Chapter 3-1

Outline of the SATREPS project "Development of genetic engineering technology of crops with stress tolerance against degradation of global environment"

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Abstract

Soybean is an important crop which is a source of food with protein and oil content, animal feed, and biofuels. Although Brazil is the second largest soybean producer in the world, yields have been severely unstable in the recent years as a result of frequent droughts. We implemented a Science and Technology Research Partnership for Sustainable Development (SATREPS) project titled "Development of genetic engineering technology of crops with stress tolerance against degradation of global environment" in cooperation with Japan and Brazil. The aim of this project was to establish a technology to develop genetically modified (GM) soybean lines with increased tolerance to environmental stresses such as drought, in Brazil. We searched for soybean genes that exhibited properties similar to those associated with drought response and tolerance in the model plant *Arabidopsis thaliana*. We also comprehensively analyzed the gene expression in soybean under stress conditions. We generated the best combination of genes that are likely to enhance drought tolerance and promoters that regulate such gene expression and introduced the resulting constructs into soybean via transformation. GM soybean lines were generated and evaluated under greenhouse and field conditions to identify drought-resistant lines in Brazil. The developed technology and the generated GM soybean lines are expected to help stabilize or increase soybean production in Brazil.

Keywords: stress-tolerant genes, stress-responsive promoters, drought-tolerance, GM soybean

Introduction

In the past decades, the global frequency of drought has increased significantly, which is likely to be related to climate change. Global soybean production in 2015/2016 was approximately 107 million tons in the United States, 100 million tons in Brazil, 59 million tons in Argentina, and 12 million tons in China (United States Department of Agriculture 2018). Over 90% of the world's soybean production areas rely on rainfed water, and only about 8.1% of soybeans are produced in irrigated areas (Portmann et al. 2010). Soybean production areas are mainly dry, and droughts often reduce yields. Approximately 40% of global soybean production has been reduced by drought (Specht et al. 1999). Drought losses during the 37 Brazilian harvest seasons from 1976/1977 to 2013/2014 were estimated at US $ 79.620 billion (Ferreira, 2016). Northeastern China is the largest soybean-producing region in China cultivated mainly by rainfed water, where the planted area and yield account for 40% to 50% of China's total soybean production. Soybean production in this region suffers from severe drought (Yang et al. 2017). All soybean producing regions in India (11.5 million ha) rely on rainfed water, with drought being the most severe hindrance to production (Bhatia et al. 2014). Salt damage is exacerbated in the arid and semi-arid regions of China and India.

Brazil is the second largest soy producer in the world after the United States, with significant losses due to drought (Nakashima et al. 2014). Due to reduced yields, direct losses to agriculture as well as to the overall economy of soy-producing regions can have serious adverse effects on the society. There are several strategies to mitigate water stress issues that affect agricultural productivity. One of the most effective approaches is the development of drought-tolerant crops. Once an effective variety has been developed, farmers need not use additional materials to reduce water stress. Therefore, the establishment and application of various breeding techniques, including biotechnology, is important for generating new varieties with improved drought tolerance. We implemented the "Development of genetic engineering technology of crops with stress tolerance against degradation of global environment biotechnology" project as the SATREPS project (JFY2009-2013) to produce drought-tolerant soybeans in Brazil. This review is based on the final report of the SATREPS project (Nakashima 2013) and related articles, including the ones published by Nakashima et al. (2014), Nakashima and Suenaga (2017), and Nakashima et al. (2018).

Molecular mechanisms involved in environmental stress responses in *Arabidopsis thaliana*

In order to develop new crops with improved tolerance to various environmental stresses, such as drought, using biotechnology, it is important to elucidate the genes and molecular mechanisms involved in environmental stress response and tolerance. Our research groups at the Japan International Research Center for Agricultural Sciences (JIRCAS), RIKEN, and the University of Tokyo have been conducting research to elucidate important genes involved in environmental stress response and tolerance mechanisms using *Arabidopsis thaliana,* a plant commonly used in plant molecular biology and molecular genetics research. We succeeded in identifying various important stress-responsive genes and revealed that stress-inducible transcription factors (TFs) such as the dehydration-responsive element-binding protein (DREB), abscisic acid (ABA)-responsive element-binding factor (AREB), and NAC (no apical meristem [NAM], Arabidopsis transcription activation factor [ATAF], and cup-shaped cotyledon [CUC]) play important roles in regulating stress response and tolerance (**Fig. 1**; reviewed in Nakashima et al. 2014, Nakashima and Suenaga 2017). High accumulation of these transcription factors in plants has been shown to enhance stress tolerance in *A. thaliana* in growth chambers.

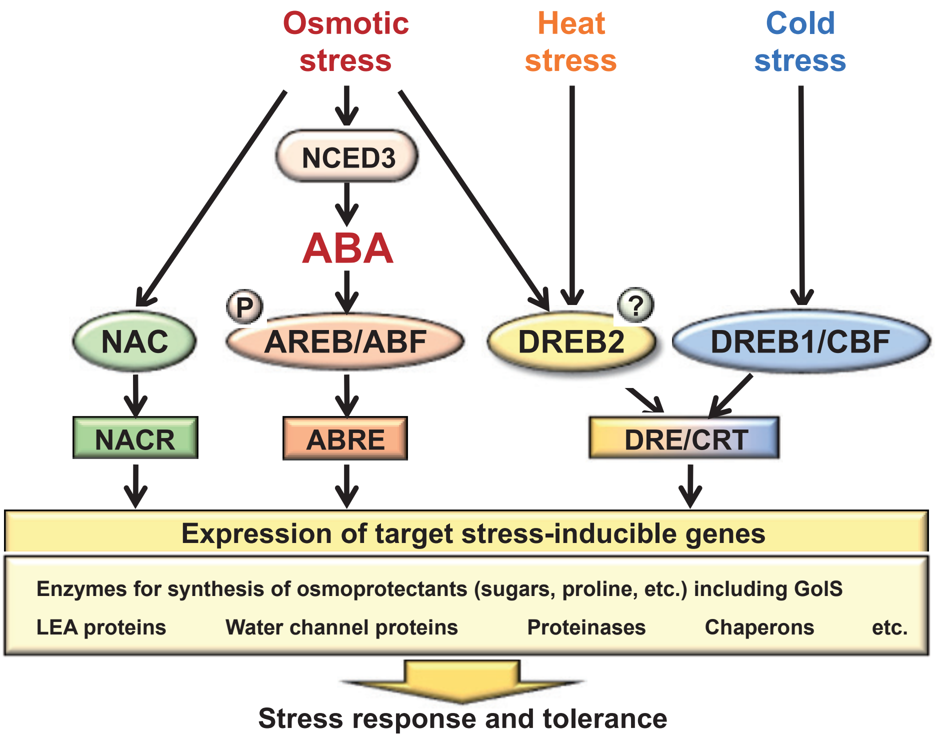

Fig. 1. Key transcriptional networks under environmental stress conditions in plants.
Environmental stresses such as osmotic stress, heat stress, and cold stress cause transcription factor biosynthesis and activation. Transcription factors bind to specific *cis*-elements and induce the expression of stress-inducible genes. Induced proteins affect stress tolerance and response. The ellipses and boxes correspond to transcription factors and *cis*-elements, respectively. This figure was adapted from Nakashima and Suenaga (2017).

The DREB1A and DREB2A TFs, which function in an ABA-independent pathway, bind to a *cis*-element containing an essential core sequence A/GCCGAC called the dehydration response element (DRE), which is present in the promoter region of the target stress-responsive genes. Binding of DREB TFs to DRE induces target gene expression and activates cell protection mechanisms under stress conditions (reviewed in Mizoi et al. 2012). Overexpression of *DREB1A* can enhance stress tolerance in various kinds of plants such as Arabidopsis (Kasuga et al. 1999), tobacco (Kasuga et al. 2004), wheat (Pellegrineschi et al. 2004), rice (Oh et al. 2005; Ito et al. 2006), potato (Behnam et al. 2007), and peanut (Bhatnagar-Mathur et al. 2007). *DREB1* homologs have been studied in many kinds of plants (reviewed in Mizoi et al. 2012). Soybean *DREB1* genes have been previously identified in the SATREPS project as described below (Kidokoro et al. 2015). In addition, overexpression of *DREB2A* improves tolerance to drought and heat stress in Arabidopsis (Sakuma et al. 2006a, b). *DREB2* homologs have been reported in various kinds of plants, including rice (Dubouzet et al. 2003) and maize (Qin et al. 2007). Soybean *DREB2* genes were identified in the SATREPS project as described below (Mizoi et al. 2013). *AREB1*, which functions in an ABA-dependent pathway, binds to a conserved *cis*-element called the ABA-responsive element (ABRE; PyACGTGG/TC) in the promoter of the ABA-inducible genes. Binding of AREB TFs to ABRE induces target gene expression and activates ABA-related cell protection mechanisms under stress conditions (reviewed in Fujita et al. 2013).

Under stress conditions, stress-related TFs such as DREB and AREB regulate the expression of the target genes encoding key metabolic proteins that protect cells from dehydration, such as late embryogenesis abundant (LEA) proteins, water channel proteins, proteases, chaperones, and enzymes for the synthesis of osmoprotectants (compatible solutes such as sugars and prolines). Galactinol synthase (GolS; **Fig. 1**), a key enzyme in the production of raffinose family oligosaccharides (RFOs), is also induced under stress conditions. RFOs, as compatible solutes, not only enhance drought tolerance by regulating osmotic potential, but also protect macromolecules such as enzymes and membranes under stress conditions. The *GolS* gene has been reported to be induced by environmental stress in many kinds of plants, including Arabidopsis (Taji et al. 2002) and soybean (Marcolino-Gomes et al. 2014, Rodrigues et al. 2015). Overexpression of *AtGolS2* has been shown to enhance environmental stress tolerance in *A. thaliana* in pot tests (Taji et al. 2002).

ABA is an important phytohormone that regulates gene expression as well as physiological responses, including stomatal closure under stress conditions. The *NCED3* gene encodes a key enzyme in the ABA biosynthesis pathway (**Fig. 1**). *NCED3* expression is strongly induced by dehydration stress in various plants, including *Arabidopsis thaliana* (Iuchi et al. 2001). Overexpression of *NCED3* increases tolerance to dehydration stress in *A. thaliana* in pot tests (Iuchi et al. 2001).

Outline of SATREPS project

We implemented the "Development of genetic engineering technology of crops with stress tolerance against degradation of global environment" project as the SATREPS project (JFY2009-2013; **Fig. 2**) with the support of the Japan Science and Technology Agency (JST) and the Japan International Cooperation Agency (JICA) to develop drought-tolerant soybeans in Brazil. The Brazilian and Japanese governments have agreed to cooperate on the SATREPS research projects involving the JST and JICA. The JST and JICA are supported by the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) and the Ministry of Foreign Affairs (MFA), respectively. This project was approved and signed by the representatives of JICA and the Division of Science and Technology of MFA on December 29, 2009. The project started on March 4, 2010, and lasted for 5 years.

The main objective of the project was to establish technology to develop genetically modified (GM) soybean lines that are tolerant to environmental stresses such as drought. This project aimed to develop a soybean line that can withstand drought by applying the results of research on model plants such as *A. thaliana* and using the information from the soybean genome to search for genes involved in drought response and tolerance in soybeans, and to elucidate the mechanisms that control these genes.

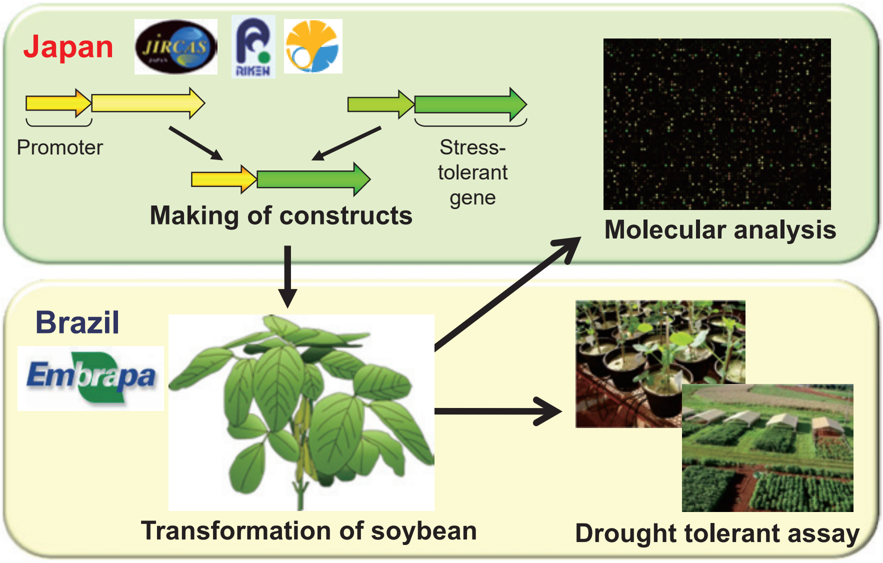

Fig. 2. Outline of the Science and Technology Research Partnership for Sustainable Development (SATREPS) project, "Development of genetic engineering technology for crops with stress tolerance to degradation of global environmental”

The aim of this project was to establish technology to develop genetically modified (GM) soybean lines that are more resistant to environmental stresses such as drought. The project aimed to establish techniques to develop GM soybean lines that are more tolerant to environmental stresses such as drought.

Researchers from the JIRCAS, RIKEN, and the University of Tokyo contributed to this project. Research activities conducted at JIRCAS, RIKEN, and the University of Tokyo in Japan were supported by JST. The Embrapa Soybean of Brazilian Agricultural Research Corporation (Embrapa) in Brazil was responsible for the project on the Brazilian side and was supported by JICA. Embrapa is the only organization that has developed a genetically modified commercial soybean variety in Brazil. All relevant biosafety studies were conducted in Brazil by Embrapa and its affiliated research institutes. In addition, to develop GM plants, Embrapa has developed and patented a technology that significantly improves the efficiency of gene gun transformation. During the SATREPS project, with the support of JICA, Embrapa Soybean deputed researchers to Japan for scientific training every year. In addition, JICA assisted in deputing Japanese researchers to Embrapa Soybean for long or short periods.

From Japan, JIRCAS, RIKEN, and the University of Tokyo participated in the SATREPS project by conducting research on stress tolerance, stress response, and stress perception, respectively (**Fig. 3**). These institutions have analyzed and provided genetic constructs containing useful genes and promoters that are expected to enhance drought tolerance in soybean. In addition, these institutions imported GM soybean lines produced in Brazil and analyzed gene expression in the promising GM soybean lines.

Embrapa Soybean contributed to the project in Brazil (**Fig. 3**). Embrapa Soybean has introduced genetic constructs into soybeans, tested drought tolerance in greenhouses, and evaluated agronomical traits in the confined field. Since soybean transformation is more difficult than that of other crops, the Brazilian side focused on soybean transformation, and a JIRCAS researcher stayed for a longer time to establish soybean transformation technology by *Agrobacterium* method in Embrapa Soybean.

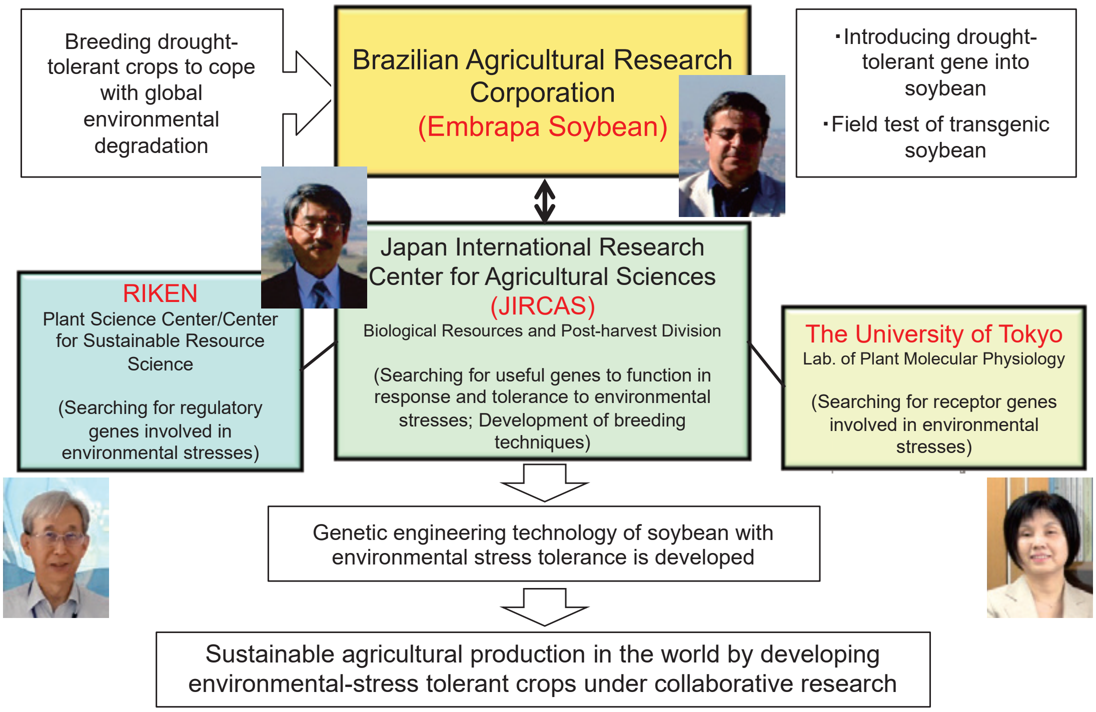

Fig. 3. Research structure of the SATREPS project, "Development of genetic engineering technology of crops with stress tolerance against degradation of global environment"

Summary of research results on the Japanese side

In this project, the following research results were obtained from the Japanese research team. JIRCAS has established a comprehensive gene expression analysis method using soybean oligo arrays (Maruyama et al. 2012). The JIRCAS team clarified the functions of GmDREB1 and GmAREB TFs involved in environmental stress response and resistance in soybean. The team also discovered excellent stress-inducible promoters such as Gm3Pro in soybean (Nakashima et al. 2018). The JIRCAS team and the team at the University of Tokyo found that the soybean DREB1/CBF-type GmDREB1 TF functions in heat- and drought-responsive gene expression as well as in cold stress-responsive gene expression (Kidokoro et al. 2015). In addition, a comprehensive gene expression analysis of GM soybeans imported from Embrapa Soybean confirmed the function of the transgene. The RIKEN team discovered the expression and function of genes encoding GmNCED, a soybean ABA synthetase. In addition, the RIKEN team and the JIRCAS team characterized soybean expression and metabolism during drought through integrated analysis of metabolome and transcriptome (Maruyama et al. 2020). The team at the University of Tokyo revealed that the functions of the histidine kinase GmHK and the TFs GmDREB2s and NF-YC10 are involved in environmental stress response and resistance in soybean (Mizoi et al. 2013, Sato et al. 2014, 2016). The Japanese teams sent 21 constructs (9 for particle gun and 12 for *Agrobacterium*) for soybean transformation to the Brazilian research institution Embrapa Soybean. **Table 1** shows the constructs sent from JIRCAS to Embrapa Soybean in the Project. JIRCAS has also improved the transformation efficiency of Brazilian soybean cultivars by establishing a transformation method using *Agrobacterium tumefaciens* in cooperation with Embrapa Soybean (Kanamori et al. 2011, 2017).

Table 1. Constructs sent from JIRCAS to Embrapa Soybean in the Project

Construct	Promoter*	Gene	Transformation method	Note
RD29A: DREB1A	RD29A (Arabidopsis)	DREB1A (Arabidopsis)	gene bombardment	
35S: DREB1A	35S (CaMV)	DREB1A (Arabidopsis)	gene bombardment	
RD29A: DREB2Aca	RD29A (Arabidopsis)	DREB2Aca (Arabidopsis)	gene bombardment	Constitutive active TF
35S: DREB2Aca	35S (CaMV)	DREB2Aca (Arabidopsis)	gene bombardment	Constitutive active TF
35S: AREB1	35S (CaMV)	AREB1 (Arabidopsis)	gene bombardment	
35S: AREB1dQT	35S (CaMV)	AREB1 (Arabidopsis)	gene bombardment	Constitutive active TF
35S: AREB1M8	35S (CaMV)	AREB1 (Arabidopsis)	gene bombardment	Constitutive active TF
Gm3Pro: DREB1A	Gm3Pro (soybean)	DREB1A (Arabidopsis)	*Agrobacterium tumefaciens*-mediated transformation	

* RD29A and Gm3Pro are stress responsive promoters; 35S is a constitutive promoter.

For details, see the original papers, the final report of the SATREPS project (Nakashima 2014) and reviews including Nakashima and Suenaga (2017) and Nakashima et al. (2018).

Summary of research results from the Brazilian team

The constructs sent to Embrapa Soybean by Japanese collaborators were sequentially introduced into soybeans to generate GM soybeans. The Embrapa Soybean team evaluated the generated GM soybeans in greenhouses and fields (**Fig. 4**; Barbosa et al. 2012; Engels et al. 2013; Fuganti-Pagliarini et al. 2017; Honna et al. 2016; Leite et al. 2014; Marinho et al. 2016; Polizel et al. 2011; Rolla et al. 2014). Improved drought tolerance at the greenhouse level has been identified in GM soybean lines generated from at least four different constructs. Under water deficit conditions in the field, a better performance was observed in the *1Ea2939* AREB line, which showed a higher performance than that of the wild type and other GM lines (Fuganti-Pagliarini et al. 2017). Interestingly, pest resistance has also been observed, and this line is expected to show biotic stress resistance as well as abiotic stress resistance. There are other lines of GM soybean that have not been tested for drought tolerance, and lines are expected to be found there (see **Chapter 3-2**). The SATREPS project has ended, but we continue to evaluate these lines.

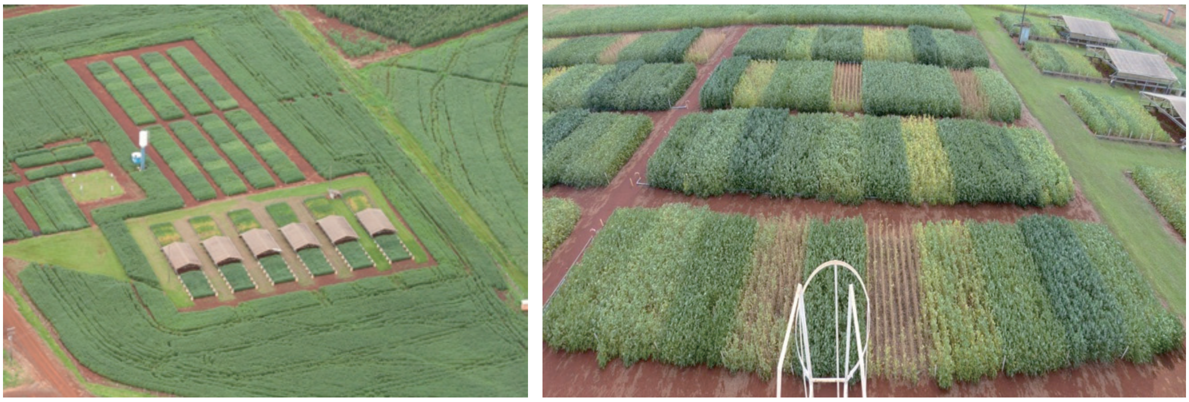

Fig. 4. Confined fields and rain-out shelters used to evaluate the drought tolerance of GM soybean lines in Embrapa Soybean in Brazil.

See **Chapter 3-2** for activities related to the development of drought-tolerant soybeans in Brazil. In this context, see **Chapter 3-3** for activities related to the development of drought-tolerant sugarcane in Brazil. For details, see the original papers, the final report of the SATREPS project (Nakashima 2014) and reviews including Nakashima and Suenaga (2017) and Nakashima et al. (2018).

Conclusion

In the SATREPS project, the Japanese team identified key genes and useful stress-inducible promoters involved in response and tolerance to drought in soybean. Furthermore, the team developed a microarray analysis technology for soybeans and established a soybean gene expression/metabolism database. These materials and technologies can be used not only as basic knowledge and techniques to understand the characteristics of soybeans, but also for the generation of environmental stress-tolerant crops. These can be used for breeding not only soybeans but also other crops.

So far, it was almost impossible to transfer genes into soybeans using the *Agrobacterium* method, but in this project, the Japanese and the Brazilian teams have improved the transformation efficiency of Brazilian soybean cultivars by establishing a transformation method using *Agrobacterium tumefaciens*. This is useful not only for introducing stress-tolerant genes, but also for introducing other useful genes into soybean. Improved transformation methods allow for the development of soybean varieties with improved and multiple beneficial properties.

The generated *AREB1*-expressing GM soybean line showed improved drought tolerance in fields in Brazil. For practical use, the transgene should be introduced into practical varieties by backcrossing and testing in various environments. When good results of GM lines are obtained in the multi-location test, a safety evaluation test is required. Developed drought-tolerant soybeans will not only stabilize and expand soybean production in Brazil, but also stabilize the soybeans in other countries, including South America (Argentina and Paraguay) and Africa, which have similar problems.

In addition, we have applied this technique to generate GM sugarcane plants that express the *DREB2A CA* gene and found that they also show good performance under greenhouse (Reis et al. 2014) and field conditions (de Souza et al. 2019) (see **Chapter 3-3**). By utilizing these GM sugarcanes, stable production of food and bioenergy is expected.

Acknowledgment

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