant into Bangladesh would result in a net benefit of $1868/ha[17]. This benefit compares to the household income per capita of $277.95 in December 2005 (https://www.ceicdata.com/indicator/bangladesh/annual-household-income-per-capita).

Unlike India, which commercialized Bt cotton in 2002, Bangladesh had not released any GE crops. Mahyco donated the EE-1 event to the Bangladesh Agricultural Research Institute (BARI) where it was incorporated into nine local eggplant lines. Breeding and efficacy trials were conducted from 2005 to 2012; subsequently, BARI applied to the National Technical Committee on Crop Biotechnology (NTCCB) to release Bt eggplant. Following the recommendation from the NTCCB, the application for release was forwarded to the National Technical Committee on Crop Biotechnology (NTCCB) Core Committee followed by the National Committee on BioSafety (NCB). The Bangladesh government granted approval for release of four varieties on 30 October 2013. On 22 January 2014, Bt seedlings were distributed among 20 farmers in four districts. The following year, demonstration trials were conducted in 108 farmer fields in 19 districts. In 2015 and 2016, demonstration trials were conducted in 230

farmer fields in 23 districts[18] and 512 farmers field in 40 districts[19], respectively. According to BARI, the performance of Bt eggplant in these demonstration trials was far superior to non-Bt eggplant, with fruit infestations in Bt eggplant ranging from 0.04 − 0.88% compared to 48 − 57% in the non-Bt eggplant[18].

The field demonstrations conducted from 2013 to 2017 clearly showed the benefit of Bt eggplant for control of EFSB, and growers were highly satisfied with their experiences (interviews with growers can be seen on the project's website, Bteggplant.cornell.edu, and readers are urged to view the 22 June 2017 video "Bt brinjal in Bangladesh: Voices from the Field."). The results from these trials are being prepared for publication. Meanwhile, additional trials were conducted in 2017, including a large-scale study by the Department of Agricultural Extension that included more than 5,000 farmers.

Good stewardship practices for Bt eggplant have been developed. As with any insect control crop, Bt eggplant must be incorporated within an Integrated Pest Management (IPM) program. The Bt protein controls the main pest, EFSB, but does not affect other eggplant pests such as leafhoppers, whiteflies, aphids, and thrips, all of which can damage eggplant. Studies are being conducted to develop thresholds for these other pests. Baseline susceptibility of EFSB to Cry1Ac is being determined as part of an Insect Resistance Management (IRM) program to ensure the long-term benefit of this technology. Farmer demonstration trials have incorporated refuges as an important component of IRM. Most importantly, farmer-training programs are being conducted and refined, since farmers are ultimately the ones who will need to protect this valuable technology.

The Philippines: Bt Eggplant in a Changing Regulatory Environment

Farmers and government regulators in the Philippines have considerably more experience with GE crops because Bt maize has been commercialized and widely cultivated (65% of the 2016 national corn acreage) since 2003[1]. As in India and Bangladesh, eggplant is an important vegetable crop in the Philippines, where it ranks as the number one vegetable crop, and it is also severely damaged by EFSB. Ex-ante studies have indicated considerable economic benefits and pesticide savings if Bt eggplant is introduced into the Philippines, resulting in increased income to farmers ranging from US$2,339 to $5,302/ ha[20,21]. Mahyco sub-licensed EE-1 to the University of the Philippines Los Baños, where the EE-1 technology was incorporated into local OP lines and hybrids. Field studies conducted from 2010 to 2012 (**Fig. 3**) demonstrated the stable expression of Cry1Ac protein and outstanding control of EFSB[22], and a lack of negative effects on non-target arthropods[23].

Figure 3. Second season confined field trial of Bt OP (open-pollinated) lines and their non-Bt comparators in UPLB Experimental Site, Bay, Laguna, Philippines. Photo shows field layout of the replicated experiment. Photo credits: ABSPII UPLB Bt eggplant team; December 14, 2010.

However, as in India, commercialization has encountered some roadblocks. Anti-biotech groups tried to stop the Bt eggplant field trials through extensive negative media campaigns and "direct action" (e.g., picketing and vandalizing field trials). A legal challenge was also launched when anti-GM activist groups filed a petition in May 2012 to the Supreme Court calling for the imposition of the *Writ of Kalikasan* and issuance of a Temporary Environmental Protection Order to stop the Bt eggplant field trials[24]. After considerable discussion, the Supreme Court decided on 8 December 2015 to permanently stop the field trials of Bt eggplant. It also declared null and void the existing biosafety regulations and temporarily stopped all biosafety

approvals for all GMOs pending promulgation of new biosafety approval guidelines. However, on 26 July 2016, the Supreme Court granted all motions for reconsideration filed by Bt eggplant proponents and other interested parties, and unanimously reversed its December 2015 decision. At the same time, a new inter-agency set of regulatory guidelines was put into place. The Bt eggplant project will prepare a regulatory package to submit to the authorities according to the new set of regulatory guidelines.

Looking Ahead

The success of large acreage GE crops has facilitated the adoption of future GE minor crops. Bangladesh has decided to allow the cultivation of Bt eggplant and in 2017, as many as 6,500 farmers are growing Bt eggplant and reaping its benefits (**Fig. 4**). For farmers, these benefits include higher income, less insecticide exposure, and increased biodiversity in their fields. For consumers and the general public, benefits include improved food safety, a more consistent supply of a highly nutritious vegetable, and less insecticide in the environment.

Figure 4. Md. Shahajahan Ali grows Bt brinjal in 2017 with high yield from his 0.16 acre field.

In Bangladesh, the Minister of Agriculture, the Honorable Agriculture Minister Begum Matia Chowdhury, MP, has been a strong supporter of biotechnology and this has made the difference for farmers across the country (**Fig. 5**). In a workshop held in March 2017 in Bangladesh, she made her position clear:

> "Development of brinjal fruit and shoot insect resistant Bt brinjal is a success story of local and foreign collaboration. We will be guided by the science-based information, not by the nonscientific whispering of a section of people. Good science will move on its own course keeping the anti-science people down. As human beings, it is our moral obligation that all people in our country should get food and not go to bed on an empty stomach. Biotechnology can play an important role in this effect." (http://bteggplant.cornell.edu/content/news/blog/workshop-bt-eggplant-brings-researchers-and-journalists-together).

Stakeholders are closely watching the success of low resource Bangladesh farmers to see how they use Bt eggplant to combat the eggplant fruit and shoot borer and reap its environmental and economic benefits.

Figure 5. Honorable Agriculture Minister of Bangladesh, Begum Matia Chowdhury, MP, providing Bt brinjal seedlings in presence of dignitaries from USAID and BARI in 2014.

References

1. ISAAA. 2016. Global status of commercialized Biotech/GM Crops: 2016. ISAAA Briefs No. 52. ISAAA, Ithaca.
2. Shelton, A. M., J. Z. Zhao, and R. T. Roush. 2002. Economic, ecological, food safety, and social consequences of the deployment of Bt transgenic plants. Annual Review of Entomology 47: 845-881.
3. Kennedy, G. G. 2008. Integration of insect-resistant genetically modified crops within IPM programs, pp. 1-26. In A. M. Shelton and G. G. Kennedy [eds.], Integration of insect-resistant, genetically modified crops within IPM programs. Springer, Dordrecht, The Netherlands.
4. Brookes, G., and P. Barfoot. 2016. GM crops: Global socio-economic and environmental impacts 1996-2014. PG Economics Ltd, Dorchester, UK.
5. Naranjo, S. E. 2014. Effects of GM crops on non-target organisms, pp. 129-142. In A. Ricroch, S. Chopra and S. Fleischer [eds.], Plant biotechnology: experience and future prospects. Springer, Dordrecht, London.
6. Tian, J.-C., J. Yao, L.-P. Long, J. Romeis, and A. M. Shelton. 2015. Bt crops benefit natural enemies to control non-target pests. Scientific Reports 5: 16636.
7. Shelton, A. M. 2012. Genetically engineered vegetables expressing proteins from Bacillus thruringiensis for insect resistance: successes, disappointments, challenges and ways to move forward. GM Crops & Food 3: 175-183.
8. Gonsalves, D., S. Ferreira, R. Manshardt, M. Fitch, and J. Slightom. 1998. Transgenic virus resistant papaya: New hope for control of papaya ringspot virus in Hawaii. APSnet Feature, American Pythopathological Society. http://www.apsnet.org/publications/apsnetfeatures/Pages/papayaringspot.aspx
9. Shelton. A. M. 2015. Tragic papaya: 12-part series. http://anthonymshelton.com/tragicpapaya/
10. Shelton, A. M., M. Fuchs, and F. Shotkowski. 2008. Transgenic vegetables and fruits for control of insect and insect-vectored pathogens. In Integration of insect-resistant, genetically modified crops within IPM programs. Eds. J. Romeis, A. M. Shelton and G. G. Kennedy, pp 249-272. Springer. Dordrecht, The Netherlands
11. Shelton, A. M., D. L. Olmstead, E. C. Burkness, W. D. Hutchison, G. Dively, C. Welty, and A. N. Sparks. 2013. Multi-state trials of Bt sweet corn varieties for control of the corn earworm (Lepidoptera: Noctuidae). J. Econ. Entomol. 106 (5): 2151-2159.
12. Bonfim, K., J. C. Faria, E. Nigueura, E. Mendez and F. Aragão. 2007. RNAi-Mediated Resistance to Bean golden mosaic virus in Genetically Engineered Common Bean (Phaseolus vulgaris). MPMI Vol. 20, No. 6, 2007, pp. 717–726
13. Gregory, P., R. H. Potter, F. A. Shotkoski, D. Hautea, K. V. Raman, V. Vijajayaraghavan, W. H. Lesser, G. Norton, and W.R. Coffman. 2008. Bioengineered crops as tools for international development: opportunities and strategic considerations. Ex. Agric. 44:277-299.
14. Hautea, D.M. V. M. Cruz, R. A. Hautea and V. Vijayaraghavan. 2011. Transgenic horticultural crops in Asia. pp 155-173. In B. Mou and R. Scorza (eds.) Transgenic horticultural crops: challenges and opportunities. Taylor & Francis, LLC, Boca Raton, FL. Book Chapter 7. https://doi.org/10.1201/b10952-8
15. Shelton, A. M. 2010. The long road to commercialization of Bt brinjal (eggplant) in India. Crop Protection 29: 412-414.
16. Alam, S.N., M.A. Rashid, F.M.A. Rouf, R.C. Jhala, J.R. Patel, S. Satpathy, T.M. Shivalingaswamy, S.Rai, I. Wahundeniya, A. Cork, C. Ammaranan, and N.S. Talekar. 2003. Development of an integrated pest management strategy for eggplant fruit and shoot borer in South Asia. Shanhua, Taiwan: AVRDC—the World Vegetable Center. Technical Bulletin No. 28. AVRDC Publication No. 03-548. 56p.
17. Islam, S. M. and G.W. Norton. 2007. Bt eggplant for fruit and shoot borer resistance in Bangladesh, pp. 91-106. In C. Ramasamy, K. N. Selvaraj, G. W. Norton and K. Vijayaraghavan [eds], Economic and environmental benefits and costs of transgenic crop: ex-ante assessment.
18. Mondal, R.I., A. K. M Quamruzzaman, K. Hasan, and D. Khanam. 2016. The journey of Bt eggplant in Bangladesh. Proceedings of the 4th Annual South Asia Biosafety Conference, Sept. 19-21, 2016. Hyderabad, India.
19. Annual Research Report of On-Farm Research Division, 2017. Performance of Bt brinjal at farmers field. Bangladesh Agricultural Research Institute, Gazipur, Bangladesh.
20. Norton, G.W. and D.M. Hautea (editors). 2009. Projected Impacts of Agricultural Biotechnologies for Fruits and Vegetables in the Philippines and Indonesia. International Service for the Acquisition of Agri-biotech Applications and SEAMEO Southeast Asian Regional Center for Graduate Study and Research in Agriculture. ISBN 978-971-93983-2-5. 184 pp.
21. Gerpacio, R.V. and A.P. Aquino (eds.) 2014. Socio-economic impacts of Bt eggplant ex-ante case studies in the Philippines. International Service for the Acquisition of Agri-biotech Applications (ISAAA), Ithaca, New York, USA and SEAMEO Southeast Asian Regional Center for Graduate Study and Research in Agriculture (SEARCA), Los Baños, Laguna, Philippines. Available for download: http://isaaa.org
22. Hautea, D., L.D. Taylo, A. P. Masanga, M. L. J. Sison, J. O. Narciso, R. B. Quilloy, R. A. Hautea, F. A Shotkoski, and A. M. Shelton. 2016. Field performance of Bt eggplants in the Philippines: Cry1Ac expression and control of the eggplant fruit and shoot borer. PLoS ONE 11(6): e0157498
23. Navasero, M.V., R.N. Candano, D. Hautea, R. A. Hautea, F. A Shotkoski, and A. M. Shelton. 2016. Assessing potential impact of Bt eggplants on non-target arthropods in the Philippines. PLoS ONE 11(10): e0165190
24. James, C. 2015. Global status of commercialized Biotech/GM Crops: 2016. ISAAA Briefs No. 51. ISAAA, Ithaca.

A. M. Shelton, K. E. Hokanson, D. M. Hautea, M. J. Hossain
M.A. Hossain, V. Paranjape, R.A. Hautea, L. McCandless, and S. H. Sarwer

Author Associations: All authors are associated with the project, Feed the Future South Asia Eggplant Improvement Partnership (Bteggplant.cornell.edu). In addition, the authors listed below have the following associations:

A. M. Shelton: Department of Entomology, Cornell/NYSAES, Geneva, New York, 14456, USA

K. E. Hokanson: Department of Horticultural Sciences, University of Minnesota, Saint Paul, MN 55108, USA

D. M. Hautea: Institute of Plant Breeding/CSC, College of Agriculture, University of the Philippines Los Baños, College, Laguna, 4031, Philippines

V. Paranjape: Sathguru Management Consultants Pvt Ltd, Plot 54, Sagar Society, Road No.2, Banjara Hills, Hyderabad, India 500034

R.A. Hautea: International Service for the Acquisition of Agri-biotech Applications c/o 3/F Khush Hall, IRRI, Los Baños, Laguna 4030, Philippines

L. McCandless: International Programs, Cornell University, Ithaca, New York, 14850, USA

Author Contributions

All authors participated in the drafting of this paper as individual subject matter experts in their fields, and the authors are solely responsible for the content. Any views expressed in this paper are the views of the authors and do not necessarily represent the views of any organization, institution, or government with which they are affiliated or employed. The authors declare no competing financial interest.

Using Biotechnology to Eliminate Mycotoxins

Monica Schmidt

Mycotoxins are compounds produced by certain fungi that are deleterious to the health of animals. They are inadvertently ingested by both livestock and humans when crops are infected with a toxin-producing fungus. Food and Agriculture Organization estimates one quarter of the world's crops are contaminated with a mycotoxin[1]. Perhaps the most problematic and widespread is the mycotoxin produced from certain species of the fungus *Aspergillus* (*A. flavus* and *A. parasiticus*) called aflatoxin.

Health concerns of aflatoxin contamination

Four aflatoxin compounds produced by some *Aspergillus* fungi are a major health concern and are responsible for massive agricultural losses world-wide. It is estimated that approximately 4.5 billion people are chronically exposed to aflatoxin through the ingestion of contaminated food items[2]. Aflatoxins are known carcinogenic compounds and have been extensively linked to liver cancer[3]. Liver cancer is the sixth leading cause of cancer worldwide with a prevalence 16-32 times higher in developing countries[3]. Additionally aflatoxin has been associated with growth impairment in children[4] and immunosuppression (for review[5]), the latter likely leading to increased incidence of secondary infections such as HIV and malaria. Populations at high risk for aflatoxin contamination are communities consisting of rural subsistence farmers in developing nations, not only because hot and humid climate conditions are ideal for fungal growth, but also because of both low risk awareness and insignificant enforcement of regulatory consumption limits. As a consequence, blood samples collected from regions of West Africa and Guangxi Province of China were over 90% positive for the aflatoxin biomarker[6].

Economic losses due to aflatoxin contamination

Aflatoxin ingestion is not considered a major health issue in developed countries as there are strict consumption limits enforced. Over 100 countries regulate the level of aflatoxin in food and animal feed for consumption. For example, the US Department of Agriculture regulates the allowable level of aflatoxin in corn destined for human food and dairy cattle feed at their most rigorous limit of 20 parts per billion (ppb). To put this number into perspective, 1 ppb is equivalent to a single drop of water in a 21,700 gallon (82,135 liter) swimming pool or from a time perspective, 1 second in 31.7 years. If a crop is measured above all allowable limits, then it is not permitted to move forward in the production stream and likely is incinerated. In the US alone, aflatoxin contamination of food/feed results in an estimated $270M agricultural loss every year[7]. Conservative estimates are that fungal toxins cost the US between

$500 million to $1.5 billion a year in lost crop revenues and expenses in monitoring[8].

Many crops such as peanuts, grains, and nuts are susceptible to *Aspergillus*-infection with subsequent aflatoxin contamination. Current aflatoxin prevention mechanisms involve breeding for fungal resistant crops[9], agronomic practices to lower the ability of the fungus to grow, biocontrol with atoxigenic fungal strains[10], improved post-harvest storage methods[11], and use of trapping agents to block uptake of aflatoxins[12]. These measures are inadequate, as millions of tons of crops are lost due to this toxin each year. Corn is the crop that suffers the most losses due to fungal toxin contamination, with an annual estimated loss of 16 million tons worldwide[13].

Biotechnology used to alleviate aflatoxin

We have genetically engineered corn to give it the ability to turn off the toxin-producing biosynthesis pathway in *Aspergillus* when the fungus infects the corn kernel[14]. This research was based on a few genetic charachertistics. First is the fact that sequence information within eukaryotic cells flows from double stranded DNA to single stranded RNA to encode for protein. Second is the knowledge that eukaryotic cells, including fungal cells, have an inherit mechanism whereby double stranded RNA molecules are deemed foreign and subsequently degraded by cells[15]. The third principle is that a somewhat new discovery that small RNA molecules readily pass between a host and its pathogen upon infection[16].

We have genetically engineered corn to give it the ability to turn off the toxin-producing biosynthesis pathway in Aspergillus when the fungus infects the corn kernel.

Using this collective information, we inserted a DNA cassette that is expressed only in the edible kernels of the corn plant. In our research, the inserted gene cassette directs the corn to produce a hairpin RNA molecule that would be degraded by the cell's own machinery into small RNA molecules. The small RNA molecules were directed to the corn kernels where they would express a sequence similar to that of a fungus *Aspergillus* gene that encodes for a key enzyme needed for aflatoxin biosynthesis. The corn kernels produced the small RNA molecule throughout corn development, and only when infected with *Aspergillus* does the small RNA molecule gain entrance into the infecting fungal cell. In the fungal cell, it will find its matching RNA sequence to the fungal toxin-encoding full RNA transcript. When the expressed small RNA is in the fungal cell, it finds and pairs with the endogenous fungal RNA sequence. The double RNA structure is recognized by the fungal cell as foreign and the fungal cell's own cellular machinery degrades it. With the fungal RNA degraded, it is not available to encode for the enzyme necessary in aflatoxin biosynthesis.

In essence, the small RNA molecules expressed in the corn enter the fungal cell and stop the fungal RNA from synthesizing a necessary toxin-producing enzyme. Corn engineered to express the RNA that was designed to target the toxin-producing enzyme was infected with toxin-producing *Aspergillus* and incubated for one month under controlled greenhouse conditions. There was no toxin detected in engineered kernels (below levels of detection in our methods) compared to control non-engineered kernels exhibiting at least 1,000s ppb toxin. Further molecular analysis showed that the targeted fungal RNA molecule was significantly suppressed in the engineered lines compared to control non-engineered kernels.

To summarize, the engineered corn kernel is directed to produce a small RNA molecule after infection by *Aspergillus*. The small RNA molecule enters the contaminating fungal cell, finds its matching RNA sequence and, in so doing, stops that transcript from encoding for a pivotal enzyme in the aflatoxin biosynthetic pathway. Research involving the use of a host organism, in this case corn plants, to suppress or silence gene expression of an infecting pathogen, such as *Aspergillus*, is known as Host Induced Gene Silencing (HIGS). HIGS has been used successfully to suppress pathogen growth in crops previously (for example[17]), but this is the first report of its successful use of the suppression of a pathogen-produced toxin.

The success of HIGS technology relies heavily on the specificity of the RNA transcript targeted for degradation. The RNA transcript that encodes for the toxin-producing enzyme that we targeted for degradation in our research was the *Aspergillus* 7 kb

polyketide synthase gene. Initially we performed a preliminary sequence search of this fungal transcript in other databases, such as in corn, humans, and pigs, as it was essential to find unique regions within the fungal transcript that we could use as the introduced expressed small RNA molecules. We chose three 200 bp regions from the single fungal transcript to introduce into corn for the production of the small RNA molecules. Simultaneously targeting three areas of the one fungal RNA transcript served two purposes: (1) it would enhance the likelihood that the fungal polyketide synthase RNA transcript would be targeted for complete degradation, and not merely be truncated; and (2) it would severely decrease the probability that the *Aspergillus* fungus could evolve resistance to this genetically engineered corn, as the fungus would have to simultaneously mutate three separate sections of the polyketide synthase gene while still encoding for a functional toxin-producing enzyme.

This aflatoxin-silencing genetically engineered corn provides proof-of-concept that a genetic suppression strategy is an effective means to prevent aflatoxin contamination in crops.

Substantial equivalence at transcript level

Because this research involves the production of an engineered corn plant, we performed preliminary transcript analysis of the corn kernels to assay for substantial equivalence. The trait inserted in the engineered corn plants was targeted only to kernels during their development. No gross morphological differences were observed in the kernels or whole plant of the engineered plants compared to the non-engineered control plants grown side-by-side under greenhouse conditions. Because the inserted trait was a small RNA molecule expressed in the engineered corn kernels, we performed RNA transcript analysis of RNA isolated from the kernels of three engineered events and two non-engineered controls. We were particularly interested to know if the inserted cassette expressed in corn kernels was causing any 'off target' affects within the overall gene expression in corn kernels. That is, did the sequence of the introduced expressed small RNA molecule targeted to the fungal toxin gene have enough sequence similarly to match RNA transcripts within the corn kernels? If so, the matched RNA would again first form double RNA structures and then cause the degradation of the matching corn transcript and, in turn, perhaps cause many undesired altered characteristics.

We compared transcripts from three engineered kernels to the transcripts from two control kernels in six pairwise comparisons. We did not detect a single transcript that was consistently and significantly expressed differentially in the engineered samples compared to the controls*. This transcript analysis indicates that the introduced fungal-targeted small RNA molecule is specific to the contaminating fungus and does not appear to alter gene expression in the corn kernels.

HIGS as a promising biotechnology tool to combat all mycotoxins

This research shows that fungal-produced toxins can be suppressed or silenced using HIGS biotechnology in corn kernels. This aflatoxin-silencing genetically engineered corn provides proof-of-concept that a genetic suppression strategy is an effective means to prevent aflatoxin contamination in crops. This technology could be incorporated into other aflatoxin-susceptible crops and in the future could be extrapolated to target other mycotoxins in crops grown globally. Mycotoxin contamination in crops threatens agricultural development, trade, food security, and human health. Our research shows biotechnology is a viable option to alleviate fungal toxins from contaminated crops.

Footnote: *This analysis was performed on full RNA transcripts that will encode for proteins to carryout numerous cellular functions and structural roles. Small RNA molecules, including the one introduced in our research, are not detected in this assay.

References

1. http://www.fao.org/food/food-safety-quality/a-z-index/mycotoxins/en/ (Aug 2012)
2. Egal S, Housa A, Gong YY, Turner PC, Wild CP, Hall AJ, Hell K and Cardwell KF (2005) Dietary exposure to aflatoxin from maize and groundnut in young children from Benin and Togo, West Africa. *Int J Food Micobiol* 104: 215-224.
3. Liu Y and Wu F (2010) Global Burden of aflatoxin-induced hepatocellular carcinoma: a risk assessment. *Environ Health Perspect* 118(6): 818-824.
4. Khlangwiset P, Shephard GS and Wu F (2011) Aflatoxins and growth impairment: A review. *Crit Rev Toxicol* 41(9): 740–755.
5. Mohsenzadeh MS, Hedayati N, Riahi-Zanjani B and Karimi G (2016) Immunosuppression following dietary aflatoxin B1 exposure: a review of the evidence. *Toxin Rev* 35: 121-127.
6. Wild CP, Hudson GJ, Sabbioni G, Chapot B, Hall A, Wogan GN, Whittle H, Montesano R, and Groopman JD (1992) Dietary intake of aflatoxins and the level of albumin-bound aflatoxin in peripheral blood in The Gambia, West Africa. *Cancer Epidemiol Prev Biomarkers* 1: 229–234.
7. Richard JL, Payne GE, Desjardins AE, Maragos C, Norred WP and Pestka JJ (2003) *Mycotoxins: risks in plant, animal and human systems.* CAST Task Force Report 139:101-103.
8. Vardon, P.J., McLauglin, C., Nardinelli, C. (2003) Mycotoxins: risks in plant, animal and human systems. In: *Potential Economic Costs of Mycotoxins in the United States.* CAST Task Force Report 139: 136-142.
9. Campbell KW and White DG (1995) 'Evaluation of corn genotypes for resistance to Aspergillus ear rot, kernel infection and aflatoxin production' *Plant Dis* 32: 1039-1044.
10. Brown RL, Cotty PJ and Cleveland TE (1991) 'Reduction in aflatoxin content of maize by atoxigenic strains of Aspergillus flavus' *J Food Protect* 54: 623-626.
11. Hell K, Fandohan P, Bandyopadhyay R, Kiewnick S, Sikora R and Cotty PJ (2008) 'Pre- and postharvest management of aflatoxin in maize: An African perspective' p. 219-229. *Myctoxins: Detection Methods, Management, Public Health and Agricultural Trade.* Leslie JF, Bandyopadhyay R, Visconti A, editors. Oxfordshire:CAB International.
12. Suzuki T, Noro T, Kawamura Y, Fukunaga K, Watanabe M, Ohta M, Sugiue H, Sato Y, Kohno M and Hotta K (2002) Decontamination of aflatoxin-forming fungus and elimination of aflatoxin mutagenicity with electrolyzed NaCl anode solution *J Agric Food Chem* 50: 633-641.
13. Schmale DG and Munkvold GP (2012) 'Mycotoxins in crops: a threat to human and domestic animal health' American Phytopathological Society (www.apsnet.org/edcenter/intropp/topics/Mycotoxins/Pages/EconomicImpact.aspx)
14. Thakare D, Zhang J, Wing R, Cotty PJ and Schmidt MA (2017) Host induced gene silencing inhibits aflatoxin production in transgenic maize when challenged with Aspergillus. *Sci Adv* 3(3) e1602382
15. Wilson RC and Doudna JA (2013) Molecular mechanisms of RNA interference. *Annu Rev Biophys* 42: 217-239.
16. Knip M, Constantin ME and Thordal-Christensen H (2014) Trans-kingdom cross talk: small RNAs on the move. *PLoS Genet* 10(9), e1004602.
17. Zhang J, Khan SA, Hasse C, Ruf S, Heckel DG and Bock R (2015) Full crop protection from an insect pest by expression of long double stranded RNA in plasmids. *Sci* 347:991-994.

Monica Schmidt
Assistant Professor - The School of Plant Sciences, BIO5 Institute
University of Arizona
monicaschmidt@email.arizona.edu

Addition of a New Nuclease Cpf1 in the Tool Box of CRISPR System

Anindya Bandyopadhyay

Summary

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) has been setting the world of genome editing ablaze due to its ease of application and the precise nature of its targeting. Even though Cas9 has emerged as the most commonly used CRISPR effector for genome editing-based experiments, certain limitations in its targeting location (targets only GC rich area) and the creation of blunt double stranded DNA breaks makes its widespread and ubiquitous use difficult. Consistent research in the complex world of CRISPR effectors has given rise to a new effector called Cpf1 that can overcome the limitations that Cas9 faces. The following will elucidate what Cpf1 is and how it can be used, and show that Cpf1 can be used not only in mammalian models but also in model and crop plants.

Introduction

CRISPR was originally discovered as an immune response for bacteria against invading nucleic acid molecules of Phage-viruses. With time however, it has become one of the most elucidated, utilized, fast growing, and diverse genome editing tools in the scientific world. An array of previously discovered nucleases are being fine-tuned and employed as per the requirements of the genome editing experiments. Major components of CRISPR are the RNA guided effectors that act as nucleases to cleave the target nucleic acid. These effectors have now been classified in two broad classes. The first class includes the effectors comprised of multi-protein complexes, whereas the second class effectors have a single effector protein, of which Cas9[1] is a well characterized example. Cas9 has been established as an efficient and precise genome editing tool and has successfully been applied in diverse of organisms from bacteria to humans, as well as in unicellular algae to higher angiosperms.

Although the efficiency of Cas9 has been well characterized and established, it still has certain limitations. First, a trans-activating crRNA (tracrRNA) is required to target DNA recognition and to trigger the processing of CRISPR-RNA (Cr-RNA) in the presence of Cas9. Next, it uses NGG as a PAM (Protospacer Adjacent Motif) site, and the target sequences of Cas9 are usually GC rich, which might have advantages in some organisms but not all (**Fig. 1**). This limits the scope of Cas9 in genome editing. Moreover, enzymatic action by the nuclease Cas9 is a blunt-end cleavage; hence it yields blunt -end products, which restricts its usage for NHEJ-mediated editing processes, leading to error prone editing. These drawbacks in Cas9 mediated genome editing could be overcome by using another Class II CRISPR system: Cpf1 as the master nuclease. Since its discovery, Cpfl is considered an alternate or possibly a complementary approach to Cas9 mediated genome editing.

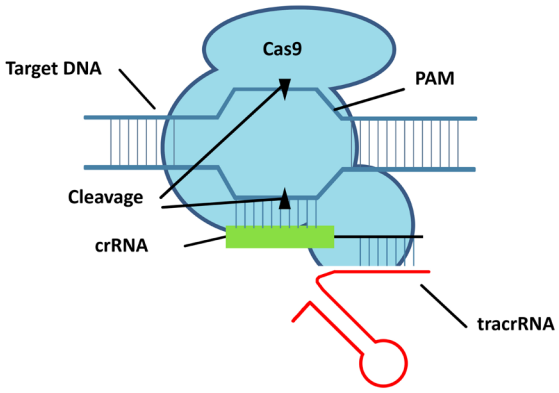


Figure 1. The structure and make up of Cas9. It relies on an NGG PAM for recognition and creates a double stranded break at the location of the target. The crRNA pairs with the target based on complementarily. Requires a tractrRNA for functioning.

Salient Features of Cpf1

Cpf1 (CRISPR from *Prevotella* and *Francisella* 1), like other CRISPR nucleases, functions as a defense molecule in the genome of a number of bacteria, with an ability to defend against plasmids or viral

Cpf1 Properties:

- **AT rich PAM, thus can target AT rich regions of the genome.**
- **Cleavage is away from the recognition/ seed sequence and PAM.**
- **Staggered cleavage resulting in overhangs.**
- **Does not require an extra tracrRNA.**
- **Can process its crRNA by itself.**

nucleic acid particles using CRISPR[2]. As stated earlier, Cpf1 has certain major advantages as a functional genome editing tool in molecular biology. The first major advantage of Cpf1 over Cas9 is it does not require a tracr-RNA to recognize the target DNA molecule. Secondly, it uses a T-rich PAM site (TTTN/ TTN), and it mainly targets the AT rich sequences in the genome (**Fig 2**). Next, the Cpf1-mediated nuclease activity provides a staggered cut to the DNA, leaving a 4- to 5-nucleotide sticky overhang[2]. Another advantage of Cpf1 is that it is reported to cleave the DNA distal to the PAM region, compared to Cas9 which cleaves near the PAM and which may not support repeated cleavage by Cas9 in the already cleaved and mutated section of the genome. Whereas with Cpf1, the targets can be repeatedly cleaved, as the target recognition region is not disrupted as such; hence Cpfl promotes repeated cleaving of the target region, thus increasing the efficiency of editing, which can also be advantageous in HDR mediated genome editing.

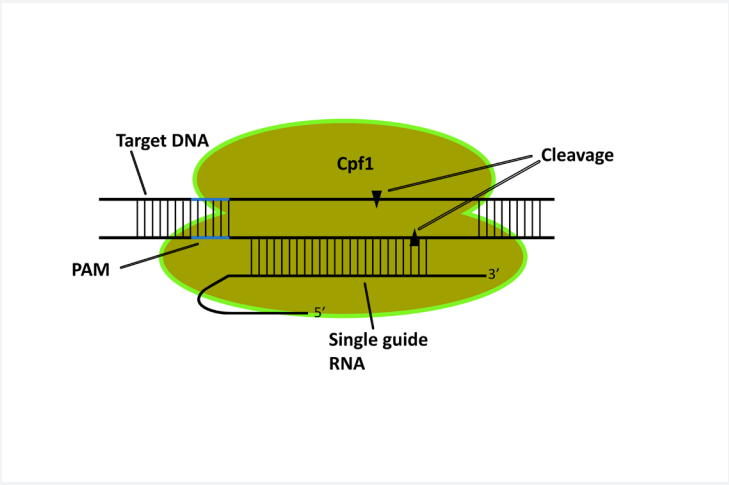


Figure 2. The structure and make up of Cpf1. It relies on a TTN PAM for recognition and creates a double stranded break at the location of the target. The crRNA pairs with the target based on complementarily. Does not require a tracrRNA.

Application and Validation of Cpf1

The first successful attempt to utilize Cpf1 as a genome editing tool was performed on the *DNMT1* gene where it successfully cleaved the PCR amplicon of the gene *in vitro*. This procedure was again tested on human embryonic kidney cells; two Cpf1 nucleases, AsCpf1 LbCpf1, efficiently produced nucleolytic cleavage resulting in insertions and/or deletions. In the same experiment, staggered cleavage by the Cpf1 molecules was also validated using Sanger sequencing. Further knockout mice were generated for *transformation related protein 53* (*Trp53*). The two Cpf1 molecules exhibited more precise genome editing[2], with high efficiency and minimum off target effects in the mice.

Application of Cpf1 in Plants

After successful validation of the nuclease in the mammalian cell system, it was subsequently tested in the plant genome. To test whether Cpf1 has any nucleolytic effect on the plant genome, two marker genes were selected (*OsPDS* and *OsBEL*). Efficient Cpf1-mediated mutations were reported in transgenic rice, which established the activity and efficiency of Cpf1 in generating stable and inheritable targeted mutations in plants[3]. Furthermore, the Cpf1 molecule has been used as a transcriptional repressor molecule in *Arabidopsis* by deactivating its nuclease domain and fusing it to three copies of the SRDX transcriptional repressor. The transcriptional repression was carried into the T1 generation[4]. Further, FnCpf1 (from *Francisella novicida*), which uses an even shorter PAM sequence (TTN), was efficiently used to induce targeted mutagenesis in two genes of tobacco, *NtPDS* and *STENOFOLIA* ortholog (*NtSTF1*), and two genes in rice, *OsDL* (Drooping leaf) an *OsALS* (Acetolactone synthase).

The FnCpf1-mediated efficiency was as high as 28.2% and 47.2% for targeted mutagenesis in tobacco and rice plants[5], respectively. Very recently, a multiplex genome editing approach has also been reported using CRISPR-Cpf1.

Four genes from the receptor-like kinase gene family (*OsRLKs*) and four members of the CYP81A gene family (*OsBEL*) were targeted by two Cpf1 nucleases (LbCpf1 and FnCpf1). There was not, however, any significant increase in the efficiency of cleavage by the multiplexing crRNA array. However no off-target effects were reported in this experiment, indicating higher fidelity in editing the Cpf1-mediated multiplex genome editing in plants[6].

Targeting Stomatal Developmental Rice Gene

At the International Rice Research Institute (IRRI), we have successfully managed to test the efficiency of the LbCpf1 system using a rice gene as a marker. The rice gene *OsEPFL9* (Epidermal Patterning factor) was used to test the cleavage efficiency of Cpf1. This gene is responsible for stomatal patterning and density in the development of a leaf. Knocking down this gene would theoretically reduce the number of stomata in an adult plant. To validate this hypothesis, exon 1 of the *OsEPFL9* gene was targeted by both Cas9 and Cpf1, respectively, to evaluate cleavage efficiencies[7] (**Fig 3**).

After the targets were cloned into the appropriate guide RNA scaffolds, constructs were transformed into rice immature embryos. The transformed plants were recovered and grown following transformation protocols. Initial screening of T0 plants for the nuclease transgene and the probable site of mutation were performed using the Surveyor assay (**Fig 4**). Subsequent sequencing analysis revealed bases were deleted in exon 1 of the gene. The deletions ranged from that of 5 bp to 63 bp in different events, resulting in truncated transcripts. To confirm the transmission of the mutation across generations, the plants were carried forward to the T1 generation. Southern blot analysis confirmed the absence of the transgene harboring the Cas9 insert, while subsequent analysis by Surveyor assay and sequencing confirmed the presence of the mutation; i.e., deletion of the bases were in the same pattern as that of the previous T0 generation. The editing efficiency of Cpf1 was higher compared to that of Cas9.

Phenotypically, the knockout plants obtained from both Cas9 and Cpf1 showed the predicted phenotype. There was a 6- to 8-fold reduction in the stomatal density of the leaves, indicating that editing was successful (**Fig. 5**). In addition, the analysis also revealed that the mutations were stable and heritable, and the plants were nuclease free and homozygous for the said mutation. With these reports of a more precise and error-free genome editing technique, the use of Cpf1 as a genome editing tool comprises a viable option for genome editing in plants and increases the horizon of CRISPR mediated gene manipulation.

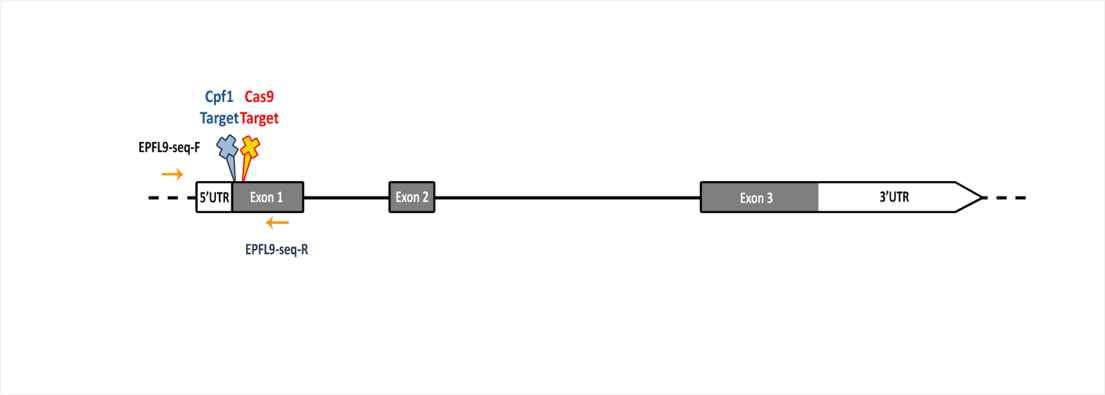


Figure 3. The location of the Cas9 and the Cpf1 targets selected in the Exon 1 of OsEPFL9 gene.

Acknowledgement: The author would like to thank Dr. Abhishek Anand, Shamik Mujumdar, Dr. Xiaojia Yin, Dr. Paul Quick from C4 rice center of IRRI and Dr. Julie E. Gray from University of Sheffield, UK.

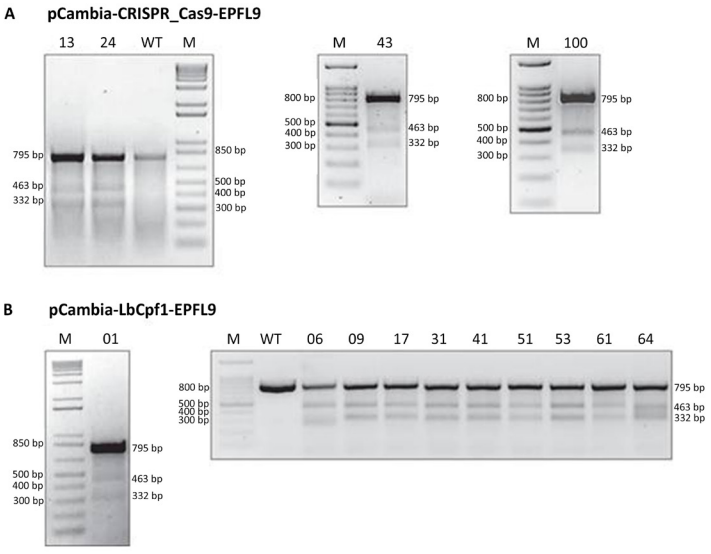
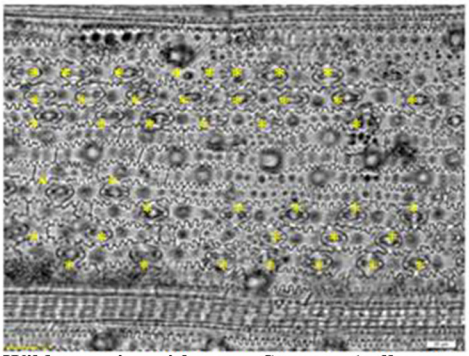
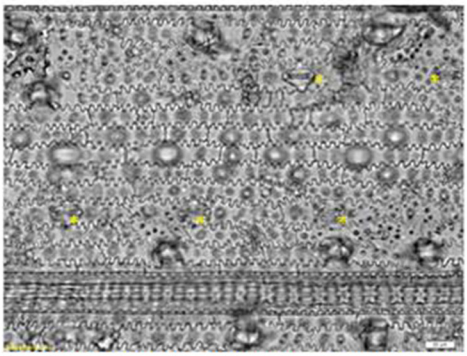


Figure 4. Surveyor Assay conducted on the TO samples of the CRISPR edited plants. The presence of multiple bands shows the presence of the mutation as the nature of the Surveyor enzyme is to cleave a double stranded DNA after encountering base pair mismatch.

Figure 5. The difference in the number of stomata between wild type and CRISPR edited rice plants. 8 fold reduction reported.



Wild type rice with many Stomata (yellow star shows stomata)



CRISPR edited rice with reduced stomata (yellow star shows stomata)

References

1. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier EA (2012) Programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 17;337(6096):816-21
2. Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, van der Oost J, Regev A, Koonin EV (2015) Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* 163(3):759-771
3. Xu R, Qin R, Li H, Li D, Li L, Wei P, Yang J (2016) Generation of targeted mutant rice using a CRISPR□Cpf1 system. *Plant Biotechnol J.* 2017 15(6):713-717
4. Tang X, Lowder LG, Zhang T, Malzahn AA, Zheng X, Voytas DF, Zhong Z, Chen Y, Ren Q, Li Q, Kirkland ER (2017) A CRISPR–Cpf1 system for efficient genome editing and transcriptional repression in plants. *Nature Plants* 3:17018.
5. Endo A, Masafumi M, Kaya H, Toki S (2016) Efficient targeted mutagenesis of rice and tobacco genomes using Cpf1 from Francisella novicida. *Sci. Rep* 6: 38169.
6. Wang M, Mao Y, Lu Y, Tao X, Zhu JK (2017) Multiplex gene editing in rice using the CRISPR-Cpf1 System. *Molecular Plant* DOI: http://dx.doi.org/10.1016/j.molp.2017.03.001
7. Yin X, Biswal AK, Dionora J, Perdigon KM, Balahadia CP, Mazumdar S, Chater C, Lin HC, Coe RA, Kretzschmar T, Gray JE, Quick WP, Bandyopadhyay A (2017) CRISPR-Cas9 and CRISPR-Cpf1 mediated targeting of a stomatal developmental gene EPFL9 in rice. *Plant Cell Reports* 36(5):745-57.

Dr. Anindya Bandyopadhyay
Scientist-II (Molecular Biology)
International Rice Research Institute,
DAPO 7777, Metro Manila, Philippines
a.bandyopadhyay@irri.org

Agriculture in a Changing Climate

Phill Jones

More than 90 percent of the world's food supply consists of agricultural crops or meat from farm animals raised on vegetarian feeds, according to The Research Council of Norway. By midcentury, the group says, farmers must produce 70 per cent more food on about the same area of farmland to keep pace with global population growth. The changing climate greatly increases difficulties in meeting this challenge. The US Department of Agriculture predicts that beyond the midcentury, climate change will detrimentally affect most crops and livestock.

Increasing amounts of carbon dioxide and other greenhouse gases increase the capacity of the atmosphere to retain heat. The higher temperature of the atmosphere heats the planet surface. Since 1900, the world's average surface temperature has risen by about 0.74°C. According to the USDA, the average temperature is predicted to warm another 1.9 to 5°C during the next century.

Effects of Climate Change on Crops

Higher maximum and minimum temperatures affect crop yields. Above a threshold, a higher air temperature adversely affects the growth of plants, as well as pollination and plant reproductive processes. Negative effects on plant growth and grain production can accelerate as the temperature rises above the optimum. A higher minimum temperature affects a plant's respiration rate at night, and can reduce crop yield and biomass accumulation. Higher temperatures also reduce productivity due to higher soil evaporation rates. The United Nation's Intergovernmental Panel on Climate Change predicts that yields of corn, wheat, and rice will probably start to drop by 2030. These three crops account for more than 60% of global food production from plants.

Unpredictable rainfall patterns are another facet of climate change. The Intergovernmental Panel on Climate Change predicts that precipitation will increase in high latitudes, and decrease in most subtropical low latitude regions. Extremes of rainfall changes – droughts and floods – clearly affect crop yield by damaging plants. Yet changes in rainfall patterns produce other deleterious effects. Excess

precipitation erodes the soil and leaches nutrients from soil, whereas a decline in precipitation decreases soil moisture.

Climate change also produces indirect effects on crops. According to the US Environmental Protection Agency, farmers probably will face new challenges as weeds, pathogens, and insect pests expand their ranges to the warming north. High levels of atmospheric carbon dioxide promote the rapid growth of invasive weeds.

Effects of Climate Change on Livestock

Increasing temperatures threaten animal health. An increase in core body temperature in excess of 2°C to 3°C can disrupt fertility, increase vulnerability to disease, and limit an animal's ability to produce meat, milk, or eggs. An increase of 5°C to 7°C can kill an animal. Higher average temperatures, hotter daily maximums, and more frequent heat waves, produce heat stress. The effects of heat stress include changes in respiration, heart rate, and hormones. Animals dealing with heat stress usually drink more water and reduce the amount of dry food that they eat. This in turn, affects the animal's health and decreases weight gain.

Although heat stress may be the most significant threat to animals, climate change creates other problems. Drought decreases the quantity and quality of available feed. Increases in carbon dioxide can decrease the quality of forage, requiring cattle to eat more to obtain the same amount of nutrients. Warmer and more humid conditions promote the prevalence of parasites and pathogens.

Adapting Agricultural Practices to Climate Change

The USDA suggests methods that farmers can use to adapt to climate change in the near-term. For example, heat stress on animals can be mitigated by energy-efficient cooling for animal housing. Changing feed rations for dairy and beef cattle can reduce effects of heat stress. Animals experiencing heat stress tend to diminish feed intake at a time when they need more energy for physiological maintenance. The agency recommends feed with increased nutritional quality and lower fiber content. "In the future," says the USDA on its Climate Hubs Website, "producers may consider selecting breeds and breed types that are genetically adapted to changed climate conditions."

To ensure productivity of their fields, the USDA offers a variety of adaptation strategies that growers can use. These include changes in cultivar selection, the timing of field operations, irrigation methods, fertilization practices, and tillage practices.

New types of genetically engineered (GE) crops provide one tactic for meeting the challenge of increasingly radical climate changes. "I don't believe that gene technology or GMOs alone will save the world, but they will be part of the solution in certain areas," Atle Bones told Biotek og mat. Bones, a professor of biology at the Norwegian University of Science and Technology in Trondheim, explained that "[s]ome changes, such as climatic ones, are going to happen rapidly, so we don't have time to wait the many years it would take with conventional selection to introduce the desired traits into our crop varieties."

Engineering Crops for a Changing Environment

Researchers have genetically engineered plants that can survive in the face of decreased rainfall, one of the abiotic stresses that accompany climate change. Plants react to drought by initiating a complex cascade of responses to protect cells against the effects of desiccation and prevent water loss. The variety of methods used by plants to manage drought stress is reflected by the range of tactics that researchers use to engineer drought-tolerant plants.

The first GE drought-tolerant crop approved for sale in multiple countries is Monsanto Company's DroughtGard® Corn (MON 87460). The GE plant has a cold-shock protein B (CSPB) gene derived from *Bacillus subtilis*. CSPB protein, an RNA chaperone, appears to support a plant's cellular functions by binding cellular RNA and unfolding untranslatable secondary structures that disrupt RNA stability and translation. As a result, CSPB protein minimizes the effects of drought on many cellular functions, including photosynthesis.

During 2015, Argentina's Ministry of Agriculture, Livestock and Fisheries approved soybeans engineered to tolerate drought. The GE soybeans are a product of Verdeca, a joint venture between Bioceres (Rosario, Argentina) and Arcadia Biosciences (Davis, CA). Cells

of the engineered soybeans overexpress sunflower Hahb-4, a homeodomain–leucine zipper transcription factor, which delays or blocks ethylene-induced senescence, while allowing ethylene to regulate leaf stomatal opening. The delay in senescence may enable plant cells to produce osmoprotectants that improve drought tolerance.

Researchers at DuPont Pioneer (Johnston, IA) also are genetically modifying ethylene production. They developed GE corn that downregulates ethylene synthesis, resulting in an increase of grain yield after exposure to drought stress.

In a plant exposed to water stress, glycine betaine acts as an osmoprotectant by stabilizing the integrity and function of cellular membranes. Indonesia's National Genetically Modified Product Biosafety Commission approved GE sugarcane that carries a *Rhizobium meliloti betA* gene, which encodes choline dehydrogenase. The enzyme converts choline into betaine aldehyde, which in turn is converted to glycine betaine by betaine aldehyde dehydrogenase.

Plants respond to drought stress by synthesizing abscisic acid, which triggers guard cells surrounding plant leaf stomata to close and limit water loss. Researchers at Performance Plants (Ontario, Canada) use RNA interference to downregulate farnesyltransferase in canola, corn, and rice. This results in abscisic acid hypersensitivity of guard cell anion-channel activation and closing of stomata. In the GE plants, RNA interference is controlled by a drought-inducible promoter. Consequently, normal stomatal function is restored after a drought has ended.

The genetic modification of plants to increase heat tolerance often focuses on methods to protect plant cells from injury. One strategy is to engineer plants that overexpress heat shock genes. Heat shock proteins stabilize or refold proteins that have become denatured during heat stress; they prevent protein aggregation. Another tactic is to alleviate the rapid accumulation of reactive oxygen species (ROS) in plant cells that occurs during high temperature stress. Transgenic plants with transgenes that express ROS detoxifying enzymes have a tolerance of high temperatures. Other targets for developing heat-tolerant transgenic plants include enzymes that regulate membrane fluidity and enzymes involved in osmolyte synthesis.

Plants are not exposed to either heat stress or drought stress in isolation. Studies reveal that plants respond to a combination of heat and drought stresses that cannot be directly extrapolated from the response of plants to each stress alone. Additional abiotic stresses, such as high salinity, and biotic stresses, such as new insect pests, complicate engineering of plants suited to the changing environment. As genetic engineers forge ahead to counter the effects of climate change on crops, they need to consider not only alterations in plant growth, but also possible changes in the characteristics of the plants. Nigel G. Halford (Rothamsted Research, Harpenden, Hertfordshire UK) and his colleagues at the Shanghai Academy of Agricultural Sciences warn that more research is required for "the identification of specific environmental stresses that affect grain composition in ways that have implications for food quality and safety."

Selected Sources

"Climate Impacts on Agriculture and Food Supply," *US EPA* Website (2012). Available at: http://www3.epa.gov.

"Focus on Livestock," *USDA Climate Hubs* Website (2015). Available at: http://climatehubs.oce.usda.gov.

Fugelsnes, Elin and Else Lie, "Food Crops Must Withstand Harsher Weather," *The Research Council of Norway* Website (October 7, 2011). Available at: http://www.forskningsradet.no.

Grover, Anil, Dheeraj Mittal, Manisha Negi, and Dhruv Lavania, "Generating High Temperature Tolerant Transgenic Plants: Achievements and Challenges," *Plant Science* 205–206: 38–47 (2013).

Halford, Nigel G., Tanya Y. Curtis, Zhiwei Chen, and Jianhua Huang, "Effects of Abiotic Stress and Crop Management on Cereal Grain Composition: Implications for Food Quality and Safety," *Journal of Experimental Botany*. 66(5):1145–1156 (2015).

Key, Nigel, Stacy Sneeringer, and David Marquardt, "Climate Change, Heat Stress, and U.S. Dairy Production," *USDA* Website (September 2014). Available at: http://www.ers.usda.gov.

Lavania, Dhruv, Anuradha Dhingra, Manzer H. Siddiqui, Mohamed H. Al-Whaibi, and Anil Grover, "Current Status of the Production of High Temperature Tolerant Transgenic Crops for Cultivation in Warmer Climates," *Plant Physiology and Biochemistry* 86: 100–108 (January 2015).

Meiszner, Peter, "Drought, Heat Take Toll on Global Crops," *The University of British Columbia* Website (January 6, 2016). Available at: http://news.ubc.ca.

"Pocket K No. 32: Biotechnology for the Development of Drought Tolerant Crops," *International Service for the Acquisition of Agri-biotech Applications* Website (March 2008). Available at: http://www.isaaa.org.

"Pocket K No. 43: Biotechnology and Climate Change," *ISAAA* Website (April 2013). Available at: http://www.isaaa.org.

Walthall, C.L., J. Hatfield, P. Backlund, R. Hauser, L. Lengnick, E. Marshall, and M. Walsh. "Climate Change and U.S. Agriculture: An Assessment of Effects and Adaptation Responses," *USDA* Website (September 2013). Available at: http://www.usda.gov.

Waltz, Emily, "Beating the Heat," *Nature Biotechnology* 32(7): 610–613 (July 2014).

Weiser, Matt, "Can Genetic Engineering Help Quench Crops' Thirst?" *ENSIA* Website (January 4, 2016). Available at: http://ensia.com.

Woolston, Chris, "These 5 Foods Will Be Harder to Grow in a Warmer World," *National Geographic* Website (April 7, 2014). Available at: http://news.nationalgeographic.com.

Phill Jones
Biotech-Writer.com
PhillJones@nasw.org