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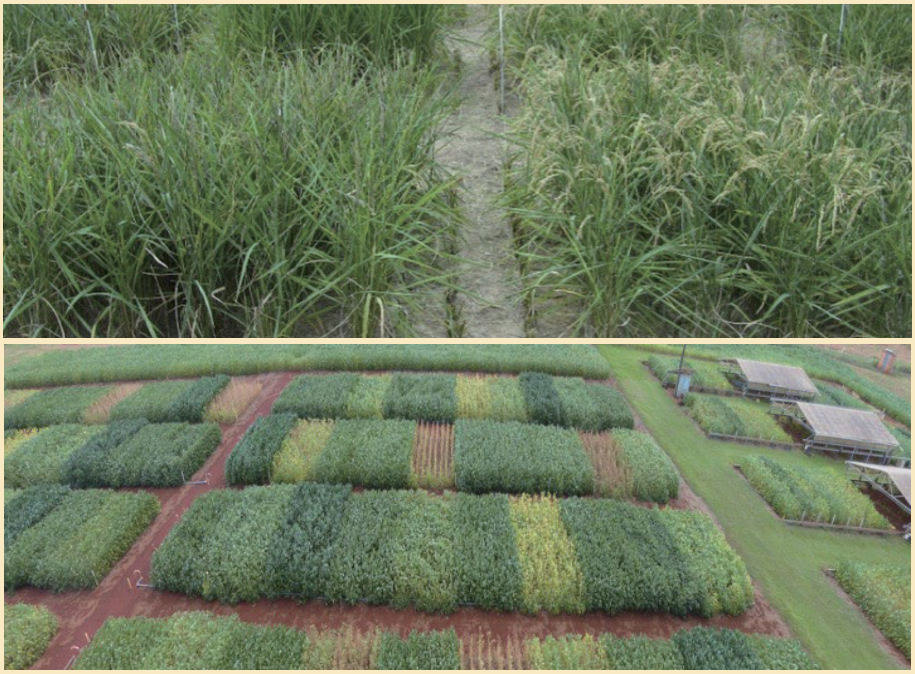
Development of biotechnologies and biotech crops for stable food production under adverse environments and changing climate conditions



Edited by NAKASHIMA Kazuo and URAO Takeshi

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Preface

Global strains on the food supply due to the ever increasing world population, chronic malnutrition in developing countries, projected economic growth in emerging countries, and the growing frequency of extreme weather events have become major concerns for mankind. According to the United Nations, the world population is projected to increase from 7.7 billion in 2019 to 9.7 billion in 2050, with the population in sub-Saharan Africa (excluding South Africa) doubling by 2050. In addition, over 800 million people, one-ninth of the population of the world, are reported to be undernourished, particularly in sub-Saharan Africa. Furthermore, in developing regions, large agricultural land areas are under adverse environmental conditions, such as insufficient fertilization and soil aridity, and are vulnerable to the adverse effects of climate change. Climate change has led to frequent droughts and other extreme weather events around the world that have threatened agricultural production. Goal 2 of the17 Sustainable Development Goals (SDGs) of the United Nations aims to end hunger, achieve food security, improve nutrition, and promote sustainable agriculture. Therefore, it is necessary to promote sustainable agricultural production activities in developing countries that have not yet reached their agricultural production potential.

In order to establish a stable and sustainable production of agricultural crops in vulnerable developing countries, the Japan International Research Center for Agricultural Sciences (JIRCAS) has been working on the development of breeding materials and basic breeding technologies for highly productive crops that are able to adapt to adverse environmental conditions through collaborative research efforts among international research centers and local institutions in developing regions. Biotechnology, especially genetic modification (GM), is expected to lead to the development of crops with increased tolerance to adverse environmental conditions like drought. JIRCAS has been promoting international joint research projects to enhance the drought tolerance of crops, such as rice, wheat, and soybeans. Throughout our projects in particular, we have shown that the overexpression of genes that encode stress-related transcription factors and enzymes may improve the drought tolerance of transgenic crops.

In this working report, we have summarized the results of our international collaborative efforts to develop genetically modified crops with enhanced drought tolerance. Chapter 1 presents the foundational research results regarding the mechanisms of environmental stress tolerance in plants that we have generated with RIKEN and the University of Tokyo in Japan as well as the applied research results of the development of drought-tolerant crops that we have generated with various local institutions and the research centers affiliated with the Consultative Group on International Agricultural Research (CGIAR). Chapter 2 introduces our international joint research efforts for the development of drought-tolerant rice and wheat with the support of the Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan. This chapter also includes two new papers from Japan and Colombia, respectively. Chapter 3 introduces our research on the development of drought-tolerant soybeans, which was implemented as a project of the Science and Technology Research Partnership for Sustainable Development (SATREPS) with the support of the Japan Science and Technology Agency (JST) and the Japan International Cooperation Agency (JICA) in Brazil. The last report of Chapter 3 presents the research on the development of drought-tolerant sugarcane in connection with the project. We have also included a presentation in the Appendix that provides an overview

of the GM research to date, which was imparted at the International Soybean Conference 2018 in Brazil. We hope that the biotechnological breeding technologies and materials produced may contribute to improved food and nutrition security in developing regions.

Finally, on behalf of JIRCAS, we would like to express our gratitude and appreciation to the Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan, the Japan Science and Technology Agency (JST), and the Japan International Cooperation Agency (JICA). We thank various local institutions, such as the Brazilian Agricultural Research Corporation (Embrapa) and the research centers affiliated with the Consultative Group on International Agricultural Research (CGIAR), such as the International Rice Research Institute (IRRI) in the Philippines, the International Tropical Agriculture Center (CIAT) in Colombia, and the International Maize and Wheat Improvement Center (CIMMYT) in Mexico, for their considerable support of our research activities. Last but not least, we thank all those who contributed to the papers in this working report.

NAKASHIMA Kazuo
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URAO Takeshi
Director, Biological Resources and Post-harvest Division, JIRCAS

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Chapter 1

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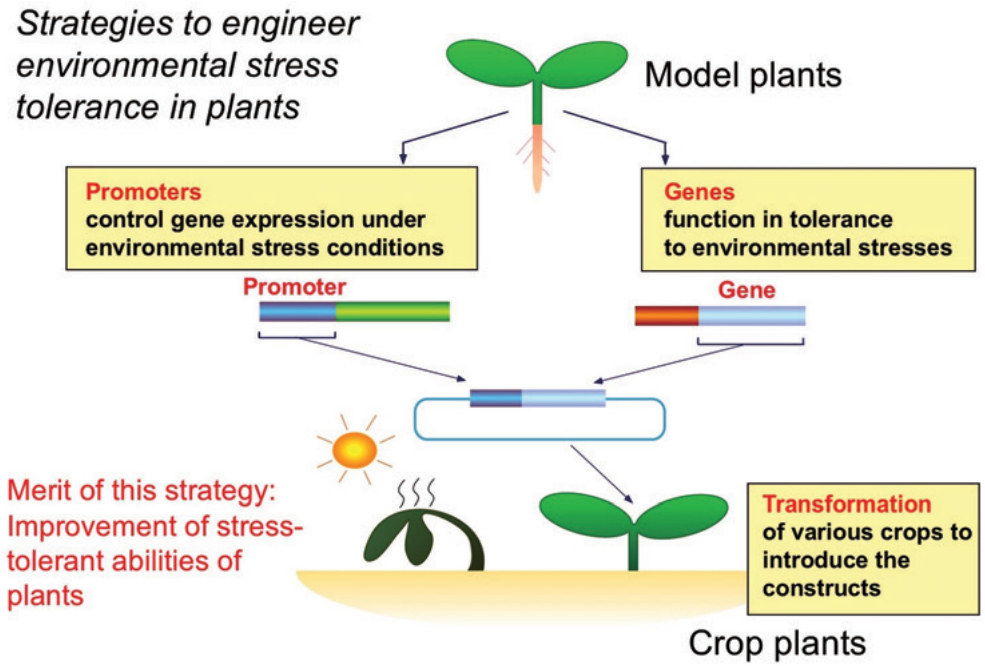

Strategy for improving the environmental stress tolerance of plants using genetic modification.

Chapter 1-1

Development of biotechnologies and biotech crops for stable food production under adverse environments and changing climate conditions

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Abstract

Developing regions, such as sub-Saharan Africa, are rapidly growing in population. In sub-Saharan Africa, 215 million people are currently malnourished. In these areas, the poor environmental conditions for agricultural production and the vulnerability to climate change make agricultural productivity low. Hence, there is a need to develop crops with improved tolerance to adverse environments and climate change. To ensure food and nutritional security, we have been working on improving crops such as rice and soybean using marker-assisted selection and biotechnology. Biotechnology, especially genetic modification, is being used to develop crops that have increased tolerance to adverse environmental conditions such as drought. We have promoted international collaborative research projects to enhance the drought tolerance of globally important crops such as rice, wheat, and soybean. Throughout the project, we have shown that overexpression of genes encoding key stress-related transcription factors and enzymes improves drought tolerance in transgenic crops such as rice and soybean. We hope that biotechnology-based agricultural research efforts and biotechnology crops can contribute to the security of food and nutrition in developing regions and globally.

1. Introduction

Food insecurity is widespread in Africa and Asia. Currently, 215 million people are malnourished in sub-Saharan Africa. Goal 2 of the United Nations' 17 Sustainable Development Goals (SDGs) aims to end hunger, achieve food security and improved nutrition, and promote sustainable agriculture (United Nations Information Centre 2018). The full potential of agriculture in developing regions, including sub-Saharan Africa, has not been realized due to adverse environmental and climatic conditions that impose environmental stress (abiotic stress) such as reduced soil fertility, and drought, and biological stress such as pests, and diseases. Haefele et al. (2014) assessed soil quality in all rice producing areas around the globe. Globally, one-third of the total rice is grown on very poor soils. Rice production in good soils is largest in Asia (47%)

while it is less common in the Americas (28%) and accounts for only 18% in Africa. They report that the most common problems pertaining to soil chemistry in rice fields are: very low inherent nutrient status (35.8 million ha), very low pH (27.1 million ha), and high P fixation (8.1 million ha); widespread physical problems of the soil, especially severe in rainfed environments are: very shallow soils and low water-holding capacity. In recent years, droughts have frequently occurred around the world, causing serious damage to agricultural production. The National Agriculture and Food Research Organization (NARO), Japan has published the geographical distribution of grain production damage in the world due to drought (Kim et al. 2019). Analysis of precipitation and grain yield data of the last 27 years (1983-2009) showed that three quarters (450 million ha) of the world's major crop (corn, rice, soybean, wheat) cultivation area had been damaged by drought, and the total grain production damage estimated from this analysis and the country's producer price (2005) was about $166 billion. In the past 27 years (1983-2009), the cultivation area of cereals that has been damaged by at least one drought is 161 million ha (75% of the world's harvested area). About 124 million ha of corn (82%), 102 million ha of rice (62%), and 67 million ha of soybeans (91%) have been damaged. The average yield loss from a single drought in 27 years was 8% for wheat (0.29 tons per hectare), 7% for corn (0.24 tons), 3% for rice (0.13 tons), and 7% for soybean (0.15 tons).

The Stable Agricultural Production Program in Japan International Research Center for Agricultural Sciences (JIRCAS) aims to improve agricultural productivity, stability, and nutrition in developing countries by utilizing stable production technology for agricultural products in adverse environments such as the tropics. We have developed technologies and crops that are highly productive and adaptable to changing adverse environmental and climatic conditions to ensure food and nutrition security in developing regions such as sub-Saharan Africa. To date, we have developed crops such as rice and soybeans that have improved resistance to adverse environmental conditions, such as high temperature, high salt, and diseases, by using marker-assisted selection (MAS). Biotechnology, especially genetically modified (GM) technology, is expected to develop GM or biotechnology crops that have increased tolerance to adverse environmental conditions, including drought. We promoted international collaborative research projects to develop drought-tolerant crops such as rice, wheat, and soybean. This chapter introduces an outline of the research on biotechnology and the development of biotechnology crops for stable food production under adverse conditions and climate change.

2. Development of biotechnology to improve drought tolerance in crops

The frequency and severity of drought in the world have increased in the recent years, and the resulting agricultural damage has been more severe than ever. Many small-scale farmers in the developing world use rain-fed cultivation for rice and other crops, however, it is affected more by drought than irrigation

cultivation. Such rainfed areas are closely linked to poor areas. Thus, drought has a major impact on social issues in developing countries. We conducted research using biotechnology to identify candidate genes to improve the drought tolerance of crops. To identify these genes, we investigated the molecular mechanisms involved in the environmental stress response in rice and Arabidopsis (*Arabidopsis thaliana*) as model plants. The results showed that many factors in gene expression were induced and/or activated for the role of stress response and tolerance in controlling stress perception, signal transduction, and drought tolerance in plants (**Fig. 1**).

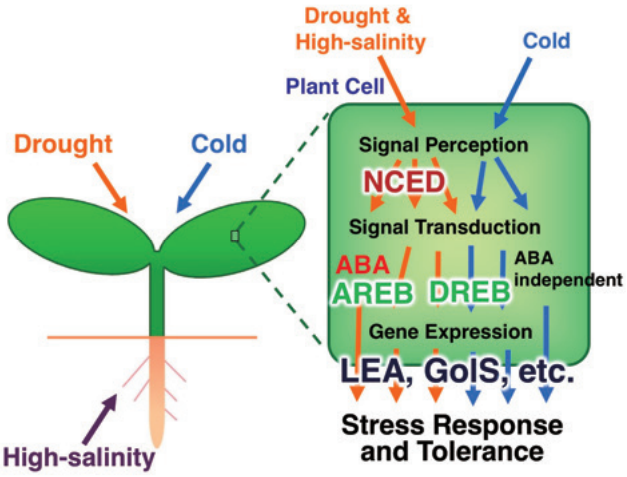

Fig. 1. Outline of the molecular network in environmental stress response and tolerance in plants

Abscisic acid (ABA) is an important plant hormone that regulates gene expression and physiological responses, including stomatal closure under environmental stresses such as drought. The *NCED3* gene encodes a key enzyme, 9-cis-epoxycarotenoid dioxygenase (NCED) 3 in the ABA biosynthetic pathway (**Fig. 1**), the expression of which is strongly induced by water-deficient stress in many kinds of plants, including Arabidopsis (Iuchi et al. 2001).

Stress-inducible transcription factors (TFs), such as the dehydration-response element (DRE)-binding protein (DREB) and the ABA-response element (ABRE)-binding factor (AREB), play important roles in regulating the stress response (**Fig. 1** to **3**; reviewed by Nakashima et al. 2014). Under environmental stress conditions, plants perceive a stress signal and transmit it to transcription factors such as DREB (**Fig. 2**). DREB TF is an important element that binds to the DRE *cis*-element of many kinds of environmental stress-responsive promoters and can switch on the expression (transcription) of stress-responsive genes. AREB can switch on gene expression in the ABA response. We showed that many types of transcription factors, including DREB and AREB, regulate different types of stress-inducible promoters through specific binding to *cis*-elements such as DRE and ABRE for plant stress response and tolerance (**Fig. 3**).

TFs regulate the expression of target genes encoding important metabolic proteins that protect cells

from dehydration, including late embryogenesis abundant (LEA) proteins, proteases, chaperones, water channel proteins, and enzymes for the synthesis of osmoprotectants (compatible solutes) such as sugars and proline. The osmoprotectants include galactinol synthase (GolS; **Fig. 1**), which is the key enzyme in the production of raffinose family oligosaccharides (RFOs). RFOs are thought to influence drought tolerance by regulating osmotic potential and protecting enzymes and membranes during exposure to environmental stresses. *GolS* genes are upregulated by abiotic stresses in many kinds of plants such as Arabidopsis (Taji et al. 2002).

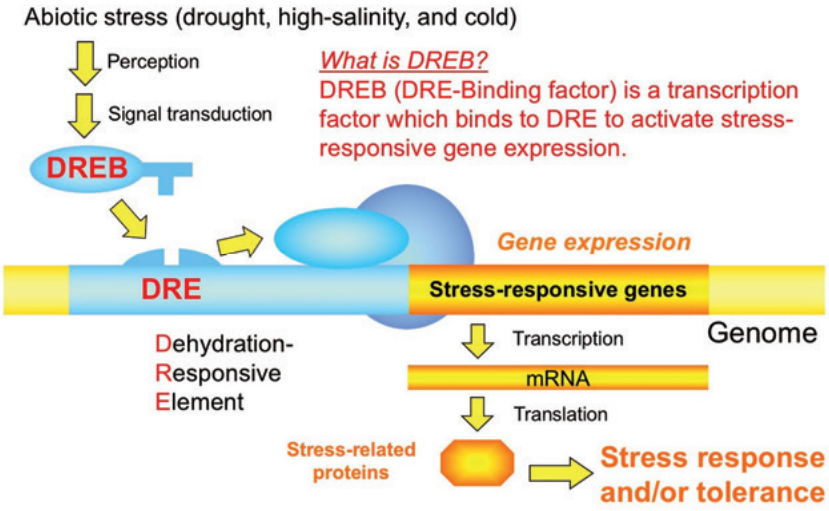

Fig. 2. Dehydration-response element-binding protein (DREB) plays an important role in regulating stress response and tolerance in plants

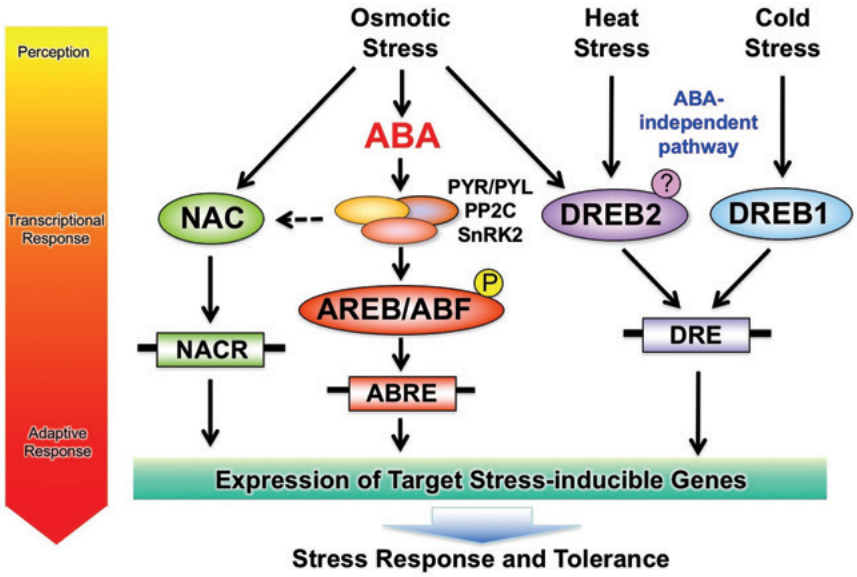

Fig. 3. Major transcriptional networks under environmental stress conditions revealed by our molecular biology studies in plants.

Environmental stresses such as osmotic stress, heat stress, and cold stress result in the expression and activation of transcription factors. The transcription factor binds to the specific *cis*-element in the promoter region of the target gene and induces the expression of the gene. Gene products function in stress response and tolerance. Ellipses indicate transcription factors. Boxes indicate *cis* elements. Details are provided in the text.

Overexpression of genes that are key to environmental stress response has been shown in greenhouse experiments to enhance stress tolerance in rice and Arabidopsis (reviewed in Nakashima et al. 2014). For example, overexpression of the *DREB1* gene increased the tolerance of Arabidopsis to drought, high salinity, and low temperature (**Fig. 4** to **6**; Liu et al. 1998; Kasuga et al. 1999). However, constitutive overexpression of *DREB1A* using the cauliflower mosaic virus *35S* promoter induced growth defects. A stress-responsive promoter such as *Arabidopsis RD29A* has been able to avoid delayed growth by *DREB1A* expression (Kasuga et al. 1999). We have worked with various research institutions to determine whether such genes can improve stress tolerance in other crops in the field (reviewed in Nakashima et al. 2014).

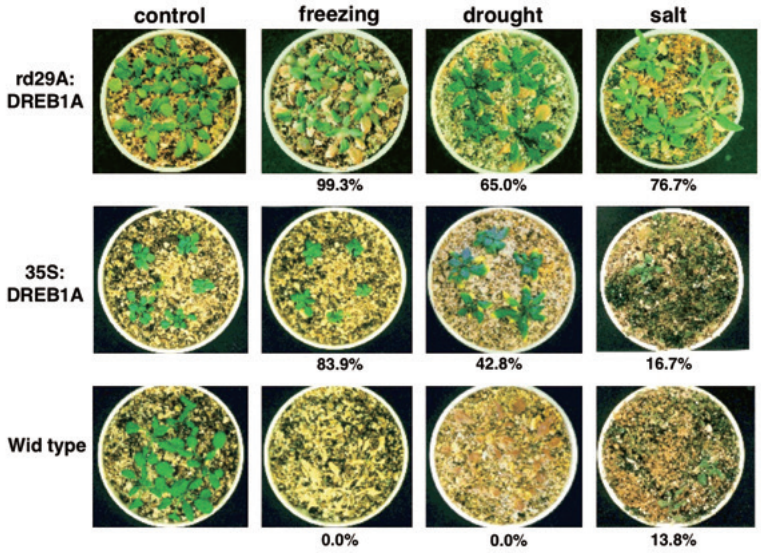

Fig. 4. Constitutive and stress-induced overexpression of *DREB1* in Arabidopsis improves tolerance to multiple stresses and alters plant growth.
Overexpression of the *DREB1* gene increased tolerance to drought, high salinity, and low temperature in Arabidopsis. However, constitutive overexpression of *DREB1A* using the cauliflower mosaic virus *35S* promoter induced growth defects. A stress-responsive promoter such as *Arabidopsis RD29A* allows the avoidance of growth defects caused by *DREB1A* expression. The figure was adapted from Kasuga et al. (1999).

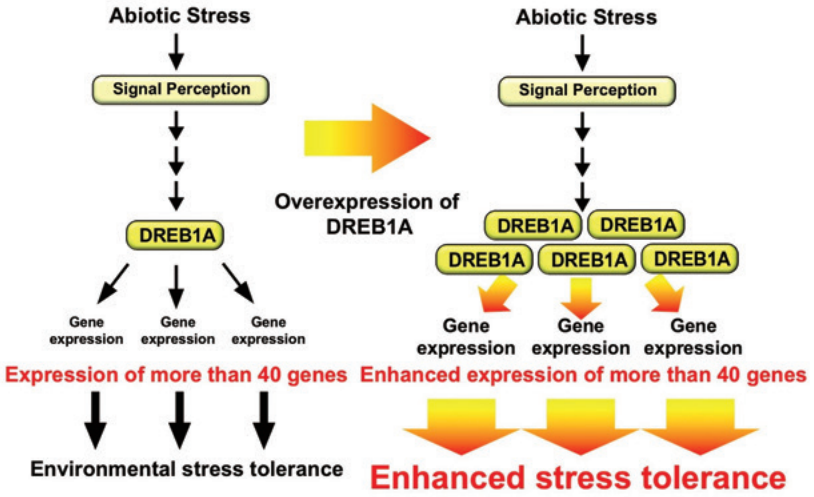

Fig. 5. Overexpression of DREB1A, a transcription factor that is key in controlling environmental stress in Arabidopsis, can enhance plant stress tolerance.

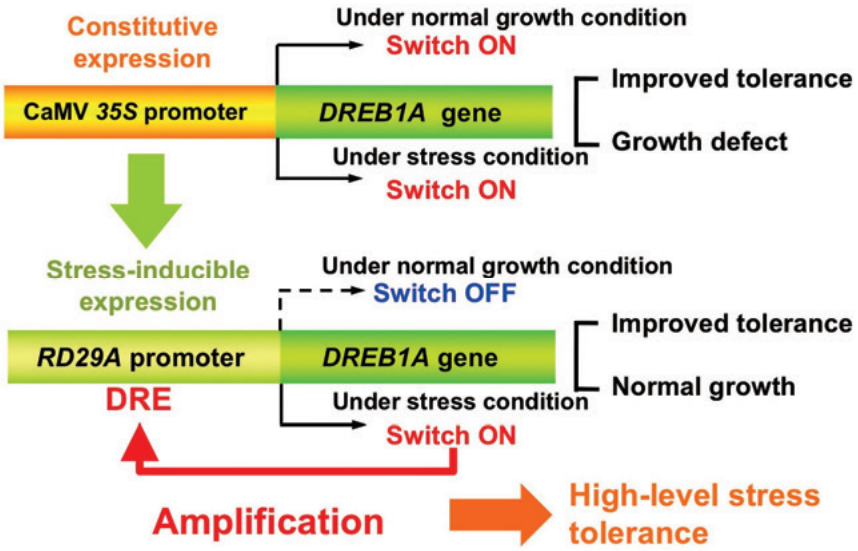

Fig. 6. *DREB1A* expression using a stress-responsive promoter, such as *Arabidopsis RD29A,* not only enhances stress tolerance but also avoids growth defects.
Constitutive overexpression of *DREB1A* enhances stress tolerance but causes plant growth defects. However, by using a stress-responsive promoter, *RD29A,* to induce high levels of *DREB1* specifically during stress conditions, growth defects can be avoided while enhancing stress tolerance. The *DREB1A* transcription factor, which is produced in large amounts during stress conditions, can exhibit a very high level of stress tolerance by amplifying gene expression through binding of the *cis*-element DRE of the *RD29A* promoter.

3. International collaboration to improve crop drought tolerance using three genetic modifications

Based on the findings presented in the previous section, we proposed a strategy to use genetic modification to improve environmental stress tolerance in plants (**Fig. 7**). There are three points to the strategy:

(1) Promoter: an expression regulatory region upstream of a plant gene that can improve gene expression under environmental stress conditions.

(2) Gene: a plant gene that enhances tolerance to environmental stress by increasing its expression.

(3) Transformation: A transformation technique for introducing a construct by combining the appropriate promoter (1) and gene (2).

Transformation methods vary by crop and variety, and hence there is a need to work with other organizations that adopt crop transformation techniques. The developed GM crop contains plant-derived DNA fragments and should be acceptable to consumers.

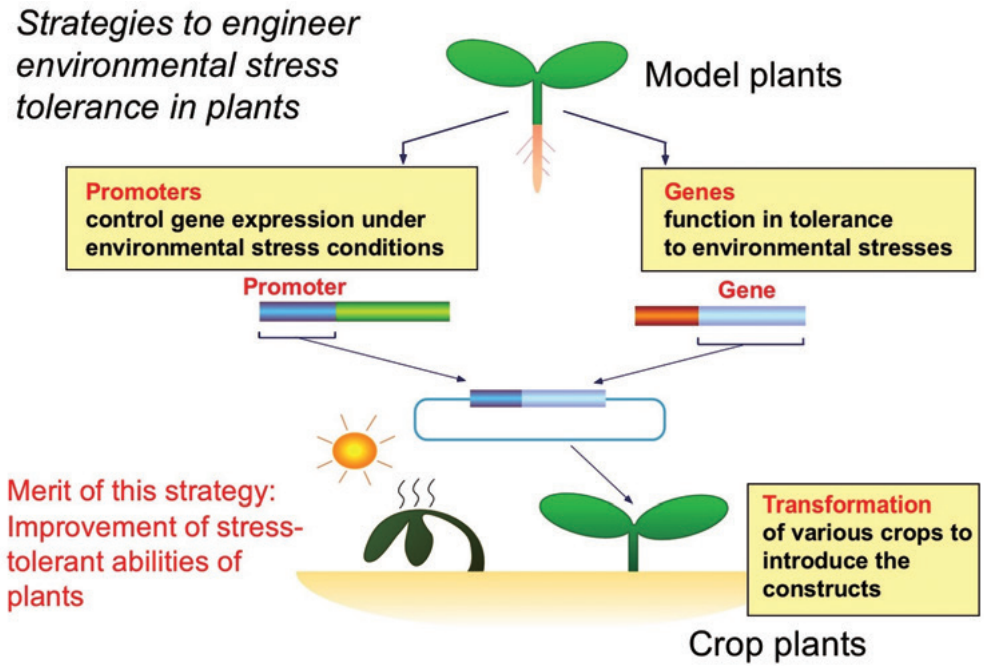

Fig. 7. Strategy for improving the environmental stress tolerance of plants using genetic modification.

Our research team has worked with research centers affiliated with the Consultative Group for International Agricultural Research (CGIAR) and national research centers in developing countries to improve the drought tolerance of some kinds of crops (**Fig. 8**; reviewed in Nakashima et al. 2014). These joint projects involve researchers from different countries specializing in diverse fields such as molecular biology, plant breeding, plant physiology, crop science, ecophysiology, and meteorology.

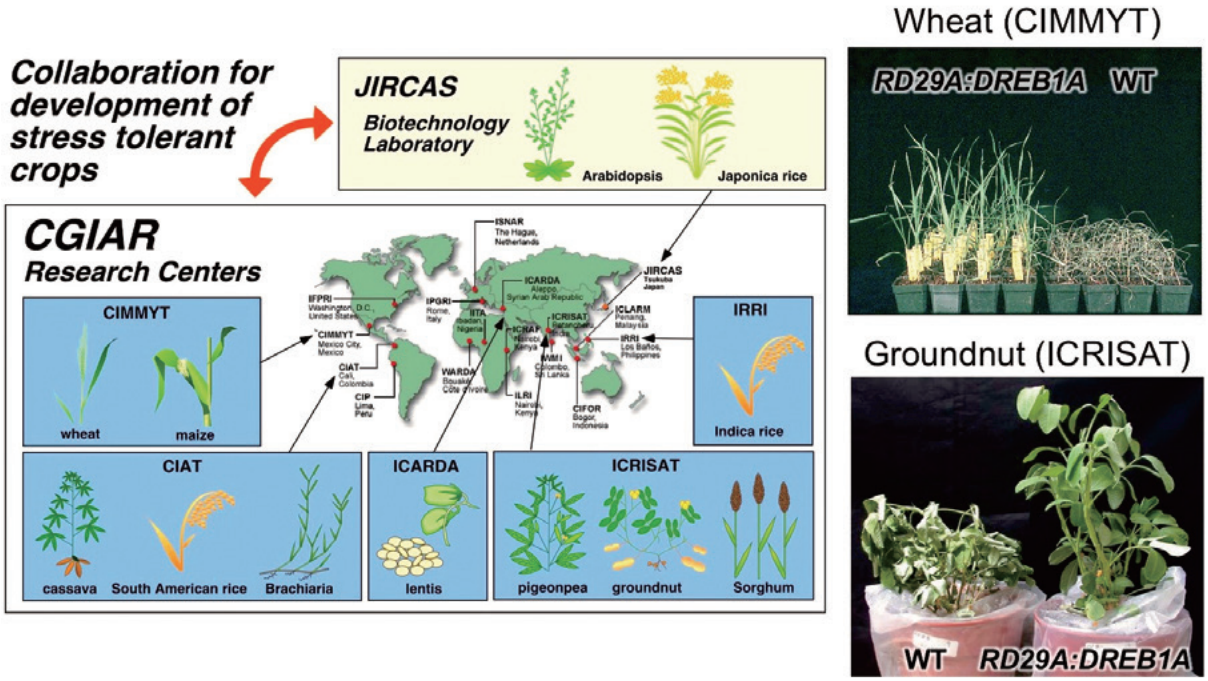

Fig. 8. International joint research with the CGIAR Research Center for the development of stress-tolerant crops.

3-1. Rice and wheat

Transgenic rice (Nipponbare) overexpressing *DREB1A* showed improved drought tolerance in greenhouses (**Fig. 9**; Ito et al. 2006). Stress-induced expression of the *DREB1A* gene in wheat delayed water stress symptoms in greenhouses (Pellegrineschi et al. 2004). Based on these results, we conducted a joint research project titled “Development of abiotic stress tolerant crops by DREB genes” (DREB Project, **Fig. 10**) supported by the Ministry of Agriculture, Forestry and Fisheries (MAFF), Japan in 2007, for a period of five years (see **Chapter 2**). This project was carried out in collaboration with research centers affiliated with the CGIAR, such as the International Rice Research Institute (IRRI) in the Philippines, the International Tropical Agriculture Center (CIAT) in Colombia, and the International Maize and Wheat Improvement Center (CIMMYT) in Mexico (Gaudin et al. 2013, Pellegrineschi et al. 2004, Saint Pierre et al. 2012). The gene was introduced into lowland rice, upland rice, and wheat, and drought tolerance was evaluated in the field. In this project, Japanese research institutes, JIRCAS and RIKEN (The Institute of Physical and Chemical Research) produced 32 combinations of constructs using 5 promoters and 14 resistance genes, and sent them to IRRI, CIAT, and CIMMYT. Approximately 350,000 calli or embryos generated more than 1,100 independent transformation events. Grain yields of the transformants under drought conditions were investigated through tests performed in greenhouses, rainout shelters, and confined fields. From the evaluation, approximately 40 elite candidate transformants were selected. A project supported by the MAFF called "Development of Drought Tolerant Crops for Developing Countries (GM Drought Tolerance Project)" was started in 2013 for five years, and the performance of these candidate lines under drought conditions was verified. The second phase of the project, aimed at developing at least 10 elite lines from the candidates selected in the DREB project, has successfully identified several promising strains. For instance, we showed that overexpression of an *Arabidopsis thaliana* galactinol synthase gene improves drought tolerance in transgenic rice and increases grain yield in the field (Selvaraj et al. 2017). Recently, we reported that the expression of the CCCH-tandem zinc finger protein gene *OsTZF5* under a stress-inducible promoter mitigates the effect of drought stress on rice grain yield under field conditions (Selvaraj et al. 2020). In this work report, we report that the expression of the *OsNAC6* transcription factor gene under a stress-inducible promoter also mitigates the effect of drought stress on rice grain yield under field conditions (see **Chapter 2-3**).

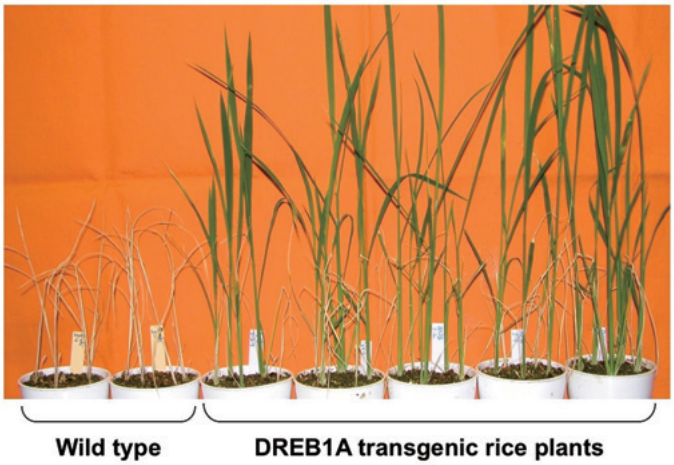

Fig. 9. Transgenic rice plants (Nipponbare) overexpressing *DREB1A* showed improved drought tolerance compared to control plants (wild type) in the greenhouse.
The figure was adapted from Ito et al. (2006).

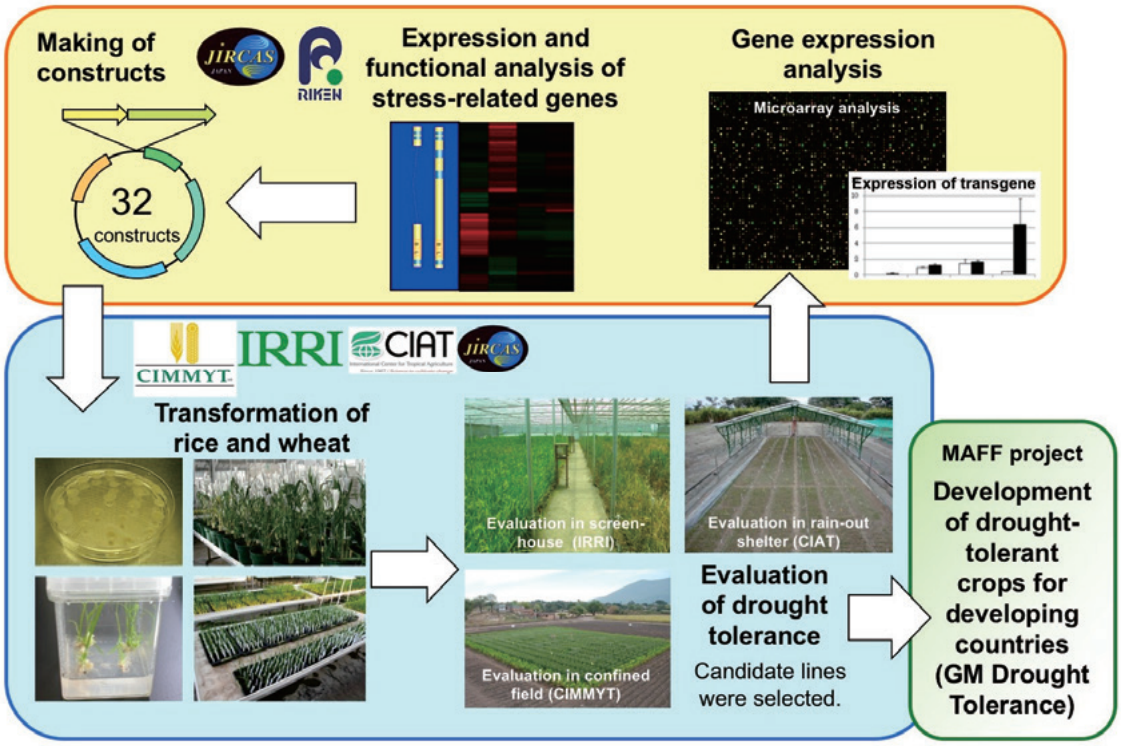

Fig. 10. Outline of the projects "Development of stress tolerant crops utilizing DREB genes" and "Development of drought-tolerant crops for developing countries" supported by the Ministry of Agriculture, Forestry and Fisheries (MAFF), Japan.
The figure was adapted from Nakashima and Suenaga (2017).

3-2. Soybean

We showed that transgenic soybean plants expressing *RD29A:DREB1A* improved drought tolerance under greenhouse conditions (**Fig. 11**; Polizel et al. 2011). We introduced stress-tolerant genes into soybean and then evaluated drought tolerance in greenhouses and confined fields in collaboration with RIKEN, the University of Tokyo, and Embrapa (Brazilian Corporation of Agricultural Research) in the Science and Technology Research Partnership for Sustainable Development (SATREPS) project supported by the Japan Science and Technology Agency (JST) and Japan International Cooperation Agency (JICA) since 2009 (**Fig. 12**; see **Chapter 3-2**). Soybeans expressing stress-related genes such as *DREB* or *AREB* were generated to examine drought tolerance under greenhouse conditions and field trials (Barbosa et al. 2012; Engels et al. 2013; Fuganti-Pagliarini et al. 2017; Leite et al. 2014; Marinho et al. 2016; Polizel et al. 2011; Rolla et al. 2014). Researchers from Japan have found important genes involved in stress response and tolerance, such as soybean DREB and AREB transcription factors, and stress-inducible promoters (Nakashima et al. 2014; see **Chapter 3-1**). Metabolite/phytohormone–gene regulatory networks in soybean organs under dehydration conditions were revealed by integration analysis (Maruyama et al. 2020). For soybean, it has been difficult to produce transformants because of the very low transformation efficiency. However, by establishing a transformation method using *Agrobacterium*, we succeeded in improving the transformation efficiency of Brazilian soybean varieties. When using the reporter β-glucuronidase gene for transformation, the transformation efficiency by the established method was 1.74%. This highly efficient method has enabled the production of transgenic soybeans at a practical level. We developed 37 different transgenic lines using the particle gun or *Agrobacterium* methods. Subsequently, in greenhouses and confined fields, we evaluated their drought tolerance. During the SATREPS period, 7 of 11 lines evaluated in the greenhouse and 1 of 4 lines evaluated in the field were tolerant to drought conditions. Under water deficit conditions in the field, a better performance was observed in the *1Ea2939 AREB* line, which showed a higher performance than the wild type and other GM lines (Fuganti-Pagliarini et al. 2017). Experiments after the SATREPS project revealed that more lines were tolerant to drought (see **Chapter 3-2**). Therefore, we can expect to produce higher yields of transgenic soybean varieties under drought conditions in the future. Considering the current situation where GM soybeans are used in more than 90% of Brazil's soybean producing regions (Rally da Safra 2016) and 80% of the world's total soybean producing regions (ISAAA 2016), drought-tolerant GM soybean varieties are expected to be used not only in Brazil but also around the world.

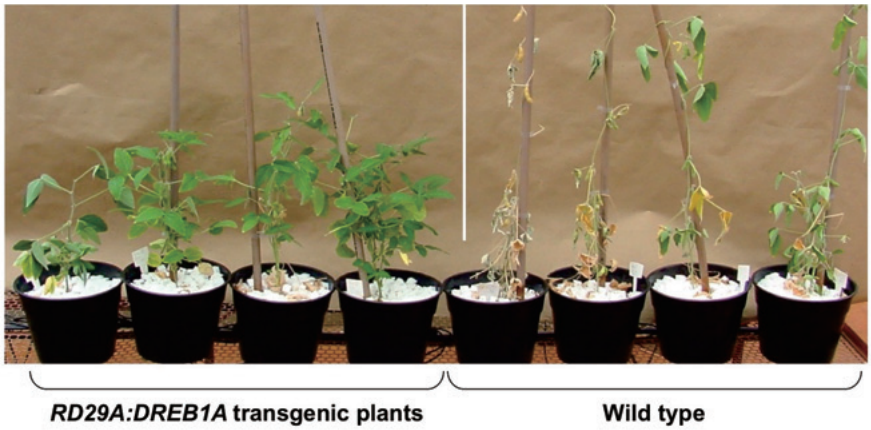

Fig. 11. Transgenic soybeans expressing *RD29A:DREB1A* showed improved drought tolerance compared to untransformed soybeans (Wild type) in a drought tolerance test performed in a greenhouse in Brazil.

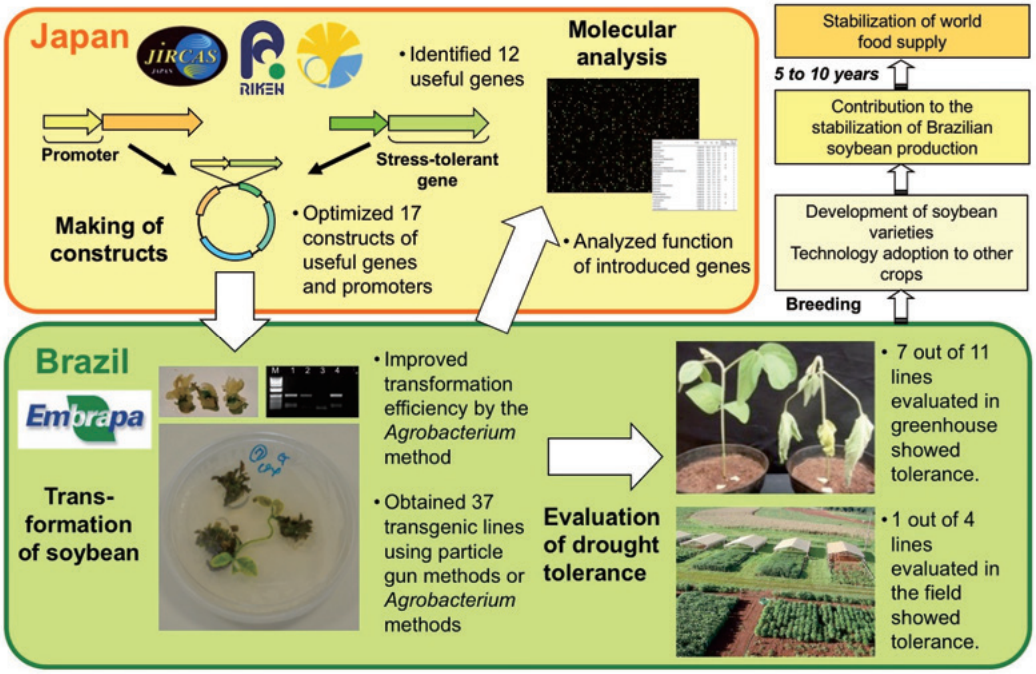

Fig. 12. To generate drought-tolerant soybean lines in Brazil, the SATREPS project for "Development of genetic engineering technology of crops with stress tolerance against degradation of global environment" has been implemented through international collaboration between Japan and Brazil.

3-3. Other crops

In collaboration with the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) in India, transgenic peanuts expressing *DREB1* have been developed (Bhatnagar-Mathur et al. 2007). *DREB1*-expressing peanut lines showed drought tolerance under greenhouse conditions, and their drought tolerance was confirmed by field tests (Bhatnagar-Mathur et al. 2007, 2013). Drought tests in experimental fields resulted in significant yield improvements of up to 24% (Bhatnagar-Mathur et al. 2013). The transgenic peanut lines had significantly higher seed loading values than the wild-type varieties.

In collaboration with Embrapa Agroenergy of Brazil, we introduced the *Arabidopsis DREB2Aca* gene into sugarcane (see **Chapter 3-3**). Dehydration assays using transgenic sugarcane expressing this gene under greenhouse conditions showed improved drought tolerance (Reis et al. 2014). The next study analyzed the performance of sugarcane transformants in fields under drought conditions and showed good results (de Souza et al. 2019). Sugarcane can propagate through buds and does not require genetic fixation. Therefore, commercializing genetically modified sugarcane varieties may be easier than crops that require transgene fixation.

4. Conclusion

To ensure food and nutrition safety, we have developed technologies and crops that are productive and can adapt to changing adverse environmental and climatic conditions. In order to develop stress-tolerant crops using biotechnology, we have promoted international joint research projects to develop crops such as rice, wheat, soybean, and sugarcane. Through these projects, overexpression of genes encoding stress-related transcription factors (e.g., DREB, AREB) and enzymes (e.g., galactinol synthase) of *Arabidopsis thaliana* have led to drought tolerance in GM crops such as rice, wheat, soybean, and sugarcane. There are four phases in the research and development (R&D) steps to bring GM crops to market (commercialization) after gene discovery (**Fig. 13**): Phase I, proof of concept; Phase II, early development; Phase III, advanced development; and Phase IV, pre-launch. To date, we have conducted international collaborative research projects to demonstrate proof of concept in Phase I through Phase III. We hope that the developed crops can move to the next stage of market launch and contribute to food and nutrition security in developing regions.

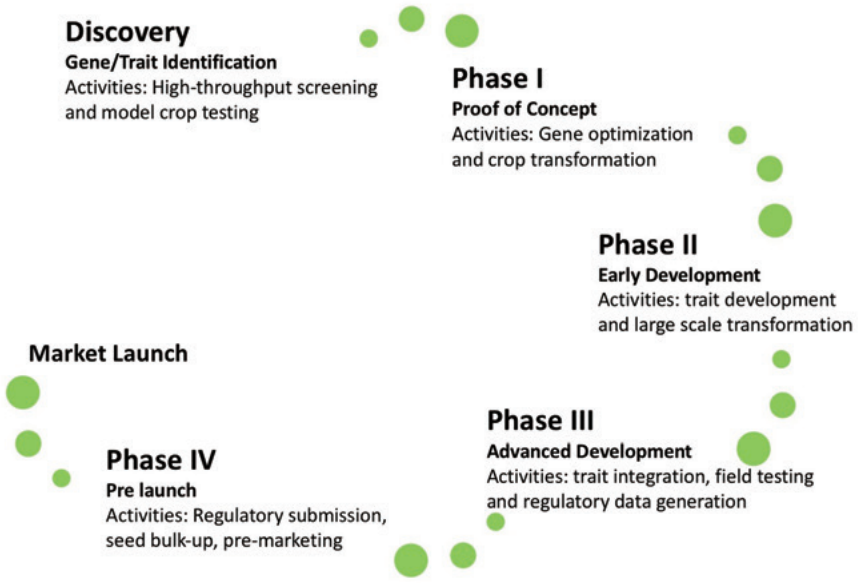

Fig. 13. Research and development steps for commercialization of GMOs.
This figure is a modified version of the original drawing of Dr. Manabu Ishitani (CIAT).

In **Chapter 2** of this work report, the achievements of an international joint research project on the development of drought-tolerant rice and wheat (the DREB Project), supported by the MAFF, Japan, will be introduced. In **Chapter 3**, the achievements of the international joint research project on the development of drought-tolerant soybean (the SATREPS Project), supported by the JST) and the JICA, and the related project on the development of drought-tolerant sugarcane will be introduced.

Acknowledgments

The international joint research project on the development of drought-tolerant rice and wheat was supported by the MAFF, Japan. The international joint research project on the development of drought-tolerant soybean was performed in the SATREPS, supported by the JST and the JICA. I thank these organizations for their support with these projects. I also express my deep appreciation to the participants of these projects. We would like to thank Editage (www.editage.com) for English language editing.

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Chapter 2

Development of drought-tolerant rice and wheat

Evaluation of transgenic rice in a confined field in CIAT, Colombia

Chapter 2-1

Outline of the Project Entitled "Development of Drought-Tolerant Crops for Developing Countries"

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Abstract

Global warming is expected to have serious effects on nature and society. The agricultural, forestry, and fishing industries have faced challenges with the increasing frequency of abnormal climate events, such as flooding and drought. These climate changes, along with soil degradation, have caused damages to crop production. In the light of these situations, in 2013, Japan International Research Center for Agricultural Sciences (JIRCAS) launched a research project on development of drought-tolerant rice and wheat for developing countries in collaboration with the International Rice Research Institute (IRRI), the International Center for Tropical Agriculture (CIAT), and the International Maize and Wheat Improvement Center (CIMMYT). We have identified 14 genes involved in the drought tolerance and three stress-inducible promoters. These genes were introduced into lowland rice variety IR64, upland rice variety Curinga and NERICA, wheat variety Fielder. In addition, in order to remove unexpected DNA fragments inserted during transformation or cultivation, we carried out a cleaning of genetic background by backcrossing with the parental variety. We also addressed to pile up drought tolerance genes or QTLs, to confer more tolerance to the promising lines. Based on physiological traits and grain yield under drought stress conditions in a greenhouse and confined field, we have so far narrowed-down following promising lines of IR64, Curinga, NERICA, and Fielder.

Outline of the project

Global food demand is expected to rise due to expanding world population in Southeast Asia and Africa, as well as chronic nutrient deficiency and rapid economic growth in emerging countries, China and India. On the other hand, food supply will decrease, with frequent abnormal weather conditions, such as water resources shortage, and soil degradation including desertification. In the medium and long term, the global food supply-demand balance will be tight, and food production will require an increase of 160% by 2050. However, we are now faced with various environmental risks, including soil and water pollution,

deforestation, and soil degradation, as well as climate change risks, such as drought, high temperature, and flooding. Climate change is expected to have serious effects on nature and society around the world. The increasing threat of climate change is manifested by extreme weather events such as high temperature, drought and heavy rainfall, which are becoming more frequent. Although climate change has had a positive effect on crop production in temperate regions. On the other hand, in tropical and subtropical regions including South-East Asia and Africa, the crop production is estimated to be reduced by 5% to 50%, even though there is a need for increase in food production (**Fig. 1**).

In the field of agriculture, forestry, and fisheries, it is forecasted to pose detrimental effects on the production of agricultural products in the world, particularly in developing countries, which are the most vulnerable to climate change.

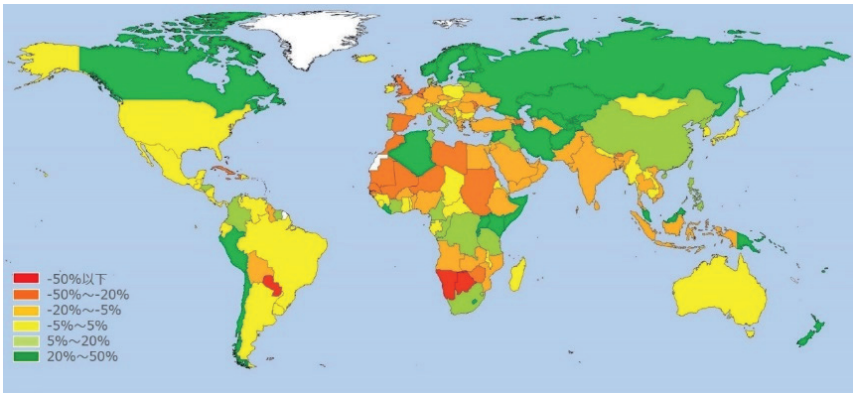

Fig. 1. Potential Effects of Climate Change on Rain-fed Cereal Production (1990 – 2050).
The data are cited from Global Agro-ecological Assessment for Agriculture in the 21st Century: Methodology and Results, FAO, 2002.

In the light of these situations, in 2013, the Ministry of Agriculture, Forestry and Fisheries in Japan established a research program on the development of adaptation and mitigation technology in response to climate change, in order to achieve a sustainable food production system that could adapt to the progressing global warming. Under this program, JIRCAS implemented a research project on the development of drought-tolerant crops for developing countries (GM Drought Tolerance Project), in collaboration with member institutes of the Consultative Group on International Agricultural Research (CGIAR).

We had previously implemented a research project on development of abiotic stress tolerant crops by DREB gene (DREB Project) in 2008. In this project, we had identified crucial genes involved in drought stress tolerance, including *Arabidopsis* transcription factor DREB1; rice putative RNA binding protein OsSCZF2 (TZF5); *Arabidopsis* AtGolS2, which is a key enzyme in galactinol biosynthesis; *Arabidopsis* ABA responsive transcription factor AREB. We had also isolated three stress inducible promoters, such as rice *Oshox 24*, *Osnac 6*, and *lip 9* promoter. Then, we introduced these genes into lowland rice variety IR64,

upland rice variety Curinga and NERICA, wheat variety Fielder. Based on physiological traits and grain yield under drought stress conditions in a greenhouse and confined field, we had selected 7 events of IR64, 16 events of Curinga, 4 events of NERICA, and 22 events of Fielder (Nakashima and Suenaga 2017).

With the help of the results obtained from previous study, we launched a research project 5 years ago in 2013. This project is aimed at developing 10 drought tolerant rice and wheat lines for developing countries, in collaboration with IRRI, CIAT, and CIMMYT. IRRI, CIAT, and CIMMYT mainly conducted field evaluation of the promising lines of lowland rice, upland rice, NERICA, and wheat, and selected 2 or 3 elite lines from each variety. On the other hand, JIRCAS carried out molecular evaluation of the promising lines selected by IRRI, CIAT, and CIMMYT (**Fig. 2**).

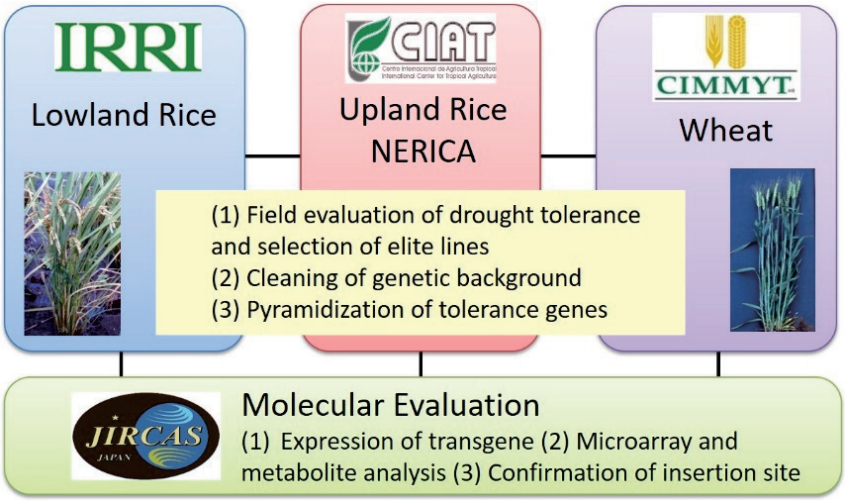

Fig. 2. Framework of GM Drought Tolerance Project.

IRRI, CIAT, and CIMMYT had 3 major research activities: (1) verification of drought tolerance by confined field trials, (2) cleaning of genetic background of the promising lines, (3) pyramiding of tolerance genes (QTLs). Each institute carried out confined large-scale field trials and investigated grain yield. They also evaluated physiological traits such as stomata conductance, harvest index, and water uptake rate, as criteria for drought tolerance. The promising lines were further evaluated in the field to verify the effects of the transgenes so that we could narrow down the number of promising lines, leaving only elite lines. A series of 3 to 4 confined field trials were performed with plot sizes larger than those in the DREB Project (**Fig. 3**).

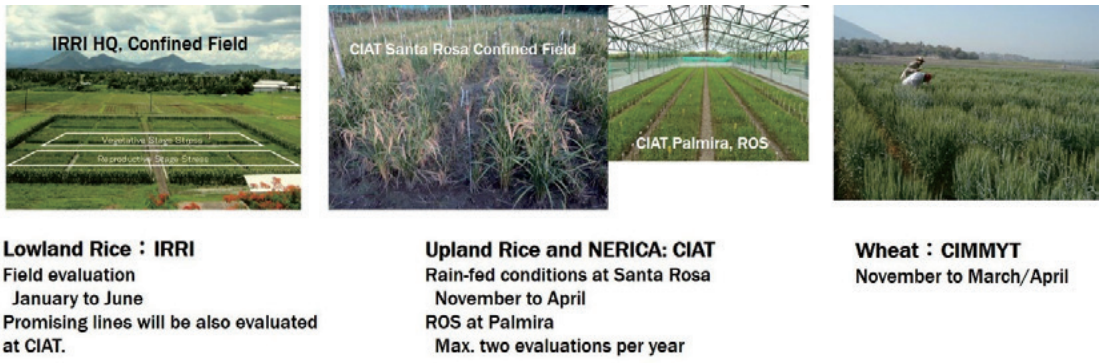

Fig. 3. Field evaluation of drought tolerance and selection of elite lines.

The 2nd activity was cleaning genetic background. Fragments of transgenes may be introduced upon transformation and remain in promising lines, which would have potentially interfered with future safety assessments. Therefore, genetic background had to be replaced to create adequate breeding material. We conducted a minimum of 3 backcross breedings, using commercial varieties, IR64, Curinga, NERICA, and Reedling as recurrent parents. Consequently, undesirable DNA fragments derived from the transformation vectors, which were at risk for being incorporated by transformation, were removed. The 3rd activity was stacking of genes for drought tolerance in candidate elite lines to confer higher drought tolerance. Major activities of JIRCAS were (1) analysis of expression of the transgenes in the promising and backcrossed lines, (2) transcriptome and metabolome analysis, (3) confirmation of flanking sequences of the insertion site in transgenic lines. JIRCAS also evaluated agronomic traits of transgenic NERICA lines in a greenhouse setting. Based on physiological traits and grain yield under drought stress conditions in a greenhouse and confined field, we were able to narrow the crop selection to 3–4 promising lines of IR64, Curinga, NERICA, and Fielder.

Recently we have succeeded in developing transgenic rice lines that overexpress *AtGolS2*, which is a candidate gene for drought tolerance encoding a galactinol synthase identified in *Arabidopsis* and presented increased grain yield in transgenic rice under drought in the field (Selvaraj et al. 2017). We generated transgenic rice lines that express *AtGolS2* in two varieties, Curinga and NERICA4. Curinga is a Brazilian local upland rice variety, and NERICA4 is a popular upland rice variety in African countries. Each transgenic line accumulated significantly higher amounts of galactinol as compared to that in non-transgenic rice plant (**Fig. 4**). The transgenic lines grown under drought had higher relative water content in leaves and higher photosynthetic activity than non-transgenic plants, leading to lesser reduction in plant growth. In order to test the performance of the transgenic lines under drought in the field, three consecutive field trials were carried out. The extent of drought varied among trial years. For instance, trial years 2012–2013 and 2013–2014 were very dry with continuous rain-free days (31 days and 39 days, respectively), including flowering periods. However, there were only 19 rain-free days after flowering in trial year 2014–2015. A transgenic Curinga

line (numbered 2580) and a transgenic NERICA4 line (numbered 1577) consistently had higher grain yield than each non-transgenic variety (**Fig. 5**). These results provide a strong evidence that *AtGolS2* is a useful biotechnological tool to reduce grain yield losses in rice under drought in the field.

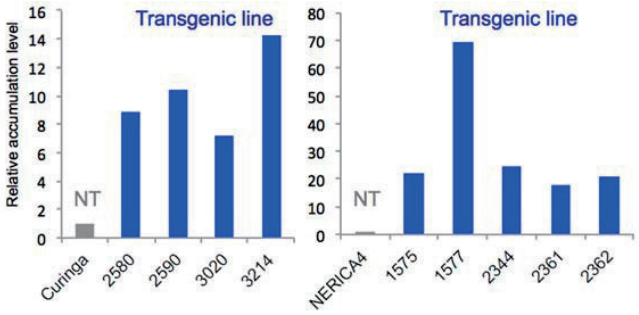

Fig. 4. Accumulation of galactinol in transgenic lines for *AtGolS2*.
Numbers indicate the identification number used for each transgenic line. NT indicates non-transgenic plants.

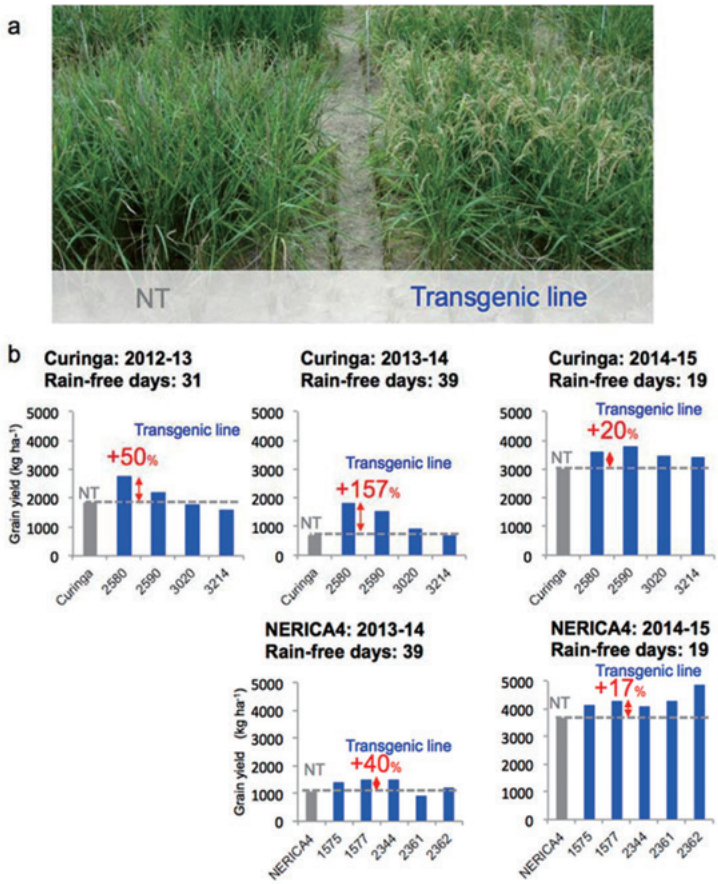

Fig. 5. Improved grain yields of transgenic lines for *AtGolS2* under drought in the field.
(a) Evaluation of transgenic rice in a confined field in CIAT. Left, non-transgenic Curinga; right, transgenic lines of Curinga (numbered 2580). (b) Grain yield of transgenic lines for *AtGolS2* in the three consecutive field trials. Numbers indicate the identification number used for each transgenic line. NT indicates non-transgenic plants.

In the final year of the project, we held an international workshop at Tsukuba, Japan. In this workshop,

the research results of the past five years and current situation and future prospects for development of GM crops were presented, and we discussed issues to be addressed for practical application of GM crops and the possibility of collaboration (**Fig. 6**). In the first session of the workshop, IRRI, CIAT, CIMMYT, and JIRCAS, reported the research results obtained over the past five years. In the second session, the invited speakers presented the status of current research and development of GM crops and their dissemination in Africa. Among the initiatives pursued was the NEWEST (Nitrogen-Use Efficient, Water-Use Efficient and Salt-Tolerant) rice program. Based on its results to date, this program could serve as a good model for practical application of GM crops. Finally, in the general discussion session, participants exchanged ideas and viewpoints on issues that need to be addressed in order to realize the practical application of GM crops in Africa. We also discussed the prospect for cooperation between JIRCAS and relevant African organizations, as well as the mutual concerns shared by both parties. We recognize the need to exert further efforts to disseminate research outputs in developing countries, in collaboration with farmers, private companies, and extension organizations. We hope that the discussion in this workshop will become the first step toward the promotion of social implementation in our research outputs.

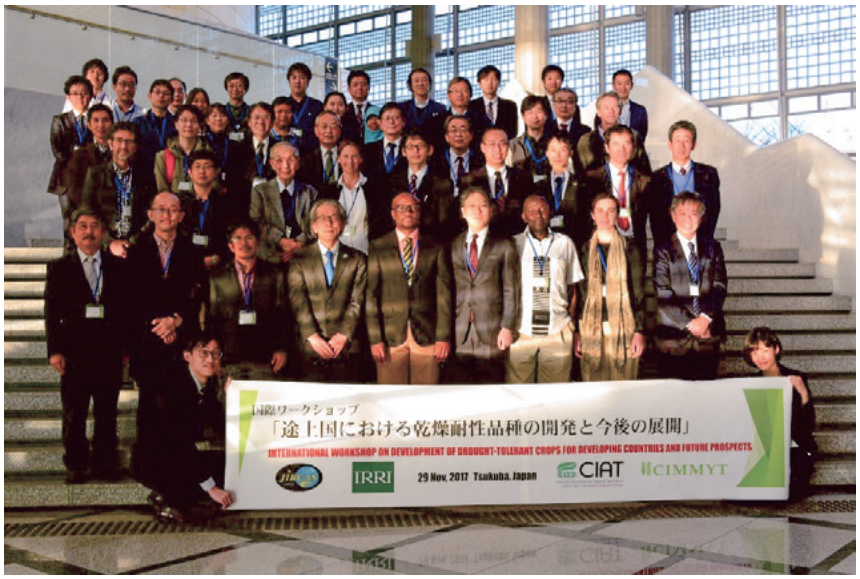

Fig. 6. All speakers, chairs, and participants in the workshop.

While preparing this manuscript, a paper titled "Expression of the CCCH-tandem zinc finger protein gene *OsTZF5* under a stress-inducible promoter mitigates the effect of drought stress on rice grain yield under field conditions" was published (Selvaraj et al. 2020).

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thaliana galactinol synthase gene improves drought tolerance in transgenic rice and increased grain yield in the field. *Plant Biotech J* **15**: 1465-1477 doi:10.1111/pbi.12731

Selvaraj MG, Jan A, Ishizaki T, Valencia M, Dedicova B, Maruyama K, Ogata T, Todaka D, Yamaguchi-Shinozaki K, Nakashima K, Ishitani M (2020) Expression of the CCCH-tandem zinc finger protein gene *OsTZF5* under a stress-inducible promoter mitigates the effect of drought stress on rice grain yield under field conditions. *Plant Biotech J.* (in press) doi:10.1111/pbi.13334

Chapter 2-2

Agrobacterium-mediated transformation system using immature embryos can effectively generate transgenic rice plants with a single copy of the transgene

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Abstract

Agrobacterium-mediated transformation in rice has been widely used for both basic and applied research. Previous reports on *Agrobacterium*-mediated transformation in rice have used mature seed-derived callus or immature embryos as starting materials and have briefly mentioned that the transgenic plants generated by these methods contained a low copy number of the transgene, which offers less labor to fix transgene genetically, to evaluate position effects, and to determine insertion site of transgene, particularly in the transgenic plants with one copy of transgene. However, most of them did not include quantitative observations regarding copy numbers of the transgene; the relationship between transgene copy numbers and starting materials, seed-derived callus, or immature embryos, has also not been elucidated. In this study, we generated transgenic rice plants from mature seed-derived callus and immature embryos of the rice variety Nipponbare and then compared the copy numbers of the transgene in transgenic plants. Transgenic plants from immature embryos had a significantly lower copy number of the transgene than those obtained from mature seed-derived callus, and the percentage of transgenic plants with one copy of the transgene was also significantly different: 25.0% from mature seed-derived callus and 46.7% from immature embryos, respectively. We generated transgenic plants in other five rice varieties—Koshihikari, NERICA1, NERICA4, Curinga, and Kasalath—by transformation using immature embryos. Transgenic plants with one copy of the transgene were generated at a high frequency in all varieties examined; this frequency ranged from 37.6 to 87.0%. We conclude that *Agrobacterium*-mediated transformation using immature embryos effectively generates transgenic rice plants with one copy of the transgene—a desirable trait in molecular breeding and in basic plant research.

Keywords: Copy number, Immature embryos, Rice, Transformation

Introduction

Since Hiei et al. (1994) established a system for genetic transformation of rice varieties mediated by *Agrobacterium*, this procedure has become routine. This technique has been widely used for both basic — i.e., functional analyses of genes—and applied research aiming to introduce desirable traits into rice. The first generation of transgenic plants (T_0) with a single copy of the transgene is desirable for molecular breeding and sometimes for basic research as well, because genetic fixation from T_0 with a single copy of the transgene is more straightforward than that from T0 carrying multiple transgene copies. The evaluation of position effects, that is, qualitative or quantitative variations in transgene expression due to different insertion sites (Matzke and Matzke 1998; Bhat and Srinivasan 2002; Schubert et al. 2004), becomes easier once selected T_0 with a single copy of the transgene. For commercialization of transgenic rice, site-specific sequence data for the entire inserted DNA along with adjacent genomic sequences near the insertion site are required to submit to regulatory agencies (Bradford et al. 2005). T_0 with a single copy of transgene is also ideal in this context.

In studies on *Agrobacterium*-mediated transformation in rice, callus derived from mature seeds (Hiei et al. 1994, Toki 1997, Mohanty et al. 1999, Rachmawati et al. 2004, Lin and Zhang 2005, Toki et al. 2006) or immature embryos (Hiei and Komari 2008, Ishizaki and Kumashiro 2008) have been used as starting materials. The advantage of the methods using mature seed-derived callus is the ease of preparation of materials. On the other hand, the methods using immature embryos have advantages regarding the range of applicable host varieties and transformation efficiencies. These reports briefly mentioned that transgenic rice plants generated by *Agrobacterium*-mediated methods contained a low copy number of the transgene; however, most of the papers did not include quantitative observations.

In this study, we compared transgene copy numbers in transgenic rice plants generated from mature seed-derived callus and immature embryos in Nipponbare variety. We also transformed several rice varieties by *Agrobacterium*-mediated transformation systems using immature embryos and investigated the transgene copy numbers obtained.

Materials and methods

Plant materials

We used six rice varieties. Nipponbare is a “model variety” of rice for which there is solid genome sequence information (International Rice Genome Sequencing Project.2005, Kawahara et al. 2013). Koshihikari has been the most popular variety in Japan for over 30 years (Nitta 2010). NERICA1 and NERICA4 are popular varieties of upland new rice in Africa (Kaneda 2007). Curinga is an elite upland rice

cultivar in South America (de Morais et al. 2005). Kasalath carries several beneficial traits such as tolerance to phosphorus deficiency (Chin et al. 2011, Gamuyao et al. 2012) and has been used for the development of a series of genetic and genomic resources (Ebitani et al. 2005, Kanamori et al. 2013). Nipponbare and Koshihikari are temperate japonica varieties; NERICA1 and NERICA4 derive from an interspecific hybrid of *Oryza sativa* L. and *Oryza glaberrima* Steud., and, together with Curinga, are tropical japonica varieties. Kasalath is an indica variety.

Production of transgenic plants

Agrobacterium tumefaciens strain LBA4404 harboring pBIG-ubi::GUS, which contained the hygromycin phosphotransferase gene (*hpt*) and the ß-glucuronidase gene (*gusA*) in the T-DNA region (Ishizaki and Kumashiro 2008), was used throughout the experiments. The transformation of Nipponbare rice from mature seed-derived callus was performed according to a method previously described (Ishizaki and Kumashiro 2008), with the modification of hygromycin concentration (50 mg/L). Transformation from immature embryos was carried out with a method previously established for NERICA varieties (Ishizaki and Kumashiro 2008), with some modifications depending on the target variety: no modifications for NERICA1, NERICA4, and Curinga; hygromycin concentration was modified from 20 to 50 mg/L for Nipponbare and Kasalath varieties. DKN medium (Daigen et al. 2000) was used as a basal medium for callus induction, selection, and regeneration for Koshihikari variety. Transformation protocols timelines are indicated in **Fig. 1**. The culture media used are listed in **Table 1**.

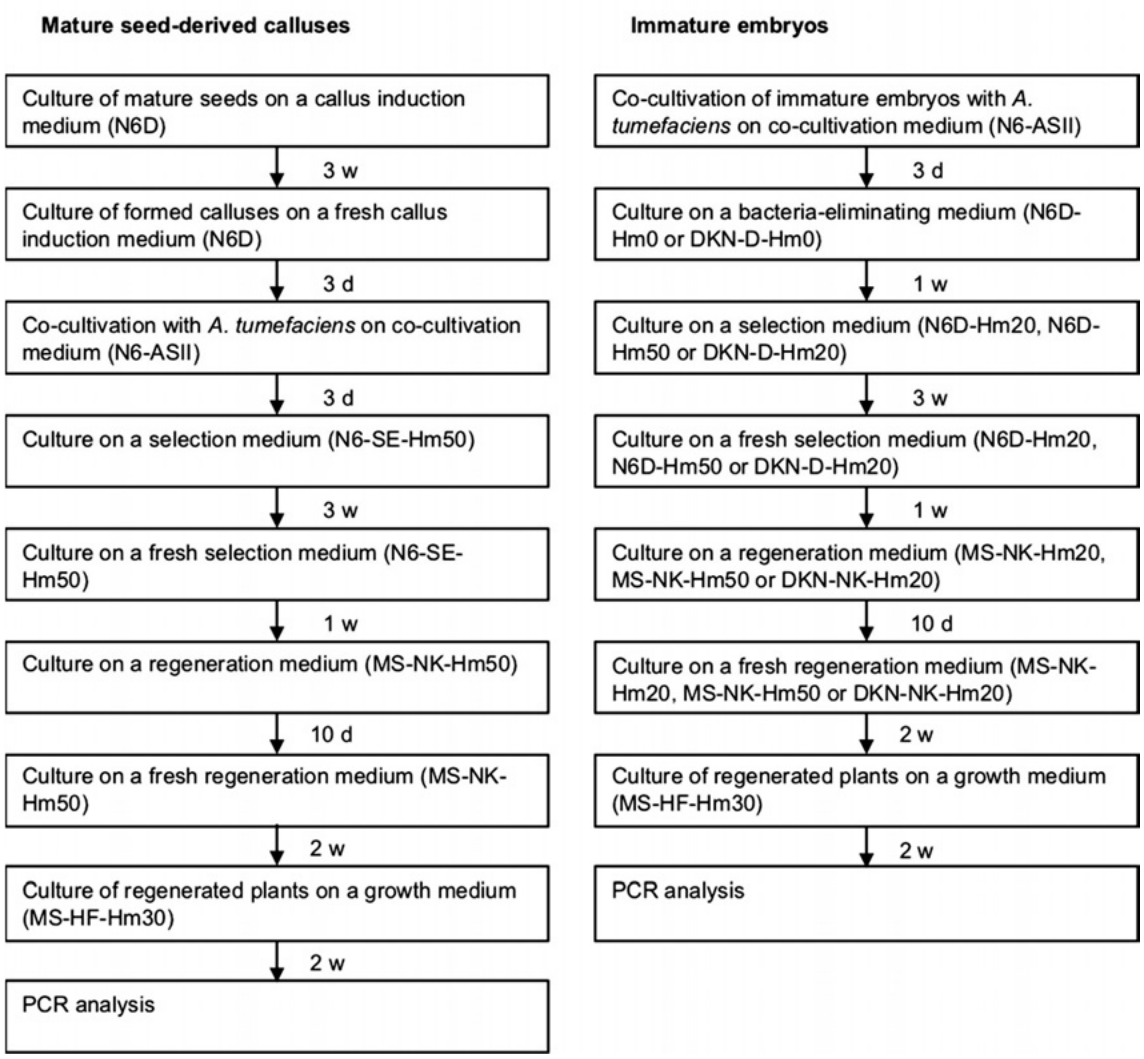

Fig. 1. Transformation protocols timelines from mature seed-derived callus and immature embryos in rice. The composition of media is indicated in **Table 1**.

Table 1. List of media used for transformation of rice varieties

Culture medium	Ingredients
DKN-D-Hm20	DKN salts and vitamins (Daigen et al. 2000), 30 g/L sucrose, 0.3 g/L casamino acids, 25 mM proline, 2 mg/L 2,4-D, 4 g/L Gelzan (Sigma, St. Louis, MO, USA), 500 mg/L cefotaxime, 20 mg/L hygromycin, pH5.8
DKN-NK-Hm20	DKN salts and vitamins, 20 g/L sucrose, 30 g/L sorbitol, 2 g/L casamino acids, 0.25 mg/L NAA, 2.5 mg/L kinetin, 4 g/L Gelzan, 250 mg/L cefotaxime, 20 mg/L hygromycin, pH 5.8
MS-NK-Hm20	MS salts and vitamins (Murashige and Skoog 1962), 20 g/L sucrose, 30 g/L sorbitol, 2 g/L casamino acids, 0.25 mg/L NAA, 2.5 mg/L kinetin, 4 g/L Gelzan, 250 mg/L cefotaxime, 20 mg/L hygromycin, pH 5.8
MS-NK-Hm50	MS salts and vitamins, 20 g/L sucrose, 30 g/L sorbitol, 2 g/L casamino acids, 0.25 mg/L NAA, 2.5 mg/L kinetin, 4 g/L Gelzan, 250 mg/L cefotaxime, 50 mg/L hygromycin, pH 5.8
N6-ASII	N6 salts and vitamins (Chu 1978), 30 g/L sucrose, 10 g/L glucose, 0.3 g/L casamino acids, 2 mg/L 2,4-D, 50 μM acetosyringone, 10 g/L Type-II agarose (Sigma), pH 5.2
N6-SE-Hm50	N6 salts and vitamins, 30 g/L sucrose, 2 mg/L 2,4-D, 4 g/L Gelzan, 500 mg/L cefotaxime, 50 mg/L hygromycin, pH5.8,
N6D	N6 salts and vitamins, 30 g/L sucrose, 0.3 g/L casamino acids, 25 mM proline, 2 mg/L 2,4-D, 4 g/L Gelzan, pH5.8
N6D-Hm0	N6 salts and vitamins, 30 g/L sucrose, 0.3 g/L casamino acids, 25 mM proline, 2 mg/L 2,4-D, 4 g/L Gelzan, 500 mg/L cefotaxime, pH5.8
N6D-Hm20	N6 salts and vitamins, 30 g/L sucrose, 0.3 g/L casamino acids, 25 mM proline, 2 mg/L 2,4-D, 4 g/L Gelzan, 500 mg/L cefotaxime, 20 mg/L hygromycin, pH5.8
N6D-Hm50	N6 salts and vitamins, 30 g/L sucrose, 0.3 g/L casamino acids, 25 mM proline, 2 mg/L 2,4-D, 4 g/L Gelzan, 500 mg/L cefotaxime, 50 mg/L hygromycin, pH5.8
MS-HF-Hm30	MS salts and vitamines, 30 g/L sucrose, 4 g/L Gelzan, 30 mg/L hygromycin, pH5.8

2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, naphthaleneacetic acid; BA, benzyladenine

DNA analyses

The presence of the introduced gene in 2-week old T0 plants was confirmed by polymerase chain reaction (PCR). The sequences of the primers used for the detection of *hpt* were 5′-TCGTGCTTTCAGCTTCGATG-3′ and 5′-TCCATCACAGTTTGCCAGTG-3′, and for the detection of *gusA* were 5′-CTGGTATCAGCGCGAAGTCT-3′ and 5′-CGATGGATTCCGGCATAGTT-3′. After confirming the presence of the transgene by PCR, transgenic plants were transferred to a 4-L pot with soil and placed in a greenhouse. Total DNA from the leaves of transgenic plants growing in the greenhouse was extracted and then subjected to Southern blot analyses, as previously described (Ishizaki and Kumashiro 2011), to determine the copy number of the transgene.

Statistical analysis

The data were analyzed by Mann-Whitney's U test and the Chi-square test of independence. Multiple comparisons were performed by Tukey's test or Steel-Dwass' test ($P < 0.05$).

Results and discussion

Transgene copy numbers of transgenic plants obtained from mature seed-derived callus and immature embryos

To compare the transgene copy numbers in transgenic plants developed from different starting materials, mature seed-derived callus and immature embryos, we generated transgenic rice plants from these materials in Nipponbare variety. Both protocols allowed the transformation of this rice variety: 190 transgenic plants were generated from 592 mature seed-derived callus and 67 transgenic plants were generated from 94 immature embryos, respectively, in total of repeated experiments. Then 40 transgenic plants from mature seed-derived callus and 45 transgenic plants from immature embryos, respectively, were subjected to Southern blot analyses to determine the copy number of the transgene in each individual. Transgenic plants from immature embryos had significantly lower copy numbers of the transgene than those obtained from mature seed-derived callus (**Fig. 2**). From both materials, transgenic plants carrying one copy of the transgene were generated at a relatively high frequency. However, only 25.0% of transgenic plants possessed one copy of the transgene when mature seed-derived callus were used as starting materials, while this ratio was significantly enhanced when immature embryos were used, viz., 46.7% of transgenic plants had one copy of the transgene. These results indicate that the transformation system with immature embryos can generate transgenic rice plants with one copy of the transgene more effectively than the system based on the use of mature seed-derived callus in Nipponbare cultivar. The mechanisms underlying the phenomenon is unclear.

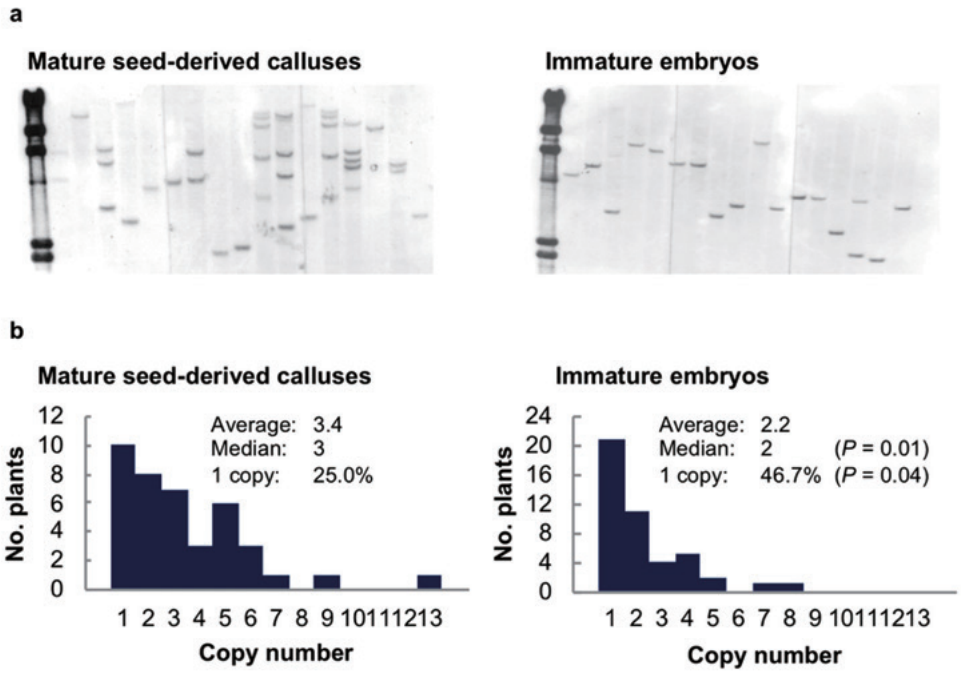

Fig. 2. Comparison of transgene copy numbers in transgenic plants obtained from mature seed-derived callus vs. immature embryos in Nipponbare variety.

(a) Examples of Southern blot analyses. The number of bands in each lane represents the transgene copy number in each individual. The leftmost lane in each blot shows lambda DNA digested with *Hin*dIII (used as a molecular marker); (b) Histograms of transgene copy numbers. Bars represent the number of plants having each transgene copy number. *P* values shown in the graph at the right side indicate significance levels of the differences between mature seed-derived callus and immature embryos. *P* value for the median was calculated by Mann-Whitney's U test. *P* value for the ratio of transgenic plants with 1 transgene copy was calculated by Chi-square test of independence. Since normality test revealed that both data sets had non-normal distribution ($P < 0.01$), statistical analysis for the differences in transgene copy number averages was not carried out.

Transgene copy numbers of transgenic plants generated from immature embryos of other rice varieties

To assess if transformation using as starting material immature embryos generates transgenic plants with one copy of the transgene at high frequency also in other rice varieties, we generated transgenic plants by several repeated experiments in five rice varieties: Koshihikari, NERICA1, NERICA4, Curinga, and Kasalath. The numbers of transgenic plants subjected to Southern blot analyses in each variety were as follows: Koshihikari, 82; NERICA1, 142; NERICA4, 92; Curinga, 57; and Kasalath, 23. Transgenic plants with one copy of the transgene were generated at a high frequency in all varieties we examined: 37.6% in Koshihikari, 42.3% in NERICA1, 42.4% in NERICA4, 40.4% in Curinga, and 87.0% in Kasalath (**Fig. 3**). Transgenic plants in Kasalath had significantly lower copy numbers of the transgene than those obtained from the other varieties examined, and the ratio of transgenic plants carrying one copy of the transgene in Kasalath was significantly higher. Overall, these results suggest that the transformation system with immature embryos can effectively generate transgenic rice plants with one copy of the transgene in several rice varieties, particularly in Kasalath.

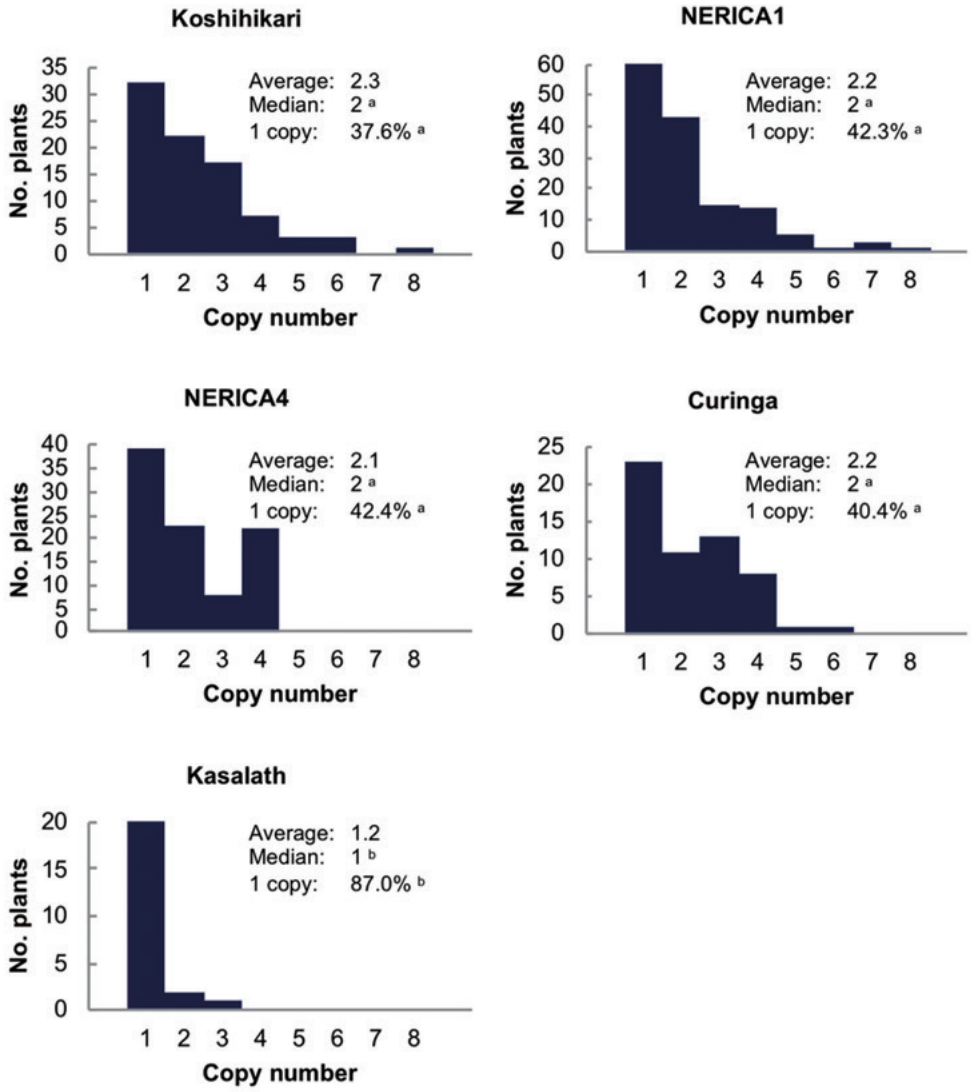

Fig. 3. Histograms showing transgene copy numbers in transgenic plants from immature embryos, in different rice varieties.

Bars represent the number of plants having each transgene copy number. Different letters denote significant differences at $P < 0.05$, as determined by Steel-Dwass' test for the median and by Tukey's test for the ratio of the transgenic plants with 1 transgene copy.

Conclusion

Higher transformation efficiencies and broader ranges of transformable varieties have been considered advantages of transformation protocols using immature embryos as starting materials, instead of mature seed-derived callus (Hiei and Komari 2008). Our results indicate that a higher frequency of transgenic plants with one copy of the transgene is also an advantage of transformation using immature embryos. Transgenic plants with a single copy of the transgene are desirable to fix transgenes genetically, to evaluate the position effects of them, and to determine their insertion sites. In target-mutagenesis technologies by

genome editing, like CRISPR/Cas9, removability of transgenes by segregation is a benefit in the context of molecular breeding (Bortesi and Fischer 2015, Hartung and Schiemann 2014). Additionally, the importance of transgene elimination by segregation to ensure stable inheritance of mutations and to avoid chimerism in later generations was also suggested (Ishizaki 2016). In this context, transgenic plants with a single copy of the transgene are ideal, since the efficiency of transgene elimination by segregation is determined by the number of loci where a transgene insertion is present: 25% (1/4) for insertion at one locus, 6.25% (1/16) for insertion at two loci, and so on. To sum up, the transformation protocol using immature embryos described in this study can be used as a reliable tool to generate transgenic rice plants with one transgene copy. Transgenic plants carrying a single transgene copy are desired in molecular breeding and in plant basic research relying on transgenic approaches and also on genome editing.

Acknowledgments

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Chapter 2-3

Towards development of drought tolerant upland rice through international research collaborations from gene discovery to trait evaluation: in case of *OsNAC6* gene as example

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Abstract

Extreme weather events such as droughts and heat waves are likely to become more common in recent warmer climate. Thus, development of drought tolerant rice varieties has been an urgent research goal since rice is the staple food of many people on planet. To speed up development of drought tolerant rice varieties, unique research collaborations between advanced institutions and international research organization were developed and led by Japan International Research Center for Agricultural Sciences (JIRCAS). This collaboration brought diverse expertise together to facilitate a product development pipeline from gene to trait evaluation under target field environments. One of genes tested in this study was *OsNAC6* gene. Twenty-eight independent transgenic lines that possessed homozygous single copy of introduced *OsNAC6* gene were generated from a commercial tropical japonica variety, Curinga. Evaluation of the transgenic lines under controlled and confined rainfed conditions in Colombia revealed superior performance of some transgenic lines in grain yield compared with non-transgenic plants. The transgenic lines of other genetic backgrounds, NERICA1 and NERICA4, also showed increased grain yield under the rainfed conditions. The results

strongly suggest that the gene works regardless of genetic background as common genetic component for drought tolerance in rice under field conditions leading to increased grain yield.

Key words: upland rice, trait evaluation, drought tolerance, transgenic approach

Introduction

Drought occur naturally but increased climate variability due to climate change accelerated to make it more extreme which increases risk of crop yield losses with many consequences. It was illustrated that globally, climate variability accounts for roughly a third of the observed yield variability, meaning that variations in temperature, rainfall or its combination explain yield variability (Ray et al. 2015). Thus, developing drought tolerant crop with minimum yield losses is crucial to meet the needs of future population growth in sustainable and environment friendly manner.

Plant's responses to water-deficit conditions have been extensively elucidated to identify drought inducible genes with various functions mainly in model plants such as Arabidopsis and a rice variety, Nipponbare (Shinozaki and Yamaguchi-Shinozaki 2007) and many of which showed increased drought tolerance under controlled conditions, meaning that most of candidate genes were still at "Gene/Trait Identification" and "Proof of Concept" (in this study demonstrating in principle with the aim of verifying gene function) phases in product development pipeline as indicated in **Fig. 1**. In this study, we moved forward from "Gene/Trait Identification" to "Early Development" and in some extent, "Advanced Development" phases to demonstrate 1) drought inducible genes work not only for drought tolerance but also for increased yield and 2) the genes work under real drought field conditions regardless of genetic background. Through research collaborations between advanced institutions and CGIAR centers (Gaudin et al. 2013), more than 15 genes and promoter combination have been evaluated among them, *OsNAC6* gene, a transcriptional activator up‐regulating stress‐inducible genes for stress tolerance was highlighted in the report since this gene showed clear evidence of better agronomic performance under drought conditions compared with other genes tested in this study.

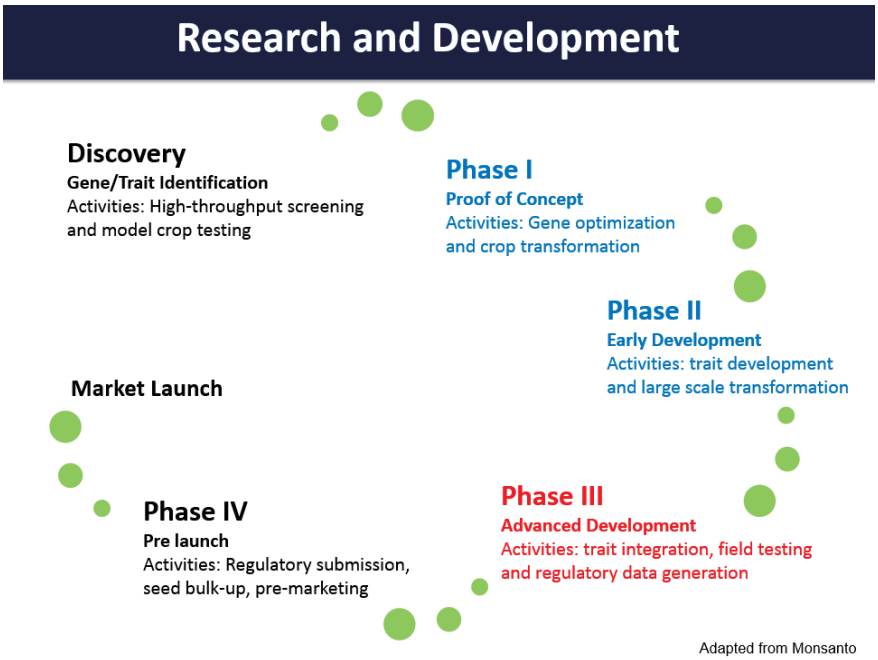

Fig. 1. Diagram of product development pipeline from Discovery to Market Launch.
Discovery and Proof of Concept work were done previously by RIKEN and JIRCAS for stress inducible genes and promoters used in for this project.

Materials and methods

QC procedure for gene constructs

Each construct provided by JIRCAS and RIKEN PSC was transformed into *Agrobacterium tumefaciens* strains EHA105 and *E. coli*, DH5. by electroporation. After gene confirmation by PCR using gene specific primers, the plasmid DNA of each construct extracted from *E. coli* DH5α was subject to sequencing for the region between T-DNA boarders except selection cassette.

Plant Material

Curinga, tropical Japonica upland rice, which showed better drought resistance compared with other varieties developed in Brazil (Breseghello et al. 2009) was used due to geological and economic importance of this genotype in Latin America, especially in Brazil when the project started in 2007. NERICA lines were produced by JIRCAS and used for agronomical performance under drought conditions at confined experimental fields managed by CIAT (Selvaraj et al. 2017). NERICA lines were reported most drought tolerant varieties among other varieties tested in water stress experiments in Uganda (Matsumoto et al. 2014).

Rice genetic transformation and isolation of transgenic events with low-copy number of the transgene

Each construct was transformed into *A. tumefaciens* (EHA105) and low-copied transgenic events are isolated as described in Selvaraj et al. (2017).

Drought trials under rainout shelter and rainfed conditions

At CIAT HQ, Palmira, two reproductive drought experiments per year were carried out one in rainy season (Feb-June) and another one in dry season (August-December) using rainout shelter in confined field. As to rainfed drought trials, confined field was established at Santa Rosa experimental fields and used to evaluate lines for agronomical performance in dry season starting in December. All the details of experimental design and agronomical analysis were described in Selvaraj et al. (2017).

Results and discussion

Isolation of single homozygous transgenic events

More than 15 constructs with gene and promoter combinations were received from RIKEN and JIRCAS in the period of 2007-2009 and one of which was pBIH-*osnac6::OsNAC6*. The *OsNAC6* was isolated as a transcriptional activator and up‐regulates stress‐inducible genes including lipoxygenase and peroxidase for stress tolerance (Nakashima et al. 2007), suggesting an important role of the gene for improved drought tolerance in rice. QC-confirmed *OsNAC6* gene construct was transformed to Curinga to generate 112 independent events and further transgene copy analysis resulted in 28 single-copy events at T_3 generations.

Establishment of confined fields for trait evaluation under drought conditions

To evaluate transgenic lines harboring gene of interest under real field drought conditions, two confined fields were prepared with permission of Colombia government. One was at CIAT-HQ under rainout shelter conditions and another was Santa Rosa experimental field where rainfall is limited from December to March during dry season. Permit of field testing of genetically modified rice for research purpose at these sites was obtained in 2008 and 2010, respectively as described in **Fig. 2**.

During the project period, CIAT established standard operating procedures to better manage transgenic materials from laboratory to field. On 2013, CIAT was certified as a member by Excellence Through Stewardship, a global not-for-profit organization that promotes quality management systems for agricultural technology products (https://www.excellencethroughstewardship.org/).

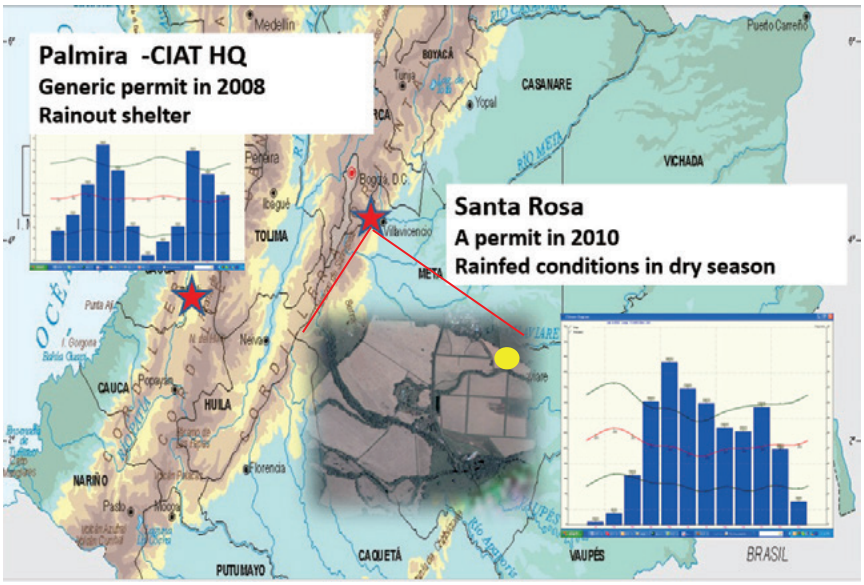

Fig. 2. Location of confined fields in Palmira and Santa Rosa and its rainfall pattern.
Star mark indicated geological location of Palmira and Santa Rosa in Colombia. Yellow dot indicated location of confined filed (40 m x 60 m) established at Santa Rosa experimental field station. Detail rainfall pattern in Colombia can be obtained at following site: https://en.climate-data.org/.

Evaluation of agronomical performance under reproductive stage drought stress conditions using rainout shelter

At first transgenic lines showing less yield and/or different plant type compared with parent genotype under well-irrigated conditions were eliminated during seed multiplication. Selected lines were further tested under reproductive stage drought stress conditions. The experiment was carried out with 52 transgenic events from Curinga and 18 transgenic events from NERICA representing 8 and 4 gene constructs respectively (data note shown). Drought was imposed by withholding irrigation when panicle initiation was around 10 mm long (63 days after sowing in the case of Curinga) for 3-4 weeks or until severe leaf rolling & drying appeared in non-transgenic control. Then the plants were re-irrigated to 90-100% field capacity till the physiological maturity. The intensity of drought was monitored through AquaPro soil moisture probes that could measure moisture in the soil profile of 0.85 m depth then plants were irrigated by boom to monitor the genetic variation in recovery from drought (**Fig. 3**).

After evaluation of agronomical performance under rainout shelter conditions, eight lines, namely, line 2967, 3008, 3012, 3074, 3080, 3085, 3270 and 3677 were selected (data not shown) based on better yield performance compared with non-transgenic plants and further tested at Santa Rosa experimental field for multiple years.

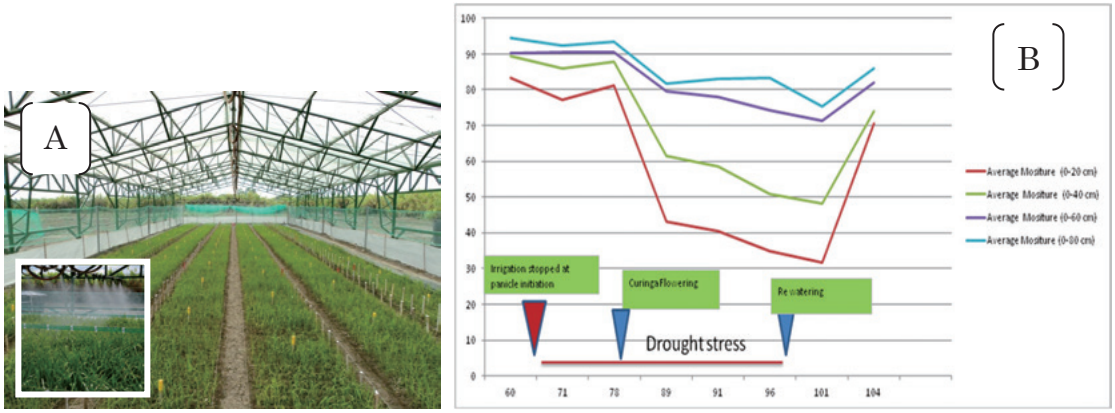

Fig. 3. Example of soil moisture profile during drought period in Feb-August 2012.
Rainout shelter at CIAT-Palmira was used for evaluation of Curinga transgenic lines under drought conditions at reproductive stage. [A] View of drought trial under rainout shelter. Photo in white boarder was boom irrigation. [B] Soil moisture profile with different depths from 20 cm to 80 cm. Three triangle markers indicated time of stopped irrigation, flowering time and re-watering.

Establishment of rainfed drought stress conditions for multiple year evaluation

Santa Rosa experimental field was used to establish the rainfed drought stress conditions. Initial field establishment was completed in 2010 after complying all the biosafety requirements (**Fig 4**). Under upland conditions, uniform seed germination was essential to establish a field trial. Therefore, initial irrigation was provided through sprinklers-irrigation till establishing the crop. During the field trial, soil moisture in different depths was monitored as indicator of severity of drought conditions.

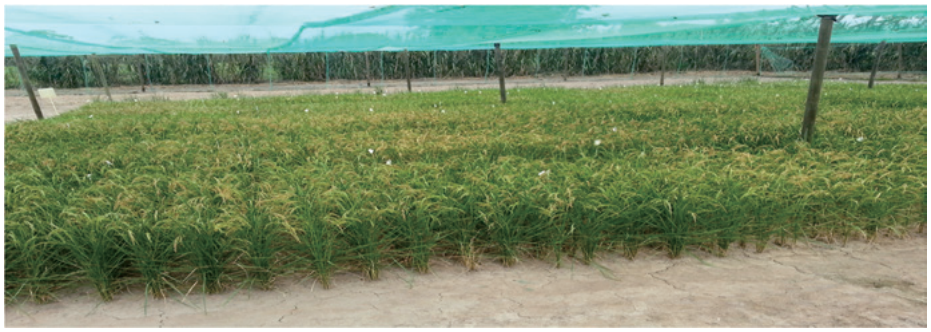

Fig. 4. A typical plot view under rainfed drought stress conditions at Santa Rosa experimental field.
Plot were covered by blue net to avoid rodents.

Fig. 5 was one example of field drought conditions from December, 2011 to March, 2012. In this trial, two dry spells were occurred, one at vegetative stage to initial panicle initiation stage (spanning about 36 days long) and one at late panicle initiation stage to flowering (19 days) (**Fig .5**). In between the two dry spells, at 54 days after sowing some rainfall around 40 mm was received to recover the drought stress. In the total crop period (sowing to harvest), plants received total of 336 mm of rainfall, it is little less against past ten-year average (388 mm). It's interesting to note that, more than 60% of the rainfall received after flowering (**Fig. 5**). The plants were under gone severe drought during late vegetative and panicle initiation stage.

Annual rainfall of this year 2011-2012 at this site is 2851 mm. Total rainfall during crop period (Dec-March) was around 336 mm. Average of maximum temperature was around 32ºC and relative humidity was around 85% during the crop period.

During vegetative stage upper layer (0-40 cm) moisture maintained around 60-68%, during the time of panicle initiation, moisture sharply declined up to 50%. The average soil moisture 0-80 cm was also declined below 60% during panicle initiation time (**Fig. 6**).

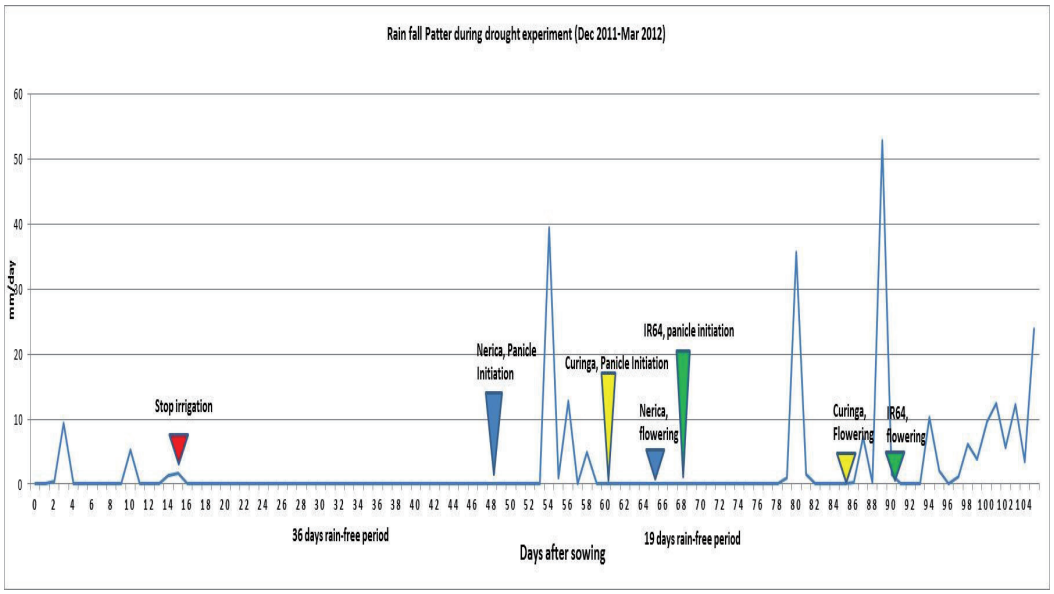

Fig. 5. Rainfall & dry spell pattern at Santa Rosa started from December, 2011.
Peak showed rainfall during the period of the experiment.

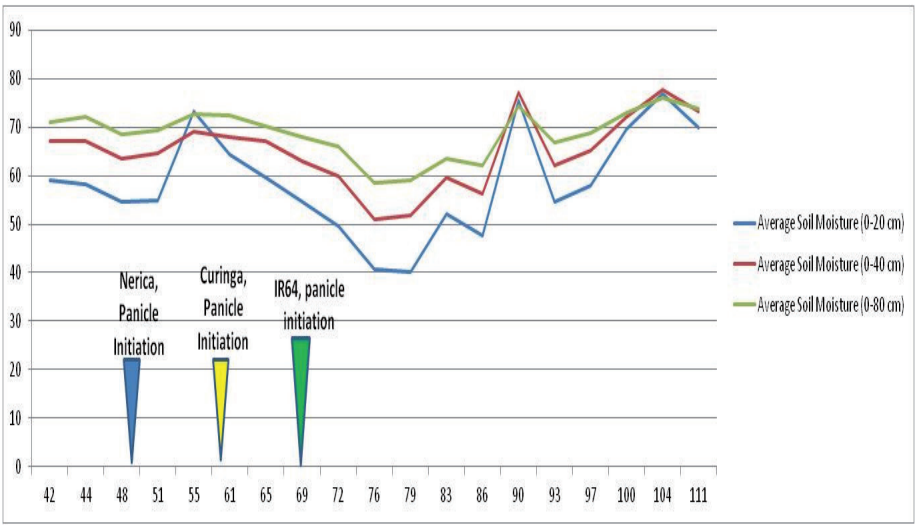

Fig. 6. Soil moisture profile during Santa Rosa rainfed trial during period of 2011-2012.
Purple line indicated moisture from 60-80 cm, Green line indicates 40-60 cm; red line indicates 20 - 40cm and blue line indicates 0-20 cm.

Third rainfed filed trial was initiated in December 2012 using eight promising lines from

osnac6::OsNAC6 as described above among other Curinga and NERICA lines (data not shown). This time the drought was very severe and 42 days rain free period was recorded and plants received most of the rainfall after flowering (data not shown). The grain yield was affected strongly among the transgenic lines. Among the 9 different constructs studied, namely *osnac6::OsNAC6*, *oshox24::OsNAC6*, *ubi::AtGolS2*, *oshox24::AREB1*, *oshox24::AREB2*, *lip9::DREB1C,* *osnac6::OsSCZF2*, *osnac6::AREB1ΔQT*, *lip9::AREB1ΔQT*, the positive effect of the constructs in terms of grain yield under stress was noticed including *osnac6::OsNAC6* and *Ubi::AtGolS2* (Selvaraj et al. 2017). The yield advantage of six lines: 2967, 3008, 3074, 3080, 3085 and 3677 over the non-transgenic Curinga ranged from 10-30% as shown in **Fig. 7**.

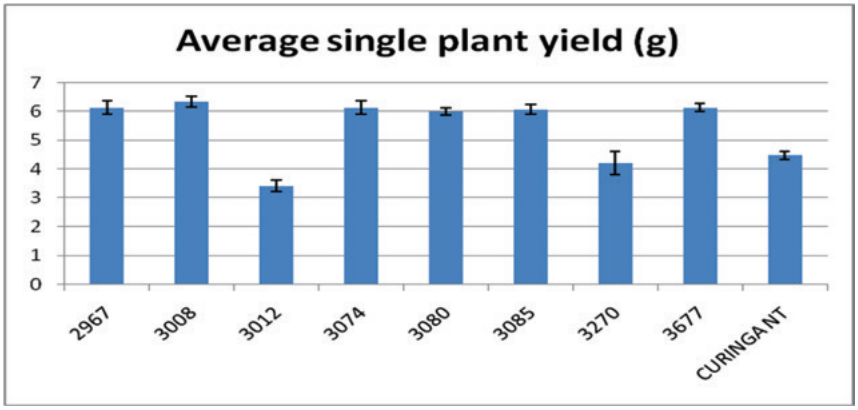

Fig. 7. Variation in single plant yield among the transgenic events of *osnac6::OsNAC6* at the end of drought stress under rainfed conditions.
Curinga NT means Curinga wild type (non-transgenic). Error bar represents ± standard error (SE) (n=15).

Furthermore, the lines 3080 and 3677 had less leaf rolling score and more numbers of productive tillers and better biomass than non- transgenic Curinga (data not shown). It was suggested that the rice OsNAC6 transcription factor is involved multiple molecular mechanisms such as root structural adaptions and nicotianamine biosynthesis for drought tolerance although root specific promoter, *RCc3* was used to ectopically express *OsNAC6* gene in Nipponbare (Lee et al. 2017). Better agronomical performance of the lines used in this study under rainfed conditions should be further confirmed by transgene expression and repeated field trials with bigger plot size.

In 2012 rainfed trial at Santa Rosa, 13 transgenic events of NERICA1 and NERICA4 backgrounds were also evaluated (data not shown). This rainfed experiment revealed that several events from the following genes, namely, *osnac6::OsNAC6* (**Fig. 8**) and *Ubi::AtGolS2* (Selvaraj et al. 2017) performed better than non-transgenic NERICA in terms of single plant yield under stress.

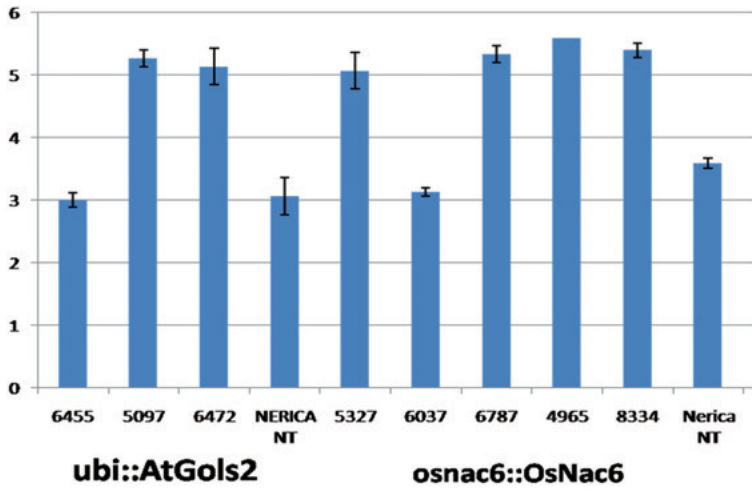

Fig. 8. Variation in grain yield among the different transgenic events of NERICA1 and NERICA4 at the end of drought stress under rainfed trial, 2012-13.
From left to right, genetic backgrounds are NERICA1 for 6455, NERICA 4 for 5097, NERICA1 for 6472, WT and 5327, NERICA 4 for 6037, 6787, 4965, 8334 and WT, respectively. Error bar represents ± standard error (SE) (n=15).

The line 6787, 4965 & 8334 of the construct *osnac6::OsNAC6* had shown more than 35 % yield increase compared to non-transgenic NERICA lines under drought stress. *osnac6::OsNAC6* (**Fig. 7**) and *Ubi::AtGolS2* (Selvaraj et al. 2017) also found to better perform in Curinga background under drought conditions. This finding strongly suggested *OsNAC6* which is to be associated common drought tolerance mechanisms (Lee et al. 2017) can improve agronomical performance under drought conditions regardless of genetic background. Furthermore, OsNAC6 lines with constitutive promoter were not performed well under the studied conditions (data not shown). This indicated suitable combinations of gene and promoter is essential for improved agronomical tolerance in rice.

Conclusion

Through international collaborative research, maximizing institutional strengths on research from basic to application, several genes with different promoter such as *osnac6::OsNAC6* and *Ubi::AtGolS2* were identified, not only improving drought tolerance but also increases grain yield under real field conditions for crop improvement.

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We thank JIRCAS for giving opportunity to conduct this study as commission research in a project funded by Ministry of Agriculture, Forestry and Fisheries of Japan (Development of Abiotic Stress Tolerant Crops by DREB Genes; Development of Drought-tolerant Crops for Developing Countries). We also thank scientists and technical staff at JIRCAS, RIKEN, IRRI and CIAT for providing technical supports and for

critical reading of the article for revisions.

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Chapter 3

Development of drought-tolerant soybean and sugarcane

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

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.

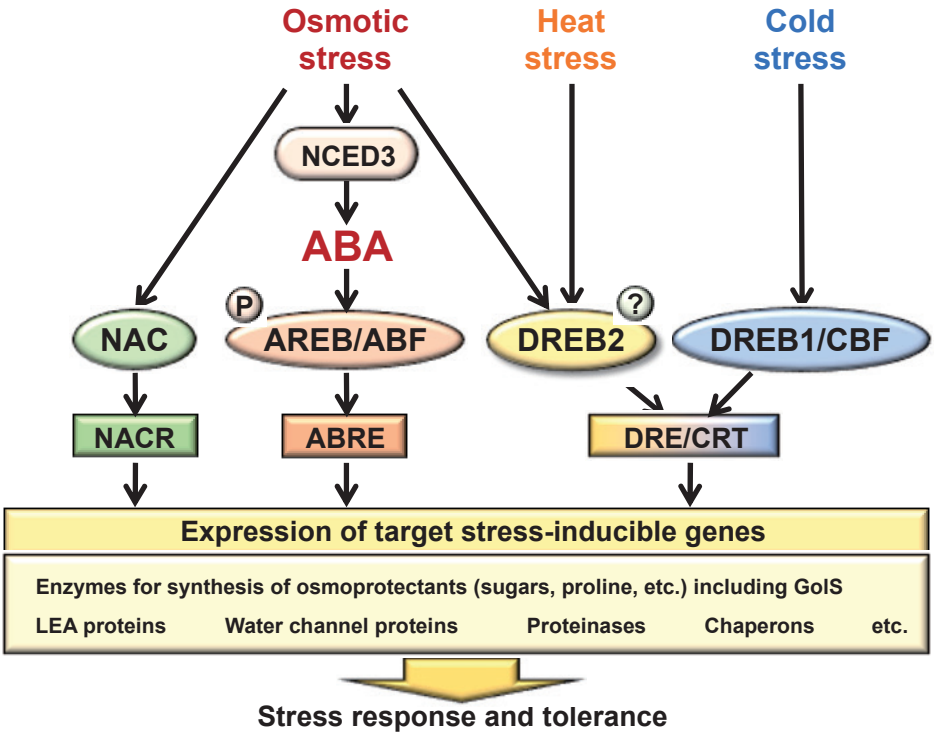

Fig. 1. Key transcriptional networks under environmental stress conditions in plants.
Environmental stresses such as osmotic stress, heat stress, and cold stress cause transcription factor biosynthesis and activation. Transcription factors bind to specific *cis*-elements and induce the expression of 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.

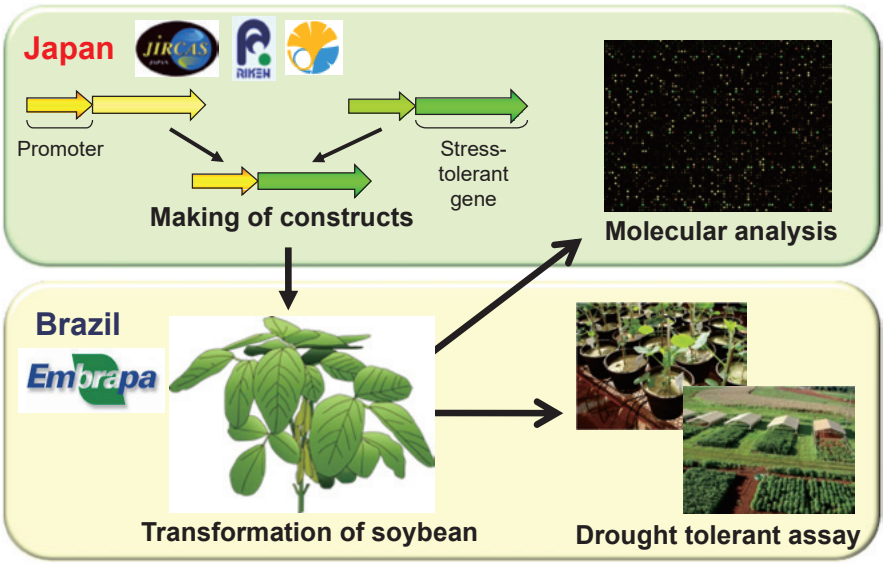

Fig. 2. Outline of the Science and Technology Research Partnership for Sustainable Development (SATREPS) project, "Development of genetic engineering technology for crops with stress tolerance to degradation of global environmental"
The aim of this project was to establish technology to develop genetically 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.

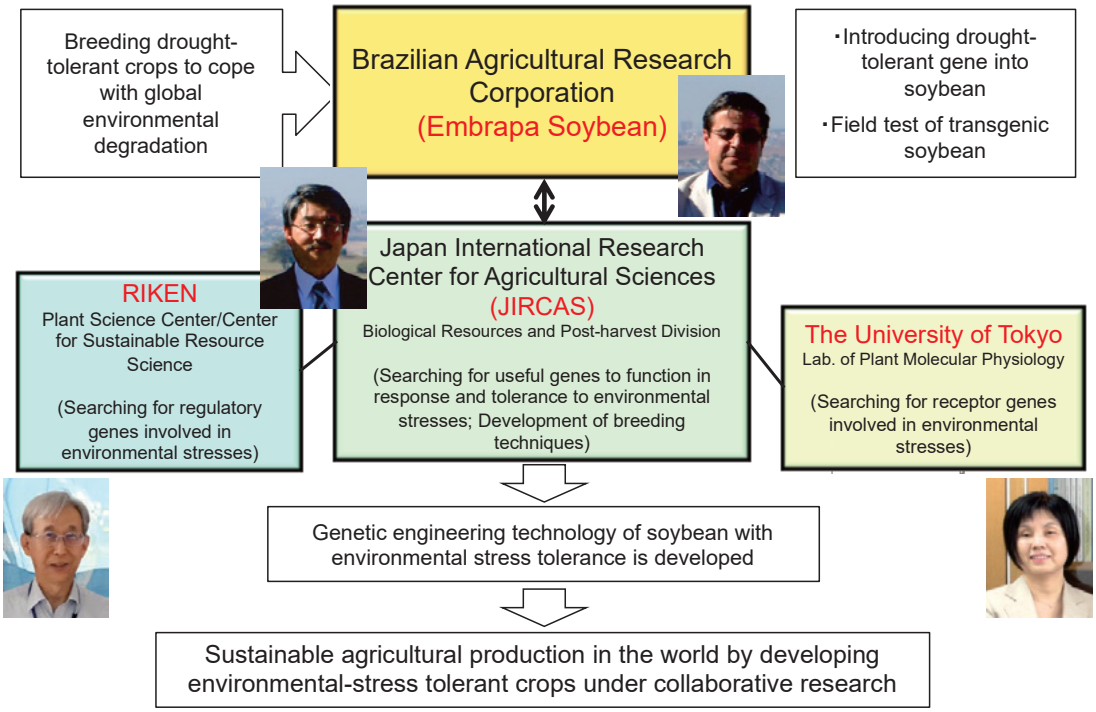

Fig. 3. Research structure of the SATREPS project, "Development of genetic engineering technology of crops with stress tolerance against degradation of global environment"

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.

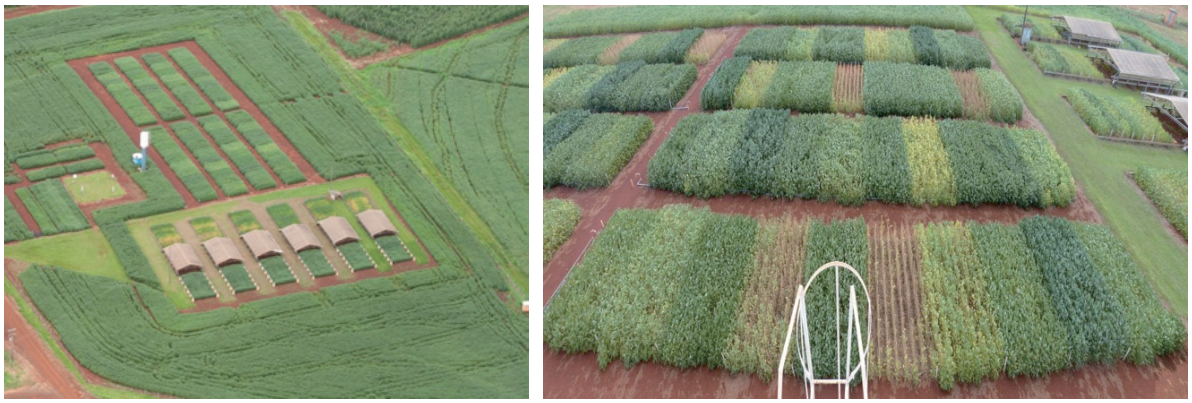

Fig. 4. Confined fields and rain-out shelters used to evaluate the drought tolerance of GM soybean lines in Embrapa Soybean in Brazil.

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.

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Chapter 3-2

Drought-tolerant soybean development: evaluation of GM lines under greenhouse and field conditions

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Abstract

Drought is one of the greatest sources of environmental stress that has resulted in both economic and yield losses in many soybean-producing regions. The use of biotechnological tools aimed to produce plants with increased drought tolerance and productivity is the result of major investments in scientific and technological research. During the last decades, transcription factors (TFs) and key genes of important drought-responsive pathways have been used to develop genetically modified (GM) plants with increased tolerance to abiotic stress. To develop soybean lines with improved drought tolerance, genes encoding dehydration-responsive element binding protein (DREB) TFs and ABA-responsive element-binding proteins (AREB) as well as the *GolS* and *NCED* genes that encode the enzyme galactinol synthase (GolS, EC 2.4.1.123) of the raffinose family of oligosaccharides (RFOs) and the key enzyme in abscisic acid (ABA) biosynthesis 9-*cis*-epoxycarotenoid dioxygenase (NCED, EC 1.13.11.51), respectively, were successfully introduced in soybean plants. After transformation, it was imperative to characterize the resultant drought tolerance of the GM lines.

Thus, the soybean GM lines containing genetic constructions to overexpress *AtDREB1A*, *GmDREB1A*, *AtDREB2A*, *AtAREB1*, *AtGolS2*, and *AtNCED3* were phenotyped based on molecular and agronomical traits, growth parameters, and survival rates under water deficit conditions in experiments carried out under greenhouse and field conditions for more than one crop season. All obtained results were compiled and are presented in this report. Overall, the GM lines are promising and show improved drought tolerance as diverse defense mechanisms aimed at surviving periods of water scarcity were targeted while retaining the productivity of the crop. These outcomes highlight soybean drought-tolerance pathways and indicate that the use of biotechnological tools in agricultural research can help producers minimize both yield and financial losses during drought-stricken crop seasons.

Keywords: *Glycine max*, transcription factor, DREB, AREB, ABA, galactinol synthase, 9-*cis*-epoxycarotenoid dioxygenase, water deficit, yield

Introduction

Drought is one of the most stressful environmental factors currently affecting crops of economic importance. Therefore, yield reductions are likely to remain ongoing and financial/economic losses are inevitable in drought-prone environments. According to a new report from the Food and Agriculture Organization (FAO) of the United Nations, natural disasters have cost the agricultural sectors of developing countries a staggering 96 billion USD in either damaged or lost crops and livestock between 2005 and 2015. Drought—which has recently battered farmers across the globe—was one of the leading culprits. Eighty-three percent of all economic losses due to drought that were documented by FAO study were absorbed by agriculture, with a price tag of 29 billion USD (FAO 2018).

As an important global commodity, soybeans are not exempt from water deficit problems and neither is Brazil, which is the second highest soybean producer worldwide and one of the few countries that could considerably increase its production over the next decades. Losses due to drought during 37 Brazilian harvests from the 1976/77 and 2013/14 crop seasons were estimated to have cost 79,62 billion USD (Ferreira 2016). To illustrate the importance of soybeans to the Brazilian economy, according to the Brazilian Institute of Geography and Statistics (IBGE), in 2015, agriculture was the only economic sector that did not reduce its contribution to the Gross Domestic Product (GDP) and instead increased its contribution 1.8% from that of the previous year, which was primarily due to the influence of soybeans and corn (FAO 2016).

Despite these positive numbers, drought periods have generated recurrent and significant losses in soybean yields. This scenario is not likely to change in the upcoming decades; instead, the climate predictions of the IPCC (Intergovernmental Panel on Climate Change) indicate that changes in both the frequency and

intensity of extreme climate events must be expected. For the coming years, it is very likely that daytime maximum and minimum temperatures will increase, accompanied by an increased frequency of hot days. It is also very likely that heat waves will become more frequent and that the number of cold waves and frost days (in applicable regions) will decline. Increases in the number of high-intensity precipitation events are also likely in many locations. In addition, the frequency of summer droughts is likely to increase in many interior continental locations, and droughts—as well as floods—associated with El Niño events are also likely to intensify. Furthermore, tropical cyclone mean and peak wind intensities and peak precipitation intensity are likely to increase. This expectancy indicates that sustainable crop production critically depends on the development of cultivars that are more tolerant to abiotic stress in general and to drought stress in particular, which may be accomplished through available genetic engineering techniques. Therefore, both the identification of genes that confer water deficit tolerance and the development of GM plants that express key responsive genes with current biotechnological tools have become a priority for agricultural research given the current global climate change conditions (Ramiro et al. 2016).

In response to environmental changes, plants, which are sessile organisms, have evolved a set of morphological, physiological, biochemical, cellular, and molecular mechanisms to metabolically cope during water deficit periods (Fang and Xiong 2015). The products of these stress-inducible genes may be classified into two groups. The first group includes proteins that are mostly associated with abiotic stress tolerance and include molecules, such as chaperones, late embryogenesis abundant (LEA) proteins, osmotins, antifreeze proteins, mRNA-binding proteins, key enzymes for osmolyte biosynthesis, water channel proteins, sugar and proline transporters, detoxification enzymes, and various proteases. The second group is comprised of regulatory proteins, which are protein factors involved in the subsequent regulation of signal transduction and stress-responsive gene expression, and include various transcription factors, protein kinases, protein phosphatases, enzymes involved in phospholipid metabolism, and other signaling molecules like calmodulin-binding protein (Hasegawa et al. 2000, Shinozaki and Yamaguchi-Shinozaki 2007). Nonetheless, drought induces the expression of abscisic acid (ABA)-dependent and ABA-independent genes (Shinozaki and Yamaguchi-Shinozaki 2000, Yamaguchi-Shinozaki and Shinozaki 2005), which points to the existence of a complex regulatory mechanism involved in the perception of abiotic stress signals (Shinozaki and Yamaguchi-Shinozaki 2000, Zhu 2001).

Among the drought stress-tolerant genes currently used, transcription factors (TFs) and key genes in drought-responsive pathways show great potential for the development of plants more resistant to water deficits. Specifically, transcription factors have shown great potential as they are able to recognize and bind to specific DNA sequences in the regulatory regions of target genes, activating and regulating the expression of the downstream genes responsible for cellular protection processes under conditions of dehydration (Shinozaki and Yamaguchi-Shinozaki 2007). The dehydration-responsive element binding protein (DREB)

TF is part of an ABA-independent drought-response pathway. By interacting with the *cis*-C-repeat/dehydration response element (CRT/DRE) [consensus sequence (A/G)CCGAC] by the AP2 DNA-binding domain, which is present in the promoter region of target genes, these TFs mediate downstream gene expression in response to environmental stress. The insertion of the TF *AtDREB1A*, which is under the control of the stress-inducible rd29A promoter, has been found to successfully improve drought tolerance responses in *Arabidopsis thaliana* (Liu et al. 1998, Jaglo-Ottosen et al. 1998, Gilmour et al. 1998), tobacco (Kasuga et al., 2004), rice (Dubouzet et al. 2003, Oh et al. 2005, Ito et al. 2006), maize (Qin et al. 2004, 2007), wheat (Pellegrineschi et al. 2004, Gao et al., 2009), and peanut plants (Bhatnagar-Mathur et al. 2004, 2007, Devi et al. 2011, Vadez et al. 2013). Also, *AtDREB1A* has been successfully introduced into soybeans with promising results for the improvement of drought tolerance (Polizel et al. 2011, Rolla et al. 2013, Fuganti-Pagliarini et al. 2017).

The DREB2A protein, which belongs to the DREB family, has been used to develop genetically modified drought-tolerant plants. In *Arabidopsis*, the overexpression of a constitutively active (CA) DREB2A form was found to result in significantly improved tolerance to drought and heat stress (Sakuma et al. 2006a, 2006b). *AtDREB2A* homologous genes have been studied in maize (Qin et al. 2007), rice (Dubouzet et al. 2003), sunflowers (Almogueva et al. 2009), wheat (Terashima and Takumi 2009), and chrysanthemums (Liu et al. 2008). In addition, *AtDREB2A* was successfully introduced into soybeans (Engels et al. 2013). Recently, Mizoi et al. (2013) identified a soybean DREB2 gene, *GmDREB2A;2,* and showed that its heterologous expression in *Arabidopsis* induced stress-inducible genes, such as *RD29A*, *RD29B*, *HsfA3*, and *HSP70*, and improved stress tolerance. Marinho et al. (2020, submitted) successfully introduced the *GmDREB2A;2* TF in soybean plants.

When considering the ABA-dependent TFs, the ABA-responsive element-binding protein (AREB) family has shown interesting results with regard to conferring drought tolerance. In *Arabidopsis*, AREB acts as the major TF family under conditions of abiotic stress (Yamaguchi-Shinozaki and Shinozaki 2005, Kobayashi et al. 2008, Lee et al. 2010, Yoshida et al. 2015) and has been reported to regulate environmental stress responses and ABA signaling during the vegetative stage (Jakoby et al. 2002, Fujita et al. 2005, Yoshida et al. 2010). These TFs target the expression of water deficit-responsive genes by binding to conserved *cis*-elements, called ABA-responsive elements (ABRE; PyACGTGG/TC) present in the promoter regions of target-genes (Barbosa et al. 2012). In *A. thaliana*, the overexpression of *AREB1* has been found to result in ABA hypersensitivity, the induction of drought-responsive genes like *RD29B*, and improved water deficit tolerance (Fujita et al. 2005). In *Glycine max*, plants overexpressing *AtAREB1* TF showed interesting results that supported the potential of *AtAREB1* TF for improving drought tolerance (Barbosa et al. 2012, Leite et al. 2014, Marinho et al. 2016, Fuganti-Pagliarini et al. 2017).

Transcription factors have not been the only focus of research to improve drought tolerance in plants,

genes have also been associated with key response mechanisms, such as *GolS* and *NCED*, which encode the enzyme galactinol synthase (EC 2.4.1.123) from the raffinose family of oligosaccharides (RFOs) and the key enzyme in ABA biosynthesis 9-*cis*-epoxycarotenoid dioxygenase (EC 1.13.11.51), respectively. Raffinose family oligosaccharides (RFOs), such as raffinose, stachyose, and verbascose, act as osmoprotectants and are known to be involved in responses to adverse environmental conditions. In drought tolerance responses, RFOs are able to regulate osmotic potential and protect both enzymes and membranes from different sources environmental stress (Panikulangara et al. 2004, Pattanagul and Madore 1999). The *GolS* genes have been reported to be upregulated by abiotic stress in many plant species, such as rice (*Oryza sativa*; Takahashi et al. 1994), grapes (*Vitis vinifera;* Pillet et al. 2012), tobacco (*Medicago falcata*; Zhuo et al. 2013), and *Salvia miltiorrhiza* (Wang et. 2012). In particular, drought tolerance has been reported for plants expressing *GolS* genes like *A. thaliana* (Taji et al. 2002), tomatoes (*Solanum lycopersicum* Mill. cv Moneymaker; Downie et al. 2003), coffee [*Coffea arabica* (Santos et al. 2011) and *Coffea canephora* (Santos et al. 2015)], *Populus tricocarpa* (Zhou et al. 2014), and soybeans (Marcolino-Gomes et al. 2014, Rodrigues et al. 2015).

As ABA is an important hormone that triggers the responses of plants to adverse environmental conditions (Barbosa et al. 2012, Cao et al. 2013, Takeuchi et al. 2014, Park et al. 2015), the genes related to its metabolism are also targets for genetic manipulation. Accordingly, several studies have reported a improved performance of plants under water deficit conditions due to the overexpression of genes that encode enzymes of the ABA biosynthetic pathway (Iuchi et al. 2001, Endo et al. 2008), specifically, the *NCED3* gene, which encodes 9-*cis*-epoxycarotenoid dioxygenase (NCED, EC 1.13.11.51), a key enzyme in ABA biosynthesis (Bhaskara et al. 2012, Behnam et al. 2013). In *Arabidopsis*, the overexpression of *AtNCED3* has been found to increase endogenous ABA levels, promote the transcription of drought- and ABA-inducible genes, and improve drought tolerance in GM plants (Iuchi et al. 2001). The *NCED3* gene has also been reported to be strongly induced under simulated drought or greenhouse conditions as well as in several economically important crops like tomatoes (Burbidge et al. 1999), common beans (Qin and Zeevaart 1999), cowpeas (Iuchi et al. 2000), avocados (Chernys and Zeevaart 2000), peanuts (Wan and Li 2006), turmeric (Ahrazem et al. 2012), citrus (Rodrigo et al. 2006, Neves et al. 2013, Pedrosa et al. 2015), and soybeans (Molinari et al. 2020).

Here, we evaluated the drought tolerance of soybean lines genetically modified for the *AtDREB1A*, *AtDREB2A*, *GmDREB2A*, and *AtAREB1* TFs and the *AtGolS2* and *AtNCED3* genes under both greenhouse and field conditions. The plants were phenotyped by drought tolerance in water-deficit and control treatments based on molecular, physiological, growth, agronomical, and survival parameters. It is important to highlight that the data obtained under greenhouse conditions indicated the potential of the DREB and AREB TFs and *GolS* and *NCED* genes to develop genetically modified drought-tolerant soybean lines. These data were generated under controlled conditions in which light, temperature, water, weed, insect, and disease levels

were monitored. According to Passioura (2012), results obtained in greenhouses may not be representative of the way in which plants behave throughout an entire season under actual field conditions. As such, this study presents a comparison of the results obtained in the field, where researchers can accurately gauge whether a technology is successful, with those obtained under greenhouse conditions. Similarly to that of other countries, field tests constitute a legal requirement of the Brazilian National Technical Biosafety Commission that must be both fulfilled and approved prior to the authorization of a commercial product.

Furthermore, the combined knowledge obtained from both greenhouse and field tests may provide new insights into the mechanisms of drought tolerance in soybean plants that may help breeders to choose the best performing lines for their breeding programs and develop cultivars that may be released to producers, which continue to face production challenges associated with drought conditions.

Soybeans genetically modified for drought tolerance

Brief description of the obtention of the soybean GM lines

Several soybean conventional cultivar backgrounds were used to obtain the GM lines containing the different genetic constructions: *rd29A:AtDREB1A*, *rd29A:AtDREB2A*, *35S:AtAREB1*, *35S:GolS2*, *35S:AtNCED3*, *35S:GmDREB2AFL*, and *35S:GmDREB2ACA*. As a standard protocol, these constructions were introduced via electroporation into the *Agrobacterium tumefaciens* strain EHA 105 (Hood et al. 1993) as described by Casali and Preston (2003). Both *rd29A:AtDREB1A* and *rd29A:AtDREB2A* were under the control of the inducible promoter *rd29A*. The other five genetic constructions were under the control of the constitutive promoter CaMV *35S* (Cauliflower mosaic virus). All vectors contained the *NOS* terminator (*A. tumefaciens* nopaline synthase) and two marker genes in the cassette structure: the *bar* gene (phosphinothricin acetyl transferase), which confers resistance to the herbicide ammonium glufosinate and was used as a selective agent; and the *NPTII* gene (Neomycin phosphotransferase), which confers resistance to the antibiotic kanamycin and was used to select the colonies containing the inserted transgene.

The genetic backgrounds (soybean conventional cultivars) were transformed using the *A. tumefaciens* method described by Paz et al. (2006). A modification was introduced that aimed to improve injury from infection; thus, each cotyledon was scratched 10 to 12 times using a stainless-steel micro brush. Seedlings developed during the selection process were transferred to a substrate/sand (1:1) mixture with the substrate containing soil/sand/organic compounds (3:2:2). Seedlings were maintained in a growing chamber and acclimated for at least 1 week. After which, the seedlings were transferred to a greenhouse and were molecularly evaluated to identify the presence of the transgenes of interest.

The confirmation of possible positive events was performed through conventional PCR assays using specific primers to identify the inserts. Thus, genomic DNA was extracted from leaf tissues (Doyle and Doyle

1987). The PCR reaction was performed in a final volume of 25 μL composed of 5 μM of each forward and reverse primer, 0.4 mM dNTPs, 2 mM magnesium chloride, 1 U Taq DNA polymerase, and 50 ng μL^{-1} DNA. Amplifications were performed in a Veritti® (Applied Biosystems, Foster City, USA) thermocycler using the following cycling parameters: an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72° C for 30 s, with a final elongation cycle of 72 °C for 5 min. The PCR products were analyzed using 1% agarose gel electrophoresis (1x SB) stained with ethidium bromide.

Plants positive for the transgenes were grown in a greenhouse for event selection with 3:1 Mendelian segregation in the T_1 generation, and the generation was allowed to progress to obtain homozygous seeds. Homozygous plants were used in the subsequent experiments to evaluate growth, survival, molecular, physiological, and agronomical parameters under greenhouse (GH) and field conditions in the water-deficit and control treatments.

Brief description of water deficit experiments

Greenhouse (GH) conditions

To phenotype the GM lines under GH conditions, several experiments were carried out. As a standard procedure, seeds from all GM events and from conventional soybean cultivars (WT plants) were treated with Vitavax® Thiram 200 SC (200 g L^{-1}; ADAPAR) for health quality purposes and then allowed to germinate on Germitest® paper for 96 h at 25 ± 1 °C and 100% relative humidity (RH). All plants used in these GH experiments had been previously identified as being positive for the target gene of interest.

In one of the experiments carried out under GH conditions with *AtDREB1A* lines, after germination, the plants were cultivated in pots containing sand and soil with 15% gravimetric humidity (GravH) for 31 days post-sowing until reaching the reproductive stage R1 (Fehr et al. 1971). Immediately after reaching R1, irrigation was withheld in the drought treatment pots until the GravH values decreased to 5% (moderate water deficit, MoWD). Twenty-nine days later, irrigation was further reduced to 2.5% GravH (severe water deficit, SWD) for approximately 30 days until harvest. The control plants were kept at 15% GravH throughout the experiment. To keep the pots at the desired GravH, they were weighed twice a day and water was added as needed (Casagrande et al. 2001). Photosynthesis (*A*), stomatal conductance (*gs*), the transpiration rate (*E*), and chlorophyll content were measured for each treatment (i.e., BR 16-treated, BR 16-control, *AtDREB1A* line P58-treated, and P58-control) under moderate and severe water deficits using a LI-6400 portable photosynthesis system (Li-Cor, Lincoln, USA) and a SPAD-502 chlorophyll meter (Sakai, Japan). Plant height was measured in each treatment. An analysis of variance (ANOVA) and Tukey post-hoc tests were performed using SAS software (Cary, USA). Anatomical analyses were also performed for samples at the R2 development stage with a MoWD (5% GravH) as well as at R4 stage with a SWD (2.5% GravH).

In another experiment conducted under GH conditions, after germination, the seedlings were

transferred to 1-L pots filled with a substrate mixture of soil:sand:organic compounds (3:2:2). Each pot contained only one seedling. All seedlings were maintained in a greenhouse at 28 ± 2 ºC, with temperature and relative humidity (RH) recorded every 5 min with a Hobo U14-002 thermistor (Onset®, Bourne, USA). The experiment was set in a complete randomized block design with a factorial arrangement of treatments, two water conditions (water deficit, WD; control, C), two plant types (GM and non-GM plants), and nine replications. Pots containing the GM lines were maintained at a 100% soil field capacity (FC) through daily irrigation with a fixed water volume sufficient to saturate the substrate until plants reached the phenological stage V4. At this stage, one day before WD induction, all pots were saturated with water at the end of the afternoon to allow the excess water to be drained overnight. The following morning, the pots were wrapped in polyethylene bags, and the central region of each pot was covered with cotton around the stem base in order to prevent water loss by evaporation. Then, the pots with control plants were maintained at 100% soil FC, while irrigation was withheld in the WD group, which was monitored daily in relation to stomatal conductance (*gs*). As a standard protocol to confirm the induction of the WD, when plants showed *gs* values less than 200 mmol H2O m^{-2} s^{-1} (Flexas et al. 2004, Salinet 2009), the gas exchange parameters (*A*, sub-stomatal CO_2 (*Ci*), *E*, and gs) were measured on the central leaflet of the third fully-expanded trifoliate leaf (apex-base direction) with an LC pro-SD portable infrared gas analyzer (ADC BioScientific, Hoddesdon, UK) in three plants. Measurements were performed inside the greenhouse at 9:00 a.m. (Brazilian daylight savings time) with 1000 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR). The intrinsic water use efficiency was obtained through the ratio *A/gs.* Thereafter, the same trifoliate leaf was sampled, wrapped in aluminum foil, immersed in liquid nitrogen, and stored at -80 ºC for the analysis of gene expression by RT-qPCR (Real-Time Quantitative Polymerase Chain Reaction).

After seven days of withheld irrigation, the number of nodes (NN) was counted in a sample of five plants, and the leaves were collected. Total leaf area was measured in this 5-plant sample using a LI-3100C leaf area meter (Li-Cor, Lincoln, USA). Then, the leaf blades, stems, petioles, and roots were dried with a forced aeration oven at 60 °C to constant weight so that shoot (leaf blades + stems + petioles) and root dry matter (per plant) could be weighed. Plant height was measured at the start (H1) and end (H2) of the WD period. The mean length of the internodes corresponded to the ratio between H2 and the number of nodes. The relative growth rate in height (RGRH) was calculated according to Eq (1):

$$RGRH\ (\%) = \frac{(H2-H1)}{H1} \times 100 \qquad \text{Eq. (1).}$$

Following the evaluation of the growth parameters and the sampling of the trifoliate leaves for the gene expression analysis, the plants were transferred to 8-L pots filled with a substrate composed of soil:sand:organic compounds (3:2:2) and maintained under continuous irrigation conditions until the end of the cycle, when the agronomical traits of the number of seeds, number of pods presenting seeds, total number of seeds, and yield were evaluated per plant.

Survival experiments were also carried out in the GH GM lines. The experimental design was completely randomized and included 10 plants. Prior to the WD initiation, all pots were saturated with water, drained overnight, and covered with plastic bags until the following morning, after which, irrigation was withheld. The monitoring of the water deprivation symptoms was performed visually and daily. When plants of the drought-sensitive cultivar BR 16 were almost 100% dry, the plants were watered to avoid death. After the plants that remained alive had recovered leaf turgor following rehydration, the number of plants that survived the water deficit was determined.

A hydroponic experiment to simulate the WD in the roots was conducted with lines containing *AtDREB2A* FL according to the protocol of Martins et al. (2008). At the V3 vegetative developmental stage, dehydration treatments lasting 0 (control, without stress), 30, 60, and 90 min under WD conditions were applied in triplicate, with each plant being considered a biological replicate. Triplicate measurements of the photosynthetic rate, stomatal conductance, transpiration, and leaf-air temperature differences were obtained during the treatments using an LI-6400 portable photosynthesis system (Li-Cor, Lincoln, USA). The data were analyzed statistically using the Duncan test at 5% significance ($p < 0.05$) in SAS software (Cary, USA).

Likewise, a molecular characterization was performed of the plants submitted to experiments under GH conditions. In addition to the expression of the transgenes introduced into the soybean genotypes, many other drought response-related genes were analyzed. In the *AtDREB1A* GM lines, the genes assayed were *GmLEA14* (late embryogenesis abundant; Glyma.18G238700), which encodes a contributor to osmotic stress protection in both embryonic and vegetative tissues; *GmGR-RBP*, which encodes a glycine-rich RNA-binding protein (Glyma.12G043000); *GmPI-PLC*, which encodes a phospholipase C (Glyma.14G059200); and *GmSTP*, which encodes a sorbitol transporter protein (Glyma.11G119500; Polizel et al. 2011).

In the *AtAREB1* GM soybean lines, the expression level of the 2C-type protein phosphatase *PP2C* (Glyma.14G195200), G*mSRK2* kinase (Glyma.02G135500), and *GmRAB18* (Glyma.09G185500) genes was assessed based on the results of the cDNA microarrays of *A. thaliana* under water deficit conditions (Fujita et al. 2009, Yoshida et al. 2010).

The GM lines for the *AtGolS2* gene were assayed to determine the expression level of the genes *GmRS1* (Glyma.03G137900) and *GmRS3* (Glyma.19G004400), which encode raffinose 1 and raffinose 3 and the late embryogenesis abundant proteins *LEA2* (Glyma.09G185500) and *LEA6* (Glyma.17G164200), respectively, (Honna et al. 2016). The *AtNCED3* lines were assessed to determine the expression levels of ABA-dependent genes, such as *GmAREB1* (Glyma.04G039300, Glyma.07G213100, and Glyma.02G131700), *GmPP2C* (Glyma.14G195200), *GmSnRK2* (Glyma.02G135500), and *GmAAO3* (Glyma.14G045100; Molinari et al. 2020). For the GM lines containing *AtDREB2A*, a molecular characterization was carried out for samples collected under field conditions (Fuganti-Pagliarini et al. 2017).

Field conditions

Before installing the experiment, all of the necessary documentation to test GM lines under field conditions were submitted and approved by the National Technical Biosafety Commission (CTNBio). The permissions to carry out the experiments were published in the Brazilian Official Journal.

The first field experiment was conducted during the 2011/2012 crop season as a "pilot screening." Subsequent experiments were carried out in the field area located at the National Soybean Research Center (23°11' S, 51°11' W, 630 m altitude; Embrapa Soja, Londrina, PR, Brazil), which is a branch of the Brazilian Agricultural Research Corporation, during the crop seasons of 2013/2014 to 2017/2018. A split-plot design was used in a complete randomized block design with four blocks. The plots corresponded to irrigated (IRR, water from precipitation + irrigation when needed) and non-irrigated (NIRR, water from only precipitation) water conditions and artificially drought simulated (DS) conditions at the vegetative (DSV) and reproductive (DSR) stages. To mimic drought conditions, the plants were sheltered from the rain by rainout shelters programmed to automatically close when rainfall was detected and to open as soon as the rain ceased.

The subplots corresponded to the conventional Brazilian soybean cultivars and GM lines for the *AtDREB1A*, *AtDREB2A*, and *AtAREB1* TFs and the *AtGolS2* and *AtNCED* genes. The area of each subplot was 220 m^2 for the IRR and NIRR treatments. The seeds were sown with 0.5-m spacing between rows and 16 plants m^{-1}. Plants of the soybean cultivar BRS 295RR were used as a 10-m wide isolation border around the experiment, following the stipulations of Brazilian legislation. The air temperature and relative humidity were monitored daily with a weather station located adjacent to the experimental area.

Soil chemical corrections and cultivations were performed according to recommendations for the crop (Embrapa 2013). During the experiment, physiological and agronomic evaluations were performed. The net CO_2 assimilation rate (*A*), transpiration rate (*E*), and stomatal conductance (*gs*) were measured from the central leaflet of the third fully-expanded trifoliate leaf (apex-to-base direction) of one plant located in the middle portion of each subplot with a LCpro-SD portable infrared gas analyzer (ADC BioScientific) calibrated for 1000 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) under sunny sky conditions between 9 and 11 a.m. (Brazilian daylight savings time). After gas exchange measurements were taken, both the instantaneous (*A*/*E*) and intrinsic (*A*/*gs*) water use efficiency (WUE) were calculated. The chlorophyll index (SPAD) was measured in one lateral leaflet from the same aforementioned trifoliate leaf using a SPAD-502 portable chlorophyll meter (Minolta). Plant height was calculated as the mean distance between the cotyledonary node and the stem apex from five plants per subplot. The mean length of the internodes corresponded to the ratio between the height per plant and number of nodes per plant. The leaf area index (LAI) corresponded to the ratio between the total leaf area, which was obtained with an LI-3100C area meter (LI-COR), and the soil area occupied by the plants. The total dry matter of the pods and seeds per plant and grain yield per plant were evaluated (10 plants per subplot) at harvest. These measurements were obtained

for all four experimental blocks at the reproductive developmental stage. The percentage of protein and oil content in the soybean grain samples at harvest was determined from whole seeds and grains using the near infrared (NIR) reflectance technique according to the methodology of Heil (2010). For lines containing DREB1A and DREB2A TFs, these parameters were assayed during the crop seasons of 2013/2104 and 2014/2015. For lines containing the *AtAREB1A* FT or the *GolS2* and *NCED3* genes, oil and protein content was sampled in four crop seasons from 2014/2015 to 2017/2018.

All residuals presented normal distributions and met ANOVA assumptions. Thus, the data were analyzed via an ANOVA, and the means were compared by a Tukey post-hoc test ($p \leq 0.05$).

Molecular analyses were performed to evaluate transgene expression under field conditions. Thus, three samples from three different blocks were collected individually based on physiological results. Samples were immediately placed into liquid nitrogen and stored in a freezer at -80 °C until RNA extraction. Total RNA was extracted from the leaf samples using Trizol® reagent. Following RNA extraction, the samples were treated with DNAse I. To verify the presence of any remaining genomic DNA, a conventional PCR was performed. cDNA synthesis was carried out using the Super Script III First Strand kit (Thermofisher Scientific, Waltham, USA) according to the instructions of the manufacturer. The expression levels of the transgenes *AtDREB1A*, *AtDREB2A CA*, and *AtAREB1 FL* were assessed by qPCR. Also, based on a search of the available literature, some genes related to drought responses were selected. These analyses were carried out only for the GM soybean event 1Ab58 (*AtDREB1A*), 1Bb2193 (*AtDREB2A CA*), 1Ea2939 (*AtAREB1)* lines and the conventional cultivar BR 16. Soybean lines containing *AtGolS2*, *AtNCED3*, *GmDREB2A;2 FL*, and *GmDREB2A2; CA* were not included in this evaluation. Thus, the expression level of the chosen genes was quantified under IRR and NIRR conditions. Genes related to drought response (i.e., stomata overture/closure and osmotic adjustment), photosynthesis, and metabolic and hormone pathways (i.e., nitrogen assimilation), drought proteins (e.g., dehydrins, DHNs), heat shock proteins, and water channels were chosen. Therefore, the selected genes were phosphatase *GmPP2C* (Glyma.14G195200), alanine aminotransferase *GmAlaAT* (Glyma.01G026700 and Glyma.07G045900), Δ-1-pyrroline-5-carboxylate synthetase (P5CS; Glyma.18G034300), galactinol/GolS (Glyma.10G145300), late embryogenesis abundant/LEA18 (Glyma.17G164200), DHN (Glyma.09G185500), heat shock protein (Glyma.17G072400), putative soybean aquaporin pip1/UDP galactose transporter (Glyma.12G066800), putative soybean aquaporin pip2/aquaporin transporter/glycerol uptake facilitator (Glyma.12G172500), ribulose-1,5-bisphosphate carboxylase/ oxygenase (small chain; Glyma.13G046200), and chlorophyll a/b binding protein (Cab21; Glyma.16G165800; Fuganti-Pagliarini et al. 2017).

Using the gene sequences obtained from Phytozome, sets of primers for each gene were designed using the Primer3Plus platform available online (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi). To verify homo- and heterodimers and hairpin formations, multiple primer analyzer software was used

(http://www.thermoscientificbio.com/webtools/multipleprimer/). Quantitative PCR reactions were carried out in biological and technical triplicate using the Platinum® SYBR Green® qPCRSuperMix-UDG kit with ROX (Thermofisher Scientific, Waltham, USA) according to the instructions from the manufacturer in a 7900HT Fast Real-Time PCR System with a 384-well block (Thermofisher Scientific, Waltham, USA). The β-actin gene (No. Access: GMU60500) was used as the reference gene (Stolf-Moreira et al. 2011).

The efficiency of the amplification reaction was estimated using five serial dilutions of cDNA (1×, 5×, 25×, 125×, and 625×). To compute the efficiency of the reaction (E), the relationship presented in Eq (2) was used:

$$E = \left[10^{\frac{-1}{slope}}\right]^{-1} \quad \text{Eq. (2)}$$

where the =SLOPE (Average Ct value range, log quantity range). Only primers with > 90% efficiency were used. The cycling parameters for the reactions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15s and 60 °C for 1 min. To evaluate the specificity of the amplified products, a dissociation curve was generated at the end of each reaction. The relative expression was determined by normalization to the reference gene β-actin. Expression was calculated by the $2^{-\Delta\Delta Ct}$ method (Bustin 2002).

Results from greenhouse and field characterization

Soybean lines containing DREB transcription factors

Different soybean lines containing the *AtDREB1A* TF were phenotyped under greenhouse conditions. One of the first studies was carried out with the P58 line (Polizel et al. 2011). In this line, *AtDREB1A* gene expression was higher in the genetically modified P58 plants under water deficit conditions, demonstrating transgene stability in the T_2 generations and the induction of the *rd29A* promoter. Drought-responsive genes, such as *GmPI-PLC*, *GmSTP, GmGRP* and *GmLEA14,* were highly expressed in plants submitted to the severe WD treatment (gravimetric humidity at 2.5%). Genetically modified plants showed higher stomatal conductance and consequently higher photosynthetic and transpiration rates. In addition, they had more chlorophyll. The overexpression of *AtDREB1A* may have contributed to a decrease in leaf thickness; however, a thicker abaxial epidermis was observed (Polizel et al. 2011).

A subsequent experiment was carried out with additional *AtDREB1A* lines. Rolla et al. (2013) evaluated GH plants from the P58 and P1142 lines in the T_8 and T_5 generations, respectively. A growth analysis of the plants under well-watered (C, control) and WD conditions showed that the *AtDREB1A* plants exhibited lower heights (C/WD), the same number of nodes (C/WD), a slightly higher number of leaves (WD), and a greater leaf area (C/WD) than that of the BR 16 plants. However, a statistical analysis of these data showed that none of these differences were statistically significant, indicating that the transformation of

soybean plants with the *AtDREB1A* gene under the control of the *rd29A* promoter did not lead to any retardation of the growth of the transformed plants. Although there were no statistically significant differences in the relative growth ratio and the percentage of growth reduction under water deficit conditions when compared to that of the control plants, the *AtDREB1A* lines exhibited a more conservative growth pattern under control conditions and slightly increased their growth rates under WD conditions compared to that of the control plants. Furthermore, although not statistically significant, *AtDREB1A* plants seemed to have a higher number of leaves and a greater leaf area than the BR 16 plants, at least in the latter stages of development (Rolla et al. 2013).

As has already been reported, previous studies under GH conditions showed that the *AtDREB1A* P58 line had a slow-wilting phenotype and was able to maintain a higher rate of photosynthesis and higher photosynthetic efficiency under water-deficit conditions than that of the control plants (Polizel et al. 2011). In this second study, GH data suggested that the higher survival rates of the *AtDREB1A* plants (70%, 60%, and 40% for GM lines P58, P1142, and the conventional cultivar BR16, respectively, after a severe water deficit) were due to lower water use resulting from lower transpiration rates under well-watered conditions in the GH experiments (**Fig. 1A**; Rolla et al. 2013).

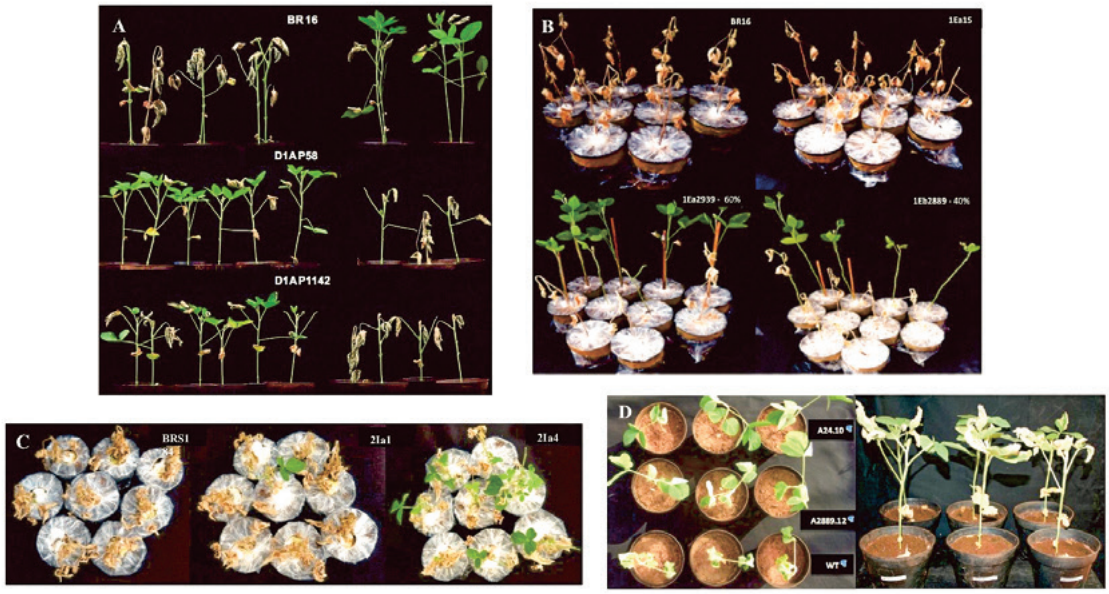

Fig. 1. Survival rate of soybean plants genetically modified with *AtDREB1A* (A), *AtAREB1A* (B and D), and *AtGolS2* (C) and their respective parental cultivar.

In A, DREB1A plants P58 and P1142 show 60% and 70% overall survival rates, respectively, while that of BR 16 is 40%. These lines were submitted to a 6-day water deficit followed by three days of recovery. In B and D, the *AtAREB1A* lines 1Ea15, 1Ea2939, A24, and A2889, and the WT cultivar, BRS 184, are shown. These lines were submitted to 17 days of withhold irrigation followed by 7 days of re-watering. In C, the survival rate of the *AtGols2* lines 2Ia1 and 2Ia4 submitted to withheld irrigation for 20 days followed by nine days of rehydration are shown. The *AtDREB1A* lines were under the control of the stress-inducible rd29A promoter, while the *AtAREB1A* and *AtGolS2* lines were under the control of the constitutive promoter 35S. Adapted from Rolla et al. 2013, Barbosa et al. 2012, and Marinho et al. 2016.

In addition to DREB1A from *Arabidopsis thaliana*, the GM soybean plants were obtained using *GmDREB2A;2 FL* and *GmDREB2A;2 CA* genes, which were under the control of the constitutive promoter CaMV *35S*. These lines were characterized for molecular, physiological, phytometric, and agronomic responses at three developmental periods (i.e., germinative, vegetative, and reproductive; Marinho et al., 2020, submitted). Therefore, three different and independent lines were submitted to WD experiments conducted under GH conditions. Molecular data showed that transgene expression was significantly higher for all three GM events under control and water deficit conditions when compared to that of the conventional background cultivar BRS 283. Drought-responsive genes were also induced in the *GmDREB2A;2 CA* and *GmDREB2A;2 FL* lines. Under the imposed water deficit, in both the vegetative and reproductive developmental stages, the expression level of *LEA6* (Glyma.17G164200), *LEA2* (Glyma.09G185500), and heat shock protein *HSP70* (Glyma.17G072400) genes was higher for all GM events when compared to that of the conventional cultivar BRS 283 (Marinho et al. 2020, submitted). LEA2 and LEA6 are Dehydrins (DHNs), which typically accumulate at the seed maturation stages and in plant tissues in response to drought, high salinity, low temperatures, or treatment with ABA (Battaglia et al. 2008). The contribution of DHNs to the abiotic stress tolerance of plants occurs mainly due to their protective effects on lipid membranes (Bao et al. 2017). Koag et al. (2009) have identified that the interaction of such proteins with lipids in the membrane or with partially denatured proteins helps to protect cells against damage caused by low water potential. Yet, DHNs play critical roles in determining desiccation tolerance by capturing water and stabilizing and protecting the structure and function of proteins and membranes in addition to acting as molecular chaperons (as heat shock proteins) and hydrophilic solutes to protect cells from damage due to water shortages (Hand et al. 2011).

Phytometric data showed a reduction in the growth rate for total seedling length and root length under osmotic conditions (-0.2 MPa of polyethylene glycol, PEG-8000); however, seedlings from the *GmDREB2A;2 FL* line presented a lower reduction in growth during germination. The physiological parameters evaluated indicated that the WD imposition resulted in a sharp reduction in all gas exchange parameters (*gs*, *Ci*, *A*, and *E*). For the *gs* and *Ci* parameters, the results indicated a lower performance of the conventional cultivar BRS 283 when compared to that of the GM events in the WD treatments; however, the interaction between genotypes and water conditions was not significant. Similarly, the *gs* of the cultivar BRS 283 also showed a lower trend compared to that of the GM lines. In contrast, the CO_2 intercellular concentration was higher in the conventional cultivars when compared to that of the GM events (Marinho et al. 2020, submitted).

The physiological responses of the GM plants, especially *A*, indicated improved physiological performance under conditions of water restriction. Drought conditions lead to a deficit in *A* caused by stomata and non-stomata limitations. Therefore, the low values of *A* found in the conventional cultivar BRS 283

might have contributed to its lower tolerance compared to that of the GM plants, which was reflected in a lower green leaf area (Marinho et al. 2020, submitted).

Under GH conditions, when WD was imposed in the vegetative and reproductive developmental stages, the GM *GmDREB2A;2 FL* line presented the highest yield after drought imposition during the reproductive period when compared to that of other materials. These data suggest that it is possible to obtain soybean GM lines with the *GmDREB2A;2* TF with minor damage to final yields, which is extremely desirable when considering a grain crop, such as soybeans. It was proposed that the *GmDREB2A;2 FL* line presented superior performance due to the high expression levels of the transgenes and drought-induced genes. These studies also indicated that the *GmDREB2A;2* TF participates in important responses to water deficits during different periods of soybean development (Marinho et al. 2020, submitted).

For the *AtDREB2A* TF, laboratory and GH studies were performed with the GM soybean lines P1397 and P2193. In a biological oxygen demand (BOD) incubator, leaflets from the T_0 and T_1 generations were dehydrated for 30 and 90 min by subjecting the detached leaves to a temperature of 30 °C and 60% humidity. Leaflets of the non-transformed conventional soybean cultivar BR 16 were used as negative controls. Results of the molecular characterization indicated a greater stability of transgene *rd29A:AtDREB2A CA* expression in the P2193 line compared to that of the P1397 line, which only exhibited notable expression in the control plants from the T_1 generation. Transgene instability in the P1397 line was further demonstrated by the possible repression of gene expression after a 90-min exposure to treatment (Engels et al. 2013).

Under GH conditions, a hydroponic experiment was carried out to simulate drought in the roots of the *AtDREB2A CA* lines. Molecular data obtained from the roots and leaves showed that both GM lines exhibited high expression of the transgene, with the roots of P2193 showing the highest expression levels during water deficit conditions. The physiological parameters examined during WD conditions confirmed the induction of stress. The *A, Ci*, *E*, and leaf-air temperature values of the *AtDREB2A CA* transgenic plants differed from those of the control cultivar BR 16 for each treatment (Engels et al. 2013). These findings confirmed the presence of stress in the hydroponic system, with a tendency to reduce the rates of stomatal conductance, photosynthesis, and transpiration (Jaleel et al. 2009).

The first experiment carried out under field conditions as an initial screening was conducted during the 2011/2012 crop season with the *AtDREB1A* lines P58 and P1142 and the genotype 09D-0077, which resulted from a cross between the P58 line and its isoline, the BR 16 cultivar. The field performance of the P58 and 09D-0077 plants was evaluated under four different water regimes: irrigated (IRR), natural rainfall (NIRR, non-irrigated), and drought simulated (DS), in which plants were sheltered from the rain in the vegetative (DSV) or reproductive (DSR) stages. The drought treatments affected plant productivity as well as their growth and yield components. Under conditions of water deficits in both the vegetative and reproductive stages, the main effects of the *AtDREB1A* gene were observed in the changes in plant height

due to a shortening of the internode, at least at the initial stages of crop growth in the field. However, there were no significant differences in the growth components between P58 and 09D-0077 plants under field conditions. No significant differences were observed in yield components among genotypes, except for the number of nodes, which was higher for the P58 line in the non-irrigated treatment and the 09D-0077 cross under a water deficit during the vegetative stage compared to that of the control plants. Although the DREB plants did not outperform their isoline (cultivar BR 16) in terms of yield, there was a clear tendency towards the superiority of the DREB line with regard to some yield components, such as the number of seeds and the total number of pods when WD was applied during the vegetative stage (Rolla et al. 2013).

The soybean *AtDREB1A* line P58 and *AtDREB2A CA* line P2193 were assayed under field conditions during the crop seasons of 2013/2014 (**Fig. 2**) and 2014/2015 for physiological and agronomical parameters (Fuganti-Pagliarini et al. 2017). The results from the crop season of 2013/2014 for instantaneous (*A*/*E*) and intrinsic (*A*/*gs*) water use efficiency and the LAI did not show any significant interactions between water conditions and plant materials. In each water condition, there were no differences between plant materials with regard to *A*/*E*. The lines 1Ab58 and 1Bb2193 showed similar behaviors to that of the wild type (WT-BR 16) plants with regard to chlorophyll content regardless of the water conditions. With regard to plant height and the mean length of internodes, the lines 1Ab58 and 1Bb2193 showed similar values relative to those of the WT genotype in the IRR and NIRR treatments. With regard to the total dry matter of pods and seeds per plant, the line 1Bb2193 showed lower values in the NIRR treatment for both agronomical traits compared to that of the control plants (Fuganti-Pagliarini et al. 2017).

Fig. 2. Field experiment during the crop season of 2013/2014.
A split-plot design was used in a complete block randomized design with four blocks. The plots corresponded to irrigated (IRR, water from precipitation + irrigation when needed) and non-irrigated (NIRR, water from only precipitation) water conditions and artificially drought simulated (DS) conditions at the vegetative (DSV) and reproductive (DSR) stages. To mimic drought conditions, the plants were sheltered from the rain by rainout shelters programmed to automatically close when rainfall was detected and to open as soon as the rain ceased. Subplots corresponded to the conventional Brazilian soybean cultivar BR 16 (genetic background of GM lines) and the GM lines for the *AtDREB1A*, *AtDREB2A* and *AtAREB1* TFs.

Yield data collected during the crop season of 2013/2014 showed that the WD caused significant losses to both DREB lines. For P58 and P2193, when a WD was applied during the reproductive stage, the yield value reached 805 and 765 kg ha^{-1}, respectively, and was more harmful to the crop compared to that when the WD was imposed during the vegetative stage (yield of 1.509 and 1.632 kg ha^{-1} for the P58 and P2193 lines, respectively). Under irrigated conditions, the values registered were 2.929 and 2.665 kg ha^{-1} for the *AtDREB1A* and *AtDREB2A CA* lines, respectively. It is important to highlight that intense water deficit periods were recorded during important developmental stages, such as flowering and pod filling, that resulted in a decrease in final yield, although the total rainfall of the crop season of 2013/2014 fell within the recommendations for the soybean crop (Fuganti-Pagliarini et al. 2017).

No differences were identified for physiological and agronomic parameters in the field experiment performed during the crop season of 2014/2015, which was probably due to the optimum rainfall volume and homogeneous distribution observed during the whole cycle, which thus resulted in a small water deficit in the plants. According to the data collected by the weather station located at the experiment site, a total rainfall of 790.8 mm was registered. The water recommendations for soybean crops vary between 450 and 800 mm/cycle, depending on the weather conditions, crop management, and the cycle duration (Embrapa 2013).

Although a short water deficit period occurred in October/2014, the experiment was sown on November 6th. Thus, no significant water deficit period occurred during the cycle, and no differences were present between GM lines and the BR 16 cultivar. As no differences were identified, no molecular analyses were performed (Fuganti-Pagliarini et al. 2017).

The gene expression analysis performed on samples collected under field conditions during the crop season of 2013/2014 showed that the expression of the transgenes *AtDREB1A* and *AtDREB2CA* was induced in the NIRR treatment for each respective transgenic line. Among these TFs, higher expression was identified for the *AtDREB1A* gene (line 1Ab58). No expression was identified for the BR 16 soybean conventional cultivar. In the P58 and P2193 lines, higher levels of Cab21 (Glyma16g165800), phosphatase *GmPP2C* (Glyma.14G195200), and putative soybean aquaporin pip1/UDP galactose transporter (Glyma.12G066800) were identified when compared to the background of BR 16, illustrating that WD response mechanisms were activated under field conditions (Fuganti-Pagliarini et al. 2017).

Neither the oil nor protein content in soybean seeds were affected by insertion of the *AtDREB1A* and *AtDREB2A CA* TFs. As no significant difference was present between WD treatments, the data were analyzed combined. During the crop season of 2013/2014, the protein content in the P58 and P2193 lines was 37.8% and 37.7%, respectively. The oil content in the seeds ranged from 20.9% (P58) to 21.4% (P2193). During the crop season of 2014/2015, the overall protein content values were higher (40.1% and 39.2% for the P58 and P2193 lines, respectively) while the oil content values were lower (19.6% for both GM lines) when compared to those of the crop season of 2013/2014, which fell within the parameters accepted by the consumer market, which are protein levels between 40-45% and lipid levels between 18-20% (Embrapa 2015).

Soybean lines containing the AREB transcription factor

Two different genetic constructions with the *AtAREB1* TF were introduced into soybean plants: *35S:AtAREB1 FL* (full-length) and *35S:AtAREB1ΔQT*, a constitutive active form of AREB1 that presents a conserved transcriptional activator P domain plus the native bZIP DNA binding domain (Fujita et al. 2005). The soybean GM lines were obtained for both strategies (Barbosa et al. 2012, Leite et al. 2014).

A GH experiment with soybean lines overexpressing *AtAREB1 FL* was performed to assay the physiological parameters under water deficit and control conditions. The GM lines, A2889.12 and A24.10, showed the ability to survive for a period of 5 days without water and exhibited no leaf damage (**Fig. 1B**). In addition, these lines remained able to grow under WD conditions, which was verified by their higher relative rates of shoot length (RRSL) compared to that of the WT plants (conventional cultivar BR 16; Barbosa et al. 2012).

Furthermore, these lines also displayed better growth and physiological performance under water deficit conditions (i.e., higher RRSL, *gs*, and *A*) when compared to that of the wild type plants, which may

have been related to the responses triggered by the transgene. Particularly, line 1Ea2939 showed a higher total number of pods and seeds and increased dry seed matter compared to WT plants. The best performance of line 1Ea2939 relative to that of the BR 16 plants might have been related to the mechanisms of drought prevention, such as reduced stomatal conductance or leaf transpiration under control conditions with no water restriction (Barbosa et al. 2012).

The other lines, 1Eb2889 and 1Ea15, were obtained with the *AtAREB1 FL* gene and additional experiments were carried out under GH conditions. *AtAREB1* expression was observed in the transgenic lines 1Ea2939 and 1Eb2889 but not in the 1Ea15 line. The phenotypic analyses of the growth parameters indicated that in the early stages of seedling development under well-watered conditions, there were no differences in plant growth among the transgenic lines 1Ea15, 1Ea2939, and 1Eb2889 when compared to that of the conventional soybean cultivar BR 16. These results allowed the authors to infer that the transformation of soybean plants with *AtAREB1*, under the control of the constitutive promoter CaMV *35S*, did not alter the growth characteristics of the transformed plants. Considering the number of nodes (NN), there was a significant interaction between genotype/transgenic lines and water conditions. Thereby, the GM lines 1Ea2939 and 1Eb2889 presented a similar NN under both water conditions, whereas the BR 16 and 1Ea15 plants presented lower values under WD conditions (Marinho et al. 2016).

Physiologically, transpiration data collected throughout the water deficit period revealed that in the first days after withholding irrigation and WD imposition (2–3 days), the transpiration rates of BR 16 and 1Ea15 plants were higher relative to that of the other two GM lines (1Ea2939 and 1Eb2889). This result was probably due to the higher *gs* of the BR 16 and 1Ea15 plants grown under well-watered conditions (C). The differences in the transpiration rates among the genotype/GM lines at the beginning of the WD period resulted in a lower water status in the substrates used to grow the BR 16 and 1Ea15 plants, and as a result, these plants presented lower transpiration rates compared to that of the plants of the GM lines 1Ea2939 and 1Eb2889 (Marinho et al. 2016).

Furthermore, under well-watered conditions, the GM lines 1Ea2939 and 1Eb2889 presented lower stomatal conductance relative to that of the cultivar BR 16, confirming that the lower transpiration rates presented by these plants were due to lower *gs* and not to lower leaf areas. However, under WD conditions, the highest values of *gs* were found for the event 1Ea2939, followed by the event 1Eb2889, when compared to that of the BR 16 and 1Ea15 plants. The differences in *gs* values among genotype/GM lines under WD conditions were likely due to differences in the manner in which the plants depleted the water from the soil throughout the experimental period. Plants that had higher *gs* (BR 16 and 1Ea15) during the initial period after withholding irrigation presented a rapid depletion of water from the soil due to their high transpiration rates. However, the opposite was observed for 1Ea2939 and 1Eb2889 plants (i.e., such plants showed slow water depletion from the substrate) that led to water conservation and the maintenance of higher gas exchange

rates when compared to that of the event 1Ea15 or the cultivar BR 16 (Marinho et al. 2016).

The decreased *gs* values in the GM lines 1Ea2939 and 1Eb2889 resulted in lower intercellular CO_2 concentrations under control conditions. However, this reduction in *gs* was not large enough to promote changes in the photosynthetic rate relative to that of the BR 16 and 1Ea15 plants (Marinho et al. 2016). A decrease in stomatal conductance is one of the first responses to water deficits and usually results in a decreased photosynthetic rate (Anjum et al. 2011), which was not observed in this report.

The expression of the transgene *AtAREB1* was observed in the GM lines 1Ea2939 and 1Eb2889 but was not detected in the line 1Ea15. These lines were obtained independently. The expression level of a transgene depends on its insertion site in the genome, the number of copy/insertions, and the occurrence of gene silencing (Li et al. 2002, Svitashev et al. 2002, Altpeter et al. 2005). The *GmRAB18* gene, which is drought-responsive, was strongly induced in BR 16 and 1Ea15 plants under WD conditions; however, in the GM lines 1Ea2939 and 1Eb2889, the expression of this gene was relatively low, which supports the observed findings of daily transpiration and stomatal conductance, indicating that subjecting the GM lines 1Ea2939 and Eb2889 to WD conditions resulted in lower stress levels compared to those of BR 16 and 1Ea15 plants (Marinho et al. 2016).

The differences in gene expression of the *AtAREB1* transgene and the physiological behavior of the GM lines 1Ea2939 and 1Eb2889 reflected the survival rates and yield components. After 17 days of withholding irrigation followed by 7 days of watering, the cultivar BR 16 and the line 1Ea15 showed 100% mortality, whereas plants of the GM lines 1Ea2939 and 1Eb2889 showed 60 and 40% survival, respectively (**Fig. 1D**). The analysis of yield components showed that the transformation of soybean plants with the *AtAREB1* gene under the control of the constitutive promoter *35S*, which has often been associated with growth abnormalities, did not impair the agronomic performance of the transformed plants. The GM line 1Ea2939 presented a larger total number of pods, higher total dry pod matter, larger number of viable seeds, higher dry matter of viable seeds, higher total dry matter of seeds, and larger total number of seeds per plant (Marinho et al. 2016).

The results obtained under GH conditions indicated that the line 1Ea2939 was the most promising with regard to improved drought tolerance. Thus, this GM line was evaluated in subsequent field experiments. During the crop season of 2013/2014 (**Fig. 2**), line 1Ea2939 showed higher intrinsic water use (*A/gs*) than that of the other plant materials in the NIRR treatment as well as a higher leaf area index (LAI). Among the different materials tested, higher plant height values were registered with line 1Ea2939, which were impaired due to severe water lodging that occurred after a plentiful rain (341.4 mm in 40 days). The results showed lower productivity for the 1Ea2939 plants in the IRR treatment (2.021 kg ha^{-1}) when compared to that of the NIRR treatment (2.153 kg ha^{-1}). This difference of approximately 140 kg ha^{-1} was due to the water lodging that occurred in the IRR treatment. Although productivity and the final potential yield numbers decreased,

data from before the abundant rainfall event showed that line 1Ea2939 exhibited a higher number of nodes (21 nodes/plant in 1Ea2939 plants, while other GM lines and WT plants presented an average ranging from 14 to 15 nodes/plant) and a higher number of pods per plant compared with those of the other plants, indicating great yield potential (Fuganti-Pagliarini et al. 2017). A positive relationship between pods, nodes, and yield or nodes, pods, and seeds has been reported (Kahlon et al. 2011, Egli 2013).

Molecular data showed higher levels of drought-responsive genes in line 1Ea2939, such as phosphatase *GmPP2C* (Glyma.14G195200), alanine aminotransferase *GmAlaAT* (Glyma.01G026700), Cab21 (Glyma.16G165800), and putative soybean aquaporin pip2/aquaporin transporter/ glycerol uptake facilitator (Glyma.12G172500), when compared to that of its genetic background, the conventional cultivar BR 16. These records suggest that the stomata closure exhibited by *AtAREB1 FL* line 1Ea2939 was probably triggered by the combination of different physiological and molecular mechanisms given that Glyma.14G195200 was up-regulated and that *GmPP2C* is closely related to this drought-responsive mechanism. The expression of the light-harvesting chlorophyll a/b-binding (LHCB) proteins (Glyma.16G165800) supported this hypothesis, as these proteins have been found to positively regulate plant drought tolerance by positively controlling stomatal movement through guard cell signaling in response to ABA in *Arabidopsis*, which was also observed for soybean plants (Xu et al. 2012). In summary, considering the molecular and physiological data obtained from the field experiment, it was suggested that the GM line 1Ea2939 targeted more than one mechanism to cope with water deficit periods and presented a combined modulation of the gene expression profile and physiological responses to conserve water and protect its cells from water starvation (Fuganti-Pagliarini et al. 2017).

The oil and protein content from *AtAREB1* lines were determined from the crop seasons of 2013/2014 until 2017/2018. The oil content values ranged from 17.9% during the crop season of 2014/2015 to 21.9% during the crop season of 2017/2018. The protein percentage reached its highest value of 41.8% during the 2014/2015 season and its lowest value during the 2017/2018 season (36.6%). Therefore, the overexpression of the transcription factor *AtAREB1* led to an improved capacity of the soybean crop to cope with drought stress without yield or nutritional losses (Barbosa et al. 2012, Marinho et al. 2016).

Soybean lines containing galactinol synthase gene (AtGolS2)

Soybean lines overexpressing the *35S:AtGolS2* construction were obtained via *Agrobacterium tumefaciens*-mediated transformation. Among these, two lines, 2Ia1 and 2Ia4, were analyzed in drought-simulated and control treatments in both GH and field conditions.

The results from the GH experiments showed that the overexpression of *AtGolS2* in GM plants led to an increase in galactinol and RFO biosynthesis transcripts, such as raffinose 1 (*GmRS1*, Glyma.03G137900) and raffinose 3 (*GmRS3*, Glyma.19G004400) genes (Honna et al., 2016). Such carbohydrate accumulation

could represent an adaptive mechanism to adverse water conditions since increased water retention in the cell can delay senescence and death (Quick et al., 1989). Therefore, the accumulation of galactinol and raffinose transcripts observed in the GM line 2Ia4 could lead to the development of plants that are more tolerant to drought due to RFO accumulation, which may act as osmoprotectors under water deficit conditions by increasing the tolerance to changes resulting from the osmotic adjustment process (Turner et al. 2001). The importance of galactinol and raffinose transcript accumulation due to changes in carbohydrate metabolism under abiotic stress conditions has been previously described by Taji et al. (2002) in *Arabidopsis*. Peters et al. (2007) also observed the accumulation of carbohydrates under adverse abiotic conditions in *Xerophyta viscosa* leaves.

In addition to the role that RFOs play in drought tolerance, it is possible that the interaction between the LEA proteins and RFOs, as suggested by Liu et al. (2010) and Wolkers et al. (2001), increased the capacity of the 2Ia4 plants to survive and recover after a period of severe drought. Both *LEA2* (Glyma.09G185500) and *LEA6* (Glyma.17G164200) presented higher expression levels under WD conditions in the GM line. According to Wolkers et al. (2001), soluble carbohydrates, such as sucrose and trehalose, and LEA proteins act jointly to form glassy structures that bind via hydrogen bonds to minimize the damage caused by abiotic stress. This network formed by carbohydrates and LEA proteins allows for a greater stability of cellular structures, while acting as an anchor for the molecular network, providing stability to macromolecular and cellular structures under extreme environmental conditions (Wolkers et al. 2001).

With regard to the gas exchange parameters assayed in the GH experiment, lower values were observed in all plants under WD conditions, regardless of the plant material evaluated (i.e., GM or conventional background). With regard to soil gravimetric moisture (GraM) content, the *AtGolS2* line 2Ia4 plants showed higher values under WD conditions compared to that of the other plant materials. The water accumulation in the substrate-sand mixture and the increase in galactinol and raffinose transcripts observed in the 2Ia4 plants probably influenced the gas exchange parameter response, thus supporting the possibility that these carbohydrates acted as osmoprotectors during osmotic adjustments under WD conditions (Honna et al. 2016).

In the GH survival experiment, the *AtGolS2* GM lines showed higher survival rates after 21 days of withholding irrigation followed by nine days of rehydration when compared to that of the genetic background (**Fig. 1C**). The conventional cultivar BRS 184 plants showed 100% mortality, while the soybean plants from the GM line 2Ia4 also showed 100% recovery after rehydration, which agrees with the data reported by Taji et al. (2002). The authors of that study demonstrated that *Arabidopsis* plants that overexpressed the gene *35S:AtGolS2* showed complete recovery after 14 days of WD followed by five days of rehydration due to a reduction in leaf transpiration, higher water accumulation in the substrate, and the accumulation of raffinose and galactinol in tissues, suggesting once again that the increased levels of these carbohydrates may have

allowed them to act as osmoprotectors.

In the experiment conducted under field conditions, a higher number of pods with seeds, number of seeds, 100-seed weight, and yield were identified in GM 2Ia4 plants in the IRR and NIRR treatments when compared to that of the conventional background. This result may have been due to the increased synthesis of RFOs, even under well-watered conditions, since a constitutive promoter (*35S*) was used. The oil and protein content in the *AtGolS2* lines was registered from the crop seasons of 2014/2015 to 2017/2018. The percentage values obtained were lower for protein content (ranging between 36-37%) and higher for oil content (ranging between 22-23%) compared to that of the standard pattern determined by the crushing market. However, through a soybean-breeding program, this may be improved as the 2Ia4 plants may be useful for the development of drought-tolerant plants (Honna et al. 2016).

Soybean lines containing the 9-cis-epoxycarotenoid dioxygenase (NCED) gene (AtNCED3)

Cotyledons from the soybean conventional cultivar BRS 184 were transformed through the *A. tumefaciens* method with the construct *35S:AtNCED3*. Two positive events were identified in the T_0 generation, 2Ha11 and 2Ha13. These lines were submitted to molecular, physiological, and agronomical characterization in WD and control treatments under both GH and field conditions (Molinari et al. 2020).

Higher expression levels of the *AtNCED3* gene and the endogenous genes *GmAREB1* (Glyma.04G039300; Glyma.07G213100; Glyma.02G131700), *GmPP2C* (Glyma.14G195200), *GmSnRK2* (Glyma.02G135500), and *GmAAO3* (Glyma.14G045100) were identified in the GM lines when compared to that of the WT plants under WD conditions (Molinari et al., 2020).

A higher expression of the genes from the ABA biosynthesis pathway suggests that the *AtNCED3* gene is involved in the drought response of soybeans. Furthermore, the ABA synthesis pathway was triggered in response to WD conditions, as observed by the higher ABA levels detected under WD conditions in the GM plants. Plants from the GM line 2Ha11 showed an ABA concentration of 166.34 pmol mL^{-1}, while the background cultivar BRS 184 plants under WD conditions and both plant materials under control conditions showed ABA concentrations under 4 pmol mL^{-1}, which is the minimum detection limit of the kit employed (Molinari et al. 2020). The increase in ABA levels and ABA biosynthesis-related genes was previously described in *Arabidopsis* (Iuchi et al. 2001). In peanut plants (*Arachis hypogaea* L.), the constitutive expression of the *AhNCED1* gene in WT *Arabidopsis* plants resulted in higher ABA accumulation in the GM plants in response to drought compared to that of the control plants (Hwang et al. 2010). Similarly, *Caragana korshinskii*, a deciduous perennial shrub of sandy grasslands and deserts, showed ABA accumulation followed by a large increase in *CkNCED1* mRNA levels in detached leaves and stems after dehydration for 4 h at room temperature (Wang et al. 2009). In addition, in *Stylosanthes guianensis*, an important forage legume and cover crop, the dehydration of leaves and roots induced the strong and rapid expression of

SgNCED1, while ABA accumulation was induced by an increase in *SgNCED1* mRNA levels under stress (Yang and Guo 2007). In soybeans, the diurnal oscillation of the *GmNCED3*, *GmNCED4*, and *GmNCED5* genes has been reported, and such oscillation appeared to be limited by light under stressful conditions, the period in which stomatal closure is needed to avoid water loss by evapotranspiration (Rodrigues et al. 2015). All these reports support a strong and direct relationship among the expression of *NCED* genes, increased ABA levels, and the activation of drought responses, such as stomatal closure, to reduce water loss under WD conditions (Molinari et al. 2020).

Likewise, as reported by Molinari et al. (2020), the expression of drought-responsive endogenous genes, such as *GmAREB1*, *GmPP2C*, *GmSnRK2*, and *GmAAO3* in soybeans, has also been described in *Arabidopsis* (Iuchi et al. 2001). Considering the genes from the ABA biosynthesis pathway, an increase in aldehyde oxidase genes (*AAO3*) under WD conditions was also found identified in peanuts (Yang et al. 2011) and peas (Zdunek-Zastocka and Sobczak, 2013). The high expression of *GmPP2C* and *GmSnRK2* identified in soybeans reflects the refined control of ABA synthesis, which is negatively regulated by the inhibition of the NCED enzyme when ABA levels exceeded cell maintenance levels (non-stressed conditions), preventing high hormone levels from indicating a metabolic disturbance (Liu et al. 2016).

Under GH conditions, the gas exchange measurements (*gs*, *Ci*, *A*, *E*) under WD conditions decreased in the GM line 2Ha11. Furthermore, GM plants showed 80% higher intrinsic water use efficiency (*A/gs*) when compared to that of the WT plants under WD conditions. The decrease in the gas exchange parameters observed in the GM event 2Ha11 has also been reported in *Arabidopsis* plants overexpressing *AtNCED3* (Iuchi et al. 2001). A reduction in transpiration was also noted in *Arabidopsis* plants overexpressing *OsNCED3* from rice (Hwang et al. 2010). Furthermore, GM tobacco lines overexpressing *SgNCED1* showed a decrease in transpiration rates and lower photosynthetic rates, which resulted from lower stomatal conductance (Zhang et al. 2008). Detached leaves from tobacco (*Phaseolous vulgaris*) that overexpressed *PvNCED1* also showed lower water loss due to transpiration compared to that of the control plants (Qin and Zeevaart 2002). Enhanced stomatal closure was also observed in *Vicia fava* lines expressing the *AtNCED3* gene (Melhorn et al. 2008). This might also have occurred with the GM event 2Ha11 since reduced gas exchange parameter values were observed under WD conditions, which was probably the result of stomatal closure triggered by an increased in ABA levels (Molinari et al. 2020).

The soybean *AtNCED3* lines were evaluated during the crop seasons of 2015/2016 and 2016/2017. In these experiments, no significant interaction between plant materials and water conditions was observed, probably due to the great amount of rainfall recorded during both crop seasons. According to data collected by the weather station located at the experiment site, a total rainfall of 1,521.4 mm and 1,147.2 mm was registered for the crop seasons of 2015/16 and 2016/17, respectively. The recommendations for soybean crop with regard to water requirements range from 450 to 800 mm/cycle, depending on weather conditions, crop

management, and cycle duration (Embrapa 2013).

Results from field assays comprised of average values from both water conditions (irrigated and non-irrigated treatments) for each cultivar/GM line. In the crop seasons of 2015/2016 and 2016/2017, the GM line 2Ha11 showed increased yield when compared to its genetic background, which was probably a result of higher 100-seed weights and the total number of pods. As observed for the GM line 2Ha11, an increase in the yield components was reported for the GM lines of creeping bent grass overexpressing *VuNCED1*, which showed an increase in plant body biomass and an increased number of tillers under WD conditions (Aswath et al. 2005).

The oil and protein content from the *AtNCED3* lines showed that when considering all crop seasons samples (from 2014/2015 to 2017/2018) overall, line 2Ha10 presented values within the range acceptable by the consumer market for oil and protein percentages in the grain (20% and 42%, respectively), indicating that the overexpression of the *AtNCED3* gene in this line did not imply in changes in the specific compositional characteristics.

Final highlights on the development of drought-tolerant soybean lines

Experiments under GH conditions give the "concept proof" of the genetic strategy; however, these tests under controlled light, temperature, water, weed, insect, and disease levels may not be representative of the way in which plants behave over the entire season in an actual field (Passioura 2012). Yet, in GH environments, plants are not able to express their full potential, as limitations due to pot size, controlled water amounts, temperature fluctuations, diseases, and pests do not challenge the organism as a whole but limit the simulation of actual environmental conditions. As such, reports containing data from field experiments are important given that it is only in the field that researchers can accurately gauge whether the technology has been successful or not in improving the characteristic of interest. In addition, when the objective is the release of a commercial variety, field tests are a legal requirement of regulatory governmental commissions.

We summarized data from soybean GM lines that were characterized in both GH and field conditions. These lines present promising indications for the improvement of soybean drought tolerance. Based on the reported results, the strategy to improve drought tolerance by inserting TFs that regulate the expression of several drought-responsive genes has proven to be an interesting approach to obtain lines that trigger mechanisms to cope with water deficits without compromising either yield or oil and protein content. In soybeans, such approaches may constitute the insertion of either ABA-independent genes, such as *DREB1* and *DREB2*, or ABA-dependent genes, such as *AREB1*, in addition to GM soybean lines overexpressing the *GolS* and *NCED* genes, which are responsible for important drought-defense pathways.

Overall, the soybean GM lines obtained in this study presented protein and oil content values that should be accepted by the crushing industry and that meet quality standards and commercial specifications

(Embrapa 2015). The maintenance of these parameters is essential when developing GM lines as it adds value to the grain and ensures the competitiveness of soy in the world market, enabling possible cultivars obtained from these lines to enter into the feed market for consumption by humans, poultry, pork, cattle, or other farm animals and pets.

Finally, these results have highlighted soybean drought-tolerance pathways and have shown that the use of biotechnological tools in agricultural science can help producers to minimize yield and financial losses during drought-stricken crop seasons.

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Chapter 3-3

Development of drought-tolerant sugarcane overexpressing the *AtDREB2A CA* gene

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Abstract

Sugarcane is considered an important economic crop not only for sugar production, but also for ethanol generation, serving as an expandable green alternative to the usage of crude oil. To take advantage of the use of sugarcane as a renewable source for bioethanol production, it is important to increase its productivity without increasing land usage, which includes sugarcane cultivation under hostile conditions, such as water-limited environments. In this study, we demonstrated that stress-inducible overexpression of the transcription factor *AtDREB2A CA* conferred drought tolerance in sugarcane subjected to water deficiency under greenhouse conditions. In addition, transgenic plants expressing *AtDREB2A CA* accumulated higher levels of sucrose than non-transgenic plants. These results indicate that the *AtDREB2A CA* gene under the control of the stress-inducible promoter *ZmRab17* represents a promising strategy for the development of new sugarcane varieties with improved drought tolerance.

Keywords: abiotic stress, *AtDREB2A CA*, *ZmRab17* promoter, *Saccharum* spp. hybrid, transcription factor

Introduction

Sugarcane is an important economic crop not only for production of sugar but also for biofuel production. The importance of sugarcane cultivation has increased in the recent years due to ethanol production, which is considered one of the most viable alternatives to fossil fuels (Savage 2011). The production of biofuels is important not only to reduce oil imports, but also to decrease CO_2 emissions contributing to global warming and mitigate climate change (Reis et al. 2014). Drought is considered the most deleterious abiotic stress affecting crop productivity worldwide, and water availability is the main factor that influences sugarcane productivity as it directly affects tillering, culm height, and sucrose production (Sugiharto 2004).

Currently, the development of drought-tolerant genotypes is one of the main objectives of sugarcane research programs. However, the achievement of this goal is hampered by the high ploidy level of modern sugarcane varieties and by the fact that drought tolerance is a multigenic quantitative trait. In addition, plant responses to drought are influenced by the time, intensity, duration, and frequency of the stress as well as by diverse plant-soil-atmosphere interactions, making breeding selection procedures difficult (Basnayake et al. 2012). Therefore, the use of different genetic engineering strategies to improve drought tolerance in sugarcane is desirable.

Dehydration-responsive element-binding proteins (DREBs) play vital regulatory roles in abiotic stress responses in plants. The transcription factor DREB2A interacts with a *cis*-acting dehydration-responsive element (DRE) sequence to activate the expression of downstream genes that are involved in abiotic stress responses in *Arabidopsis thaliana* (Sakuma et al. 2006). In the present study, we evaluated the effects of stress-inducible overexpression of *AtDREB2A CA* in sugarcane. The results demonstrated that sugarcane transgenic lines presented significantly enhanced drought tolerance without yield penalty under greenhouse conditions. These promising results prompted us to evaluate these sugarcane plants in field trials conducted in Brazil, and the results obtained from the field trials will be briefly discussed here.

Materials and Methods

Plant material and transformation

Sugarcane *ZmRab17::AtDREB2A CA* lines were generated and analyzed at the molecular level as described by Reis et al. (2014). Sugarcane embryogenic calli were generated from immature leaf segments

of 6-8-month-old plants of the RB855156 variety and bombarded with the expression vector pBract 302, containing the *A. thaliana DREB2A CA* (Sakuma et al. 2006), which is driven by a stress-inducible promoter Rab17 from *Zea mays*. pBract 302 also contains the bar cassette used as a selective marker. Regenerated plantlets were transferred to planting trays containing a commercial propagation substrate (Plantmax^{TM}) and grown under controlled greenhouse conditions.

Molecular analysis

The presence of the *AtDREB2A CA* transgene in leaf samples from regenerated sugarcane plantlets was confirmed by standard polymerase chain reaction (PCR) using specific primers. Positive PCR plants and non-transgenic plants were sprayed with 1% (v/v) glufosinate ammonium and evaluated 8 days after spraying, obtaining eight independent events that were further evaluated under drought conditions.

Screening of transgenic sugarcane events under water-deficit conditions

One-month-old plants from the eight events obtained from plant transformation were subjected to 21 days of water-deficit stress to select the best events for further analysis. One out of five events demonstrated outstanding drought tolerance, as suggested by reduced leaf rolling and decreased senescence when compared with non-transformed (NT) plants. Therefore, this independent event was further characterized in detail under drought stress conditions.

Physiological measurements of sugarcane under water-deficit conditions

Eight-month-old plants were grown in 28-L PVC cylinders (25 cm diameter, 150 cm height). Water stress trials were carried out in a randomized block design (RBD) with five replications for both transgenic and non-transgenic plants. The water tension in the xylem was measured daily (8:00 – 11:00), using a Scholander pressure chamber, in the fully expanded photosynthetically active leaf (+4 leaf). These measurements were assumed to represent the leaf water potential (Ψ_L). The relative water content (RWC) was estimated in the +5 leaf (base, middle, top) by measuring the fresh and dry weights, and the RWC was calculated as: RWC (%) = [(FM - DM)/ (TM - DM)] × 100, where FW, DM, and TM are the fresh, dry, and turgid weights, respectively.

The net photosynthetic rate (A), intercellular CO_2 concentration (C_i), stomatal conductance (g_s), and transpiration (E) were assessed using an open gas exchange system with a 6 cm^2 clamp-on leaf cuvette (LI-6400XT, LICOR, Lincoln, NE, USA). Leaf gas exchange was evaluated in the middle third of the second fully expanded leaf with visible ligule (+2 leaf). These measurements were taken between 8:00 and 11:00 hours, for 4 days after withholding water every day. The photosynthetic photon flux density (PPFD) was fixed at 1.500 μmol m^{-2} s^{-1}, using a red-blue LED light source built into the leaf cuvette, although other

environmental factors, such as air humidity and temperature, were not controlled; in other words, natural variation was permitted. The vapor pressure deficit in the cuvette was maintained below 2.5 kPa to prevent stomatal closure due to the low air humidity effect. The air collected outside the greenhouse was passed through a buffering zone and then pumped into the system, with a mean CO_2 concentration of 380 μmol mol $^{-1}$.

Agronomic characterization of sugarcane plants under water-deficit conditions

The agronomic characterization of the 8-month-old transgenic (T) and non-transgenic (NT) sugarcane plants was performed after four days of withholding water. The agronomic parameters consisted of shoot dry weight, root dry weight, internode length, culm length, sucrose content, and bud sprouting rate. Shoot and root dry weights were determined after drying the leaves or roots in an oven at 80 °C until a constant weight was obtained. The culm length, diameter, and number and length of internodes were measured using a graduated meter ruler and a digital caliper Vonder model (PPV-1506). Sucrose content was measured at the end of the experiment, when aliquots of cane juice from the last, penultimate, and antepenultimate internodes were collected and the sucrose content was determined as prescribed by Reis et al. (2014). Briefly, the juice was separated by high-performance liquid chromatography (HPLC) and subsequently quantified using refractive index detection (RID). HPLC-RID was conducted with an Agilent 1260 Infinity system (Agilent Technologies, Palo Alto, CA) equipped with an Aminex HPX-87H anion-exchange column, 300 × 7.8 mm (Bio-Rad, Hercules, CA). Samples were diluted with 9 volumes of H_2O, injected into the HPLC-RID system (50-μl injection volume), and eluted isocratically with 0.02 N H_2SO_4 at a flow rate of 0.5 mL min^{-1} (RID flow cell, 45°C; column, 50°C). Reference sucrose (Fisher Scientific) was diluted in H_2O and used to generate a standard curve.

Bud sprouting rate was measured after harvesting the middle portion of each stalk from 9-month-old transgenic (T) and non-transgenic (NT) sugarcane plants that were subjected to water deprivation as described above. Each measurement consisted of 24 buds grown in vermiculite substrate on plastic trays under greenhouse conditions. At the end of 30 days, the bud sprouting rate was calculated and expressed as percentage.

Results

Screening of drought-tolerant sugarcane lines under greenhouse conditions

Eight independent events of sugarcane *AtDREB2A CA* plants and non-transgenic plants (NT) were subjected to 6 days of water deprivation, which demonstrated a better performance of transgenic lines than NT plants (**Fig. 1a**). From these lines, event 24.2 presented the best characteristics to be considered an elite

event, that is, a very strong "stay-green" phenotype, compared to NT plants (**Fig. 1b** and **1c**) and high levels of *AtDREB2A CA* expression under water-deficit conditions (**Fig. 1d**). Therefore, this event was chosen for further detailed analysis, and from this point onwards, it will be designated as a transgenic event (T).

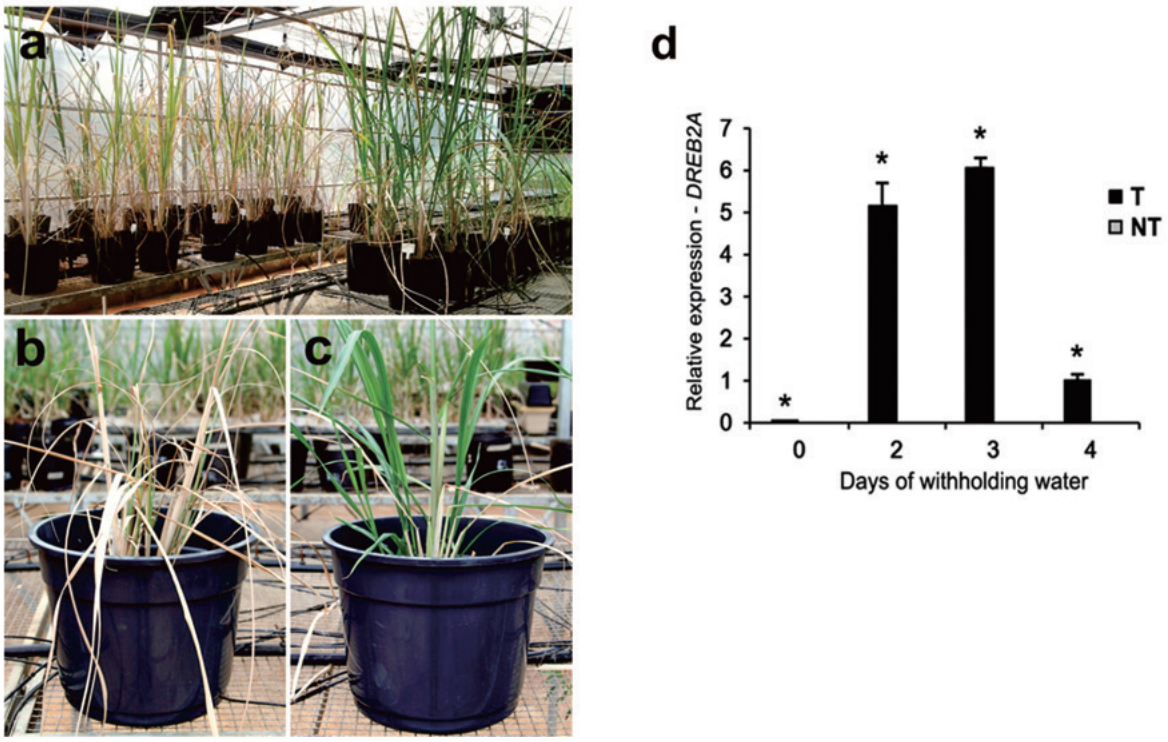

Fig. 1. Sugarcane with drought-inducible expression of *AtDREB2A CA* during a 'survival' drought tolerance test.
a – Three-month-old plants subjected to 6 days of water deprivation. Left side: non-transgenic plants; right side: eight independent transgenic events. **b** and **c**: Independent transgenic event (24.2) and control (non-transgenic plants) sugarcane plants that are 1 month old grown in 8-L plastic pots and subjected to 21 days of water deficit stress period. **d** – Transcript abundance of *AtDREB2A CA* during drought treatment. The geometrical mean of *GAPDH* and *25S rRNA* was used as a reference to measure the relative quantification, which corresponds to the mean of three biological repetitions ± SE. Statistical differences (*$p < 0.05$) were analyzed with ANOVA, followed by Tukey's test (Figure modified from Reis et al., 2014 with permission).

Plant water status and gas exchange analysis

Under well-watered conditions, it was verified that the difference in leaf water potential (Ψ_L), relative water content (RWC), net photosynthesis rate (A), concentrations of CO_2 in the substomatal chamber (Ci), transpiration (E), and stomatal conductance (g_s) were not statistically significant between transgenic (T) and non-transgenic (NT) plants (**Fig. 2**). However, under drought stress, the Ψ_L and RWC in T and NT plants were similar until day 3 (i.e., after the start of water deficit), with mean values of −1.41 MPa and 74%, respectively. In addition, on day 4 of water deprivation, the Ψ_L and RWC of the NT plants dropped to nearly −2 MPa and 60%, respectively, whereas the Ψ_L and RWC of the T plants increased to −1.42 MPa and 75%, respectively. In general, the gas exchange measurements related to A, g_s, and E were higher for T plants than for NT plants, and the Ci was smaller for T plants than for NT plants. However, statistical significance was

only found for *A*, g_s, and *Ci* at days 2 and 3 of withholding water (**Fig. 2**). By the end of day 4 of water deprivation, the NT plants appeared completely wilted and exhibited leaf rolling, while the leaves of T plants were still turgid and expanded (data not shown). These results suggest that differential responses to the drought stress of T and NT plants were observed only on the 2nd and 3rd days when the plants experienced moderate levels of stress (RWC = 80% and water potential ~ −1.5 MPa for the NT and T plants) or a more severe stress (RWC = 70% and water potential lower than −1.5 MPa for NT plants).

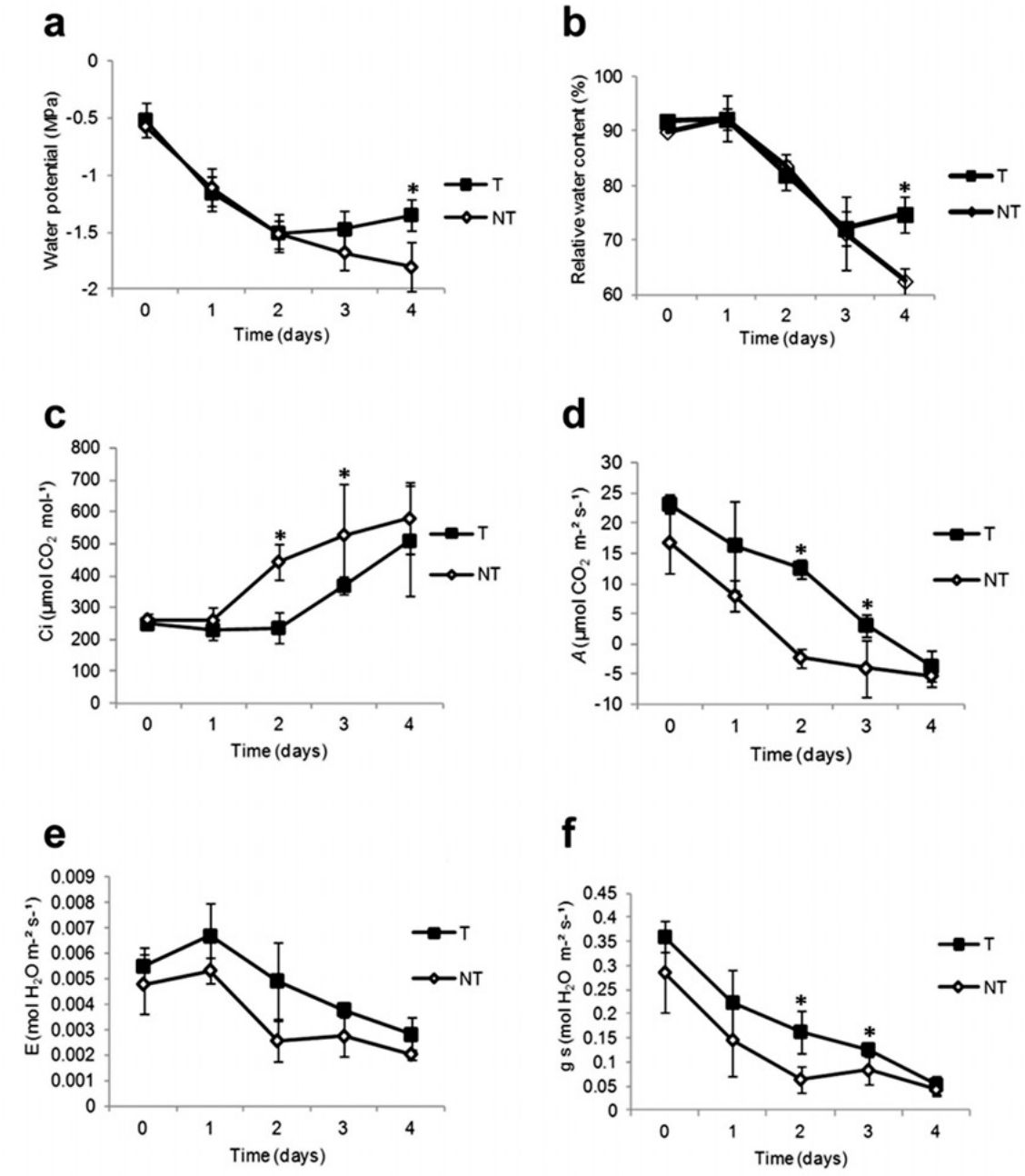

Fig. 2. Physiological characterization of transgenic (T) and non-transgenic (NT) sugarcane plants under water deprivation for four days of withholding water.

a) Water potential; **b**) relative water content (RWC). **c - f** Gas exchange parameters including: **c** - concentrations of CO_2 in the substomatal chamber (*Ci*), **d** is the net photosynthesis rate (*A*), **e** is the transpiration (*E*), and **f** is the stomatal conductance (*gs*). Values are the mean ± S.E. (n = 5). Statistical differences (*$p < 0.05$) were analyzed with ANOVA followed by Tukey's test (Figure modified from Reis et al., 2014 with permission).

Agronomic characteristics of transgenic sugarcane under greenhouse conditions

As observed in **Fig. 3**, we found no significant differences between T and NT plants in the shoot and root dry weight after a period of water deprivation for 4 days (**Fig. 3a** and **3b**). However, statistical differences were found in the culm length and internode length (**Fig. 3c** and **3d**), which were higher for the T plants. **Fig. 3e** demonstrates that the sucrose content in the culms of T plants was 33.8% higher than that in NT plants, while the bud sprouting rates of T plants that descended from plants subjected to water deprivation were 82% higher than that of NT plants (**Fig. 3f**).

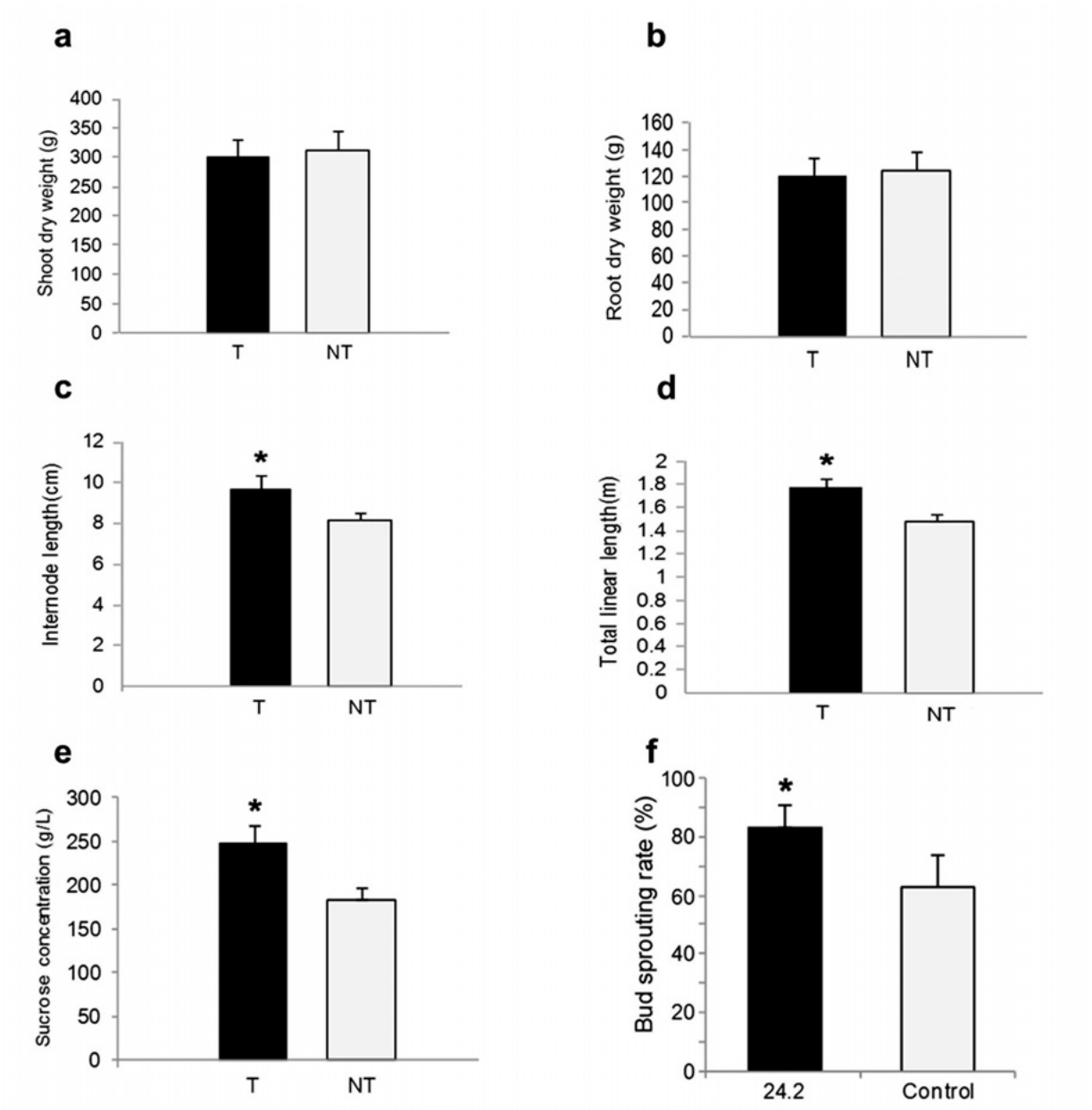

Fig. 3. Agronomic characterization of 8-month-old transgenic (T) and non-transgenic (NT) sugarcane plants after four days of withholding water.

Shoot dry weight **(a)**, root dry weight **(b)**, internode length **(c)**, culm length **(d)**, sucrose content **(e)** and bud sprouting rate **(f)**. Values are the mean ± S.E. (n = 5 for a-e and n = 24 for f). Statistical differences (*$p < 0.05$) were analyzed with ANOVA followed by Tukey's test (Figure modified from Reis et al., 2014 with permission).

Discussion

AtDREB2A is a transcription factor involved in drought stress responses, and its native form is not sufficient for the activation of drought-responsive genes because of the presence of a negative regulatory domain (NRD). In *Arabidopsis thaliana*, the deletion of the central region in the NRD makes DREB2A constitutively active (*DREB2A CA*) (Sakuma et al. 2006). The constitutive expression of *DREB2A* leads to severe growth defects, but the overexpression of the transcription factor under a stress-inducible promoter, such as ZmRab17, allows regular plant development (Sakuma et al. 2006, Engels et al. 2013, Reis et al. 2014). Here, we used the construct *ZmRab17::AtDREB2A CA* to transform the drought-sensitive sugarcane RB855156 variety as background. Under greenhouse conditions, our results demonstrated that *ZmRab17::DREB2A CA* plants subjected to drought stress displayed better performance related to increased sucrose content and bud sprouting rate verified with sugarcane descended from plants subjected to water deprivation (**Fig. 3**). In addition, the physiological measurements showed that plants transformed with the *ZmRab17::DREB2A CA* construct presented higher stomatal conductance and photosynthetic and transpiration rates, even under normal water regimes (**Fig. 2c**, **2d,** and **2f**).

As discussed by Reis et al. (2014), one of the most important characteristics of sugarcane varieties is their capacity for initial sprouting. The RB855156 variety used in the present study has high yields and sucrose levels; however, low initial sprouting is a major concern for this variety, especially after a period of water deprivation (Silva et al. 2007). It is possible that physiological and molecular adaptations obtained by *AtDREB2A CA* plants are not only during water stress trials but also during all survival tests performed previously, and during the whole plant cycle with the plants under control conditions, as shown in **Fig. 1d**, revealing the basal activity of the *ZmRab17* promoter in the leaves of the transgenic plants, could be responsible for higher rates of bud germination and effects observed in transgenic plants. The higher photosynthetic rates of transgenic plants prompted us to speculate that induced overexpression of *AtDREB2A CA* gene was capable of activating the sucrose synthesis pathways, as these plants demonstrated increased accumulation of sucrose. However, at this point, we are not able to rule out a plausible explanation for the increased sucrose accumulation in transgenic plants. Thus, additional studies on gene expression analysis would be required to investigate the role of *DREB2A CA* overexpression in the modulation of sugar biosynthesis pathways.

The promising results obtained from this study prompted us to evaluate these sugarcane transgenic lines under field conditions. Two distinct lines, including line 24.2, were analyzed in two seasonally independent dry regions of Brazil. As presented by de Souza *et al.* (2019), *AtDREB2A CA* sugarcane lines demonstrated higher yield and productivity than non-transformed plants under drought conditions. The

agronomical performance of these lines was measured in terms of the content of soluble solids (°Brix), sugar content in the culm juice (Pol%), tons of cane per hectare (TCH), and tons of Pol% per hectare (TPH). In both the dry locations, the sugarcane lines performed better, demonstrating increased levels of °Brix, %Pol, TCH, and TPH corresponding to 11.6%, 18.0%, 20.3%, and 41.7%, respectively, than NT plants. The performance of *AtDREB2A CA* sugarcane lines in the field under drought conditions was comparable to that of a high-yield and drought-tolerant elite variety grown in Brazil (CTC9001). These results corroborate that overexpression of *AtDREB2A* in sugarcane might be incorporated as a new biotechnological strategy for the development of drought-tolerant varieties.

Conclusions

The results presented here show that transformation of sugarcane with the *AtDREB2A CA* gene under the control of the *ZmRab17* promoter enhanced the tolerance of the sugarcane RB855156 variety to water deficiency. Transgenic sugarcane had improved initial bud sprouting, increased culm and internode lengths, and higher sucrose content (33.8%), than non-transgenic plants under water deficiency in greenhouse conditions. The results described here were presented by Reis et al. (2014) and extracted with permission. As the success of any biotechnological strategy would ultimately be determined by the final yield under field conditions, the analysis of two sugarcane transgenic events overexpressing *AtDREB2A CA* in two different dry regions of Brazil. These results indicated that these lines presented higher productivity and yield than control plants under drought conditions. Therefore, the results obtained from field trials corroborated that the overexpression of *AtDREB2A CA* in sugarcane might be a useful strategy for the development of new drought-tolerant varieties.

Acknowledgment

We would like to thank Editage (www.editage.com) for English language editing.

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47572013000400015
Reis RR, da Cunha BADB, Martins PK, *et al*. (2014). Induced overexpression of AtDREB2A CA improves drought tolerance in sugarcane. *Plant Science* **221–222**: 59–68.
Sakuma Y, Maruyama K, Qin F, Osakabe Y, Sek, M, Shinozaki K, Yamaguchi-Shinozaki K (2006) Dual function of an *Arabidopsis* transcription factor DREB2A in water stress-responsive and heat-stress-responsive gene expression. Proc. Nat. Acad. Sci. USA **103**:18828-18833.
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Silva MA, Jifon JL, Da Silva JAG, Sharma V (2007) Use of physiological parameters as fast tools to screen for drought tolerance in sugar. *Brazilian Journal of Plant Physiology* **19**: 193-201.
Sugiharto, B (2004) Biochemical and molecular studies on sucrose-phosphate synthase and drought inducible-protein in sugarcane (*Saccharum officinarum*). *ILMU Dasar* **5**: 62–67.

Appendix

Development of technologies and crops for stable food production under adverse environments and changing climate conditions

Kazuo Nakashima [1*]

1 Japan International Research Center for Agricultural Sciences (JIRCAS), Tsukuba, Japan

***** *Corresponding author; E-mail: kazuo.nakashima@affrc.go.jp*

The file "Development of technologies and crops for stable food production under adverse environments and changing climate conditions" presented at the International Soybean Conference in Brazil on June 14, 2018, is attached at the end of this article. This presentation file contains information on other research related to the development of drought-tolerant GM crops. The file can be found at the following site: https://www.cbsoja.com.br/images/cbsoja2018/docs/palestras/Kazuo_Nakashima.pdf.

VIII CONGRESSO BRASILEIRO DE SOJA

June 14, 2018

Development of technologies and crops for stable food production under adverse environments and changing climate conditions

Kazuo Nakashima
Program Director, Stable Agricultural Production Program
Japan International Research Center for Agricultural Sciences

JIRCAS

1

Contents

1. Introduction
2. Marker-assisted selection (MAS)
3. Genetic modification (Biotech)
4. New breeding technology (NBT)
5. Conclusion

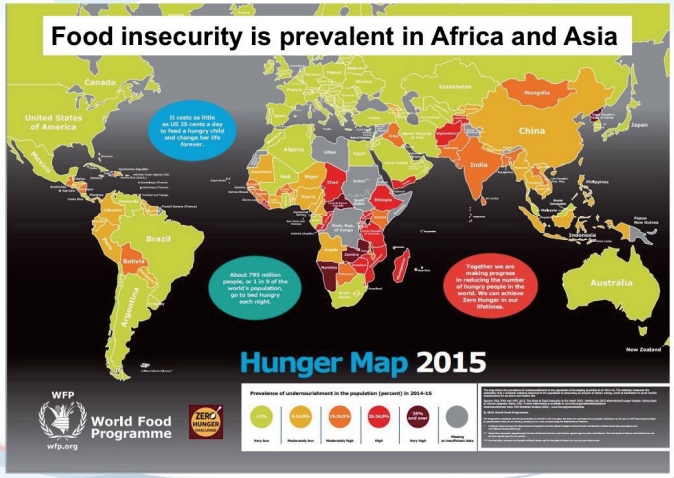
Japan International Research Center for Agricultural Sciences (JIRCAS)

JIRCAS conducts research to develop improved technologies for the agriculture, forestry, and fishery industries in developing regions.

It plays a central role in international contribution and cooperation initiatives in the field of agriculture, forestry, and fishery research in Japan, with the aim of providing solutions to global environmental problems, food insecurity, and extreme poverty.

2

Introduction



3

Drought occurs around the world

Worst drought and heat in decades disrupts life in Southeast Asia's Mekong region in 2016

http://www.nbcnews.com/slideshow/worst-drought-decades-disrupts-life-southeast-asia-s-mekong-region-n562166

4

Toward the stable agricultural production and improvement of nutrition in developing regions

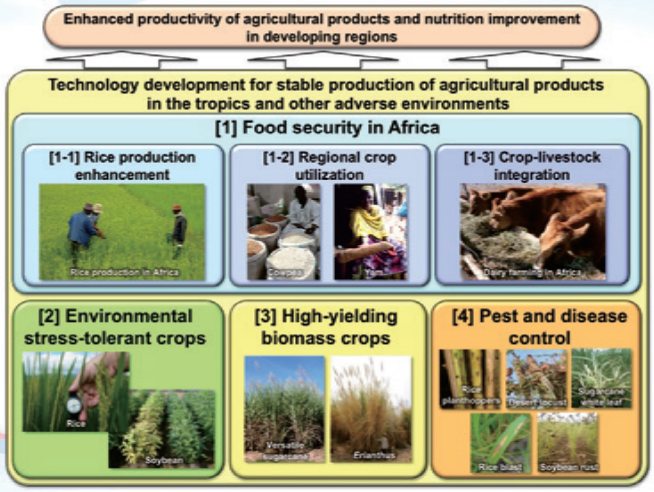
JIRCAS

- In developing regions including Africa, agricultural production potential has not been sufficiently realized because of adverse conditions such as low soil fertility and drought.
- Consequently, food and nutrition security has remained relatively low.
- The second of the 17 Sustainable Development Goals (SDGs) adopted by the UN General Assembly calls on all nations to "end hunger, achieve food security and improved nutrition, and promote sustainable agriculture."
- In Stable Agricultural Production Program, we aim to enhance agricultural productivity and improve nutrition in developing countries through technology development for stable production of agricultural products in the tropics and other adverse environments.

5

Stable Agricultural Production Program

JIRCAS



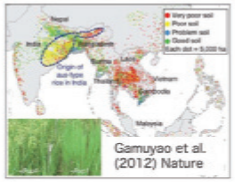
6

Marker-assisted selection (MAS)

JIRCAS

The growth of NERICA 4 (front side) of phosphorus fertilizer 0 is extremely bad compared to 50 kg / ha (back) (Madagascar).

Limitation of yield due to phosphorous deficiency



We introduce phosphorous deficiency tolerance gene *Pup1* and so on to local varieties.

Restriction on yield due to nitrogen deficiency

Obara et al. (2014) Plant Biotech Rep

We use QTL (qRL) which efficiently promotes root elongation corresponding to nitrogen concentration. Root elongation may contribute to drought avoidance.

Contributing to the development of varieties with improved traits such as phosphorous use and nitrogen use by MAS

Contributing to alleviate climate change by reducing GHG N_2O emissions through development of varieties with improved nitrogen usage

7

Breakthrough in Nutrient Use Efficiency for Rice by Genetic Improvement and Fertility Sensing Techniques in Africa (SATREPS)

JIRCAS

Madagascar is the largest rice producing country in Sub-Saharan Africa

Weathered soil + fertilizer shortage (Low input / low fertility environment)

Poverty and hunger due to the stagnation of rice crops (staple food and business)

Integration of JIRCAS rice research to solve the problem

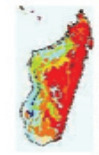
① Evaluation of the fertility of the field

② Utilization of breeding materials with excellent nutrient absorption utilization efficiency

③ Sustainable rice cultivation technology with excellent nutrient balance

Yield enhance-ment

Impact assessment on income and nutrition improvement ④



Weathered soil with poor nutrient supply in Madagascar (red)

Social Implementation

Improve rice yield and farmer's income and nutrition of Madagascar by using genetic resources and fertilization technology excellent for nutrient use

8

Rice near-isogenic line (NIL) with early-morning flowering trait for improvement of heat tolerance

Early-morning flowering is effective in heat escape at flowering

9

Development of salinity tolerant soybean

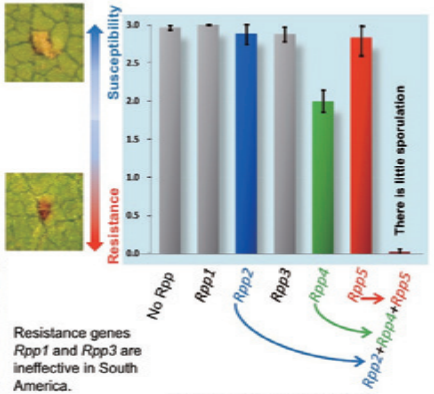
Field performances of *Ncl* near isogenic lines (NILs) in a saline field condition in Japan. *Ncl* could increase soybean grain yield in saline field conditions.
Do et al. (2016), Scientific Reports.

10

Development of soybean rust resistant varieties is required

- Climate change may affect the occurrence of pests and diseases.
- It is estimated that the damage of soybean rust in the world is as much as 5% of soybean production, and a large amount of fungicides is used for control.
- In South America, it is already confirmed that the efficacy of fungicides has been reduced, and the demand for resistant varieties is large.
- By accumulating three kinds of resistance genes in soybeans, it is possible to impart high resistance.

Spore production by pathogenic rust fungus



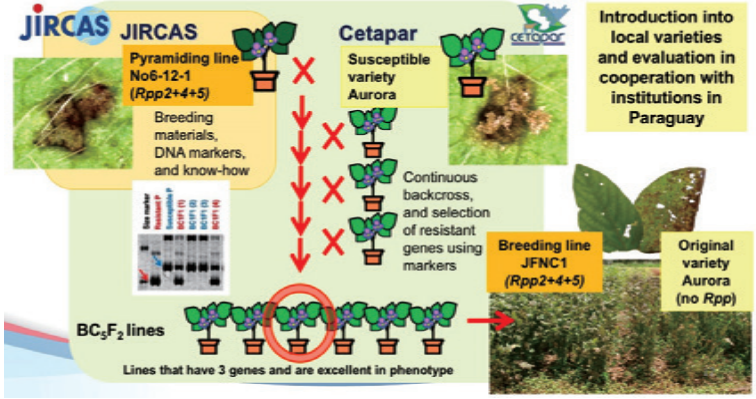
Resistance genes *Rpp1* and *Rpp3* are ineffective in South America.

Varieties with resistance genes

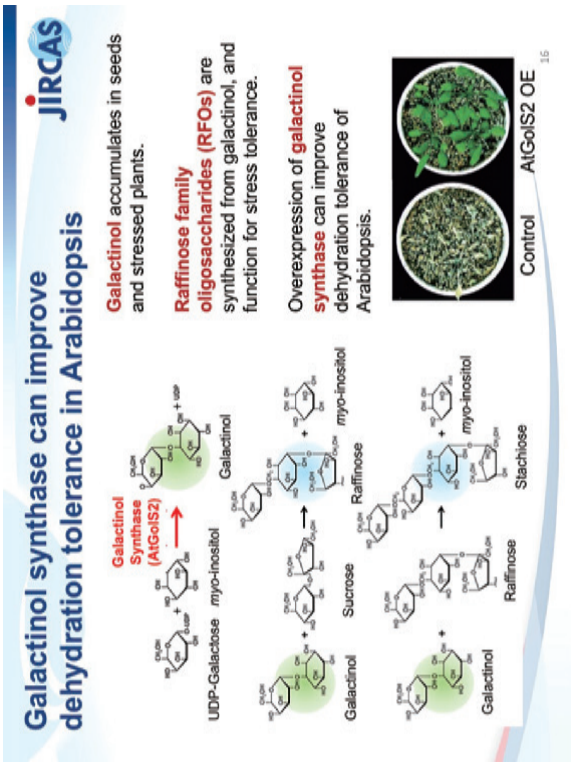
11

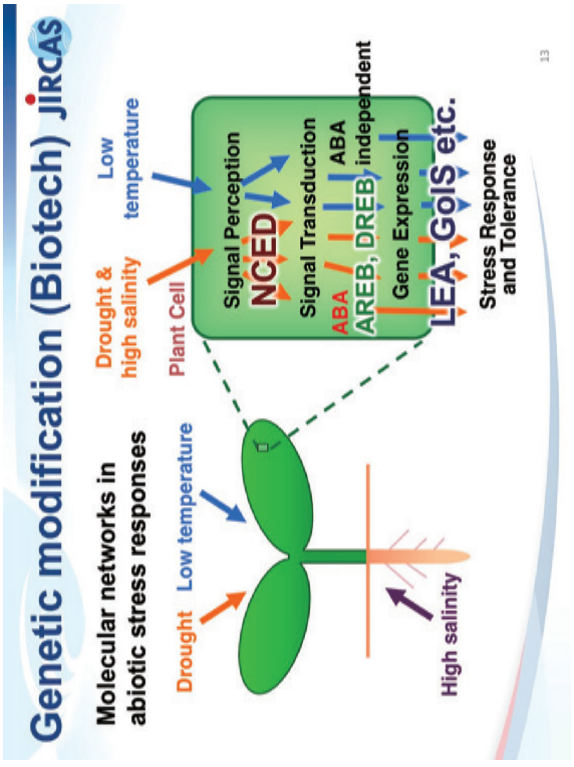
Registration of soybean rust resistant varieties by MAS-based backcross breeding (Paraguay)

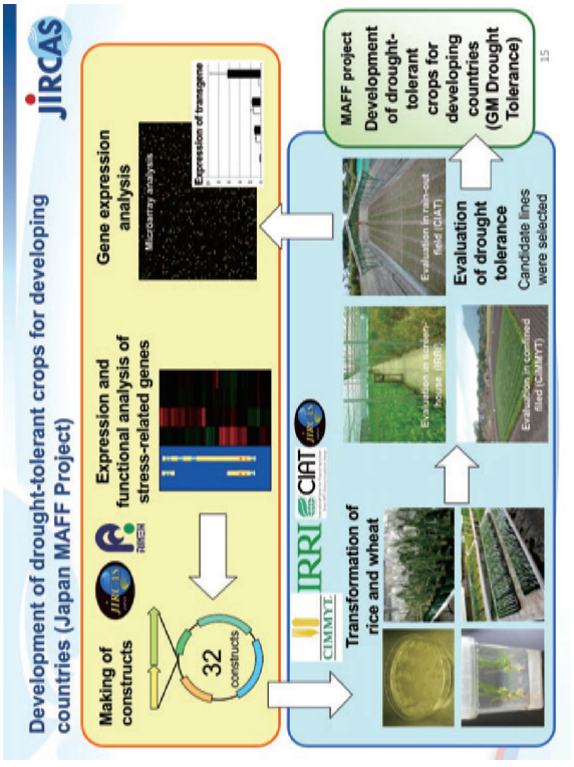
- Three soybean rust resistance genes were introduced into Aurora which is a local variety susceptible to rust by MAS-based backcross breeding.
- The line JFNC1 with high resistance and agronomic traits similar to Aurora was registered as a subclass of Aurora in collaboration with Nikkei-Cetapar in Paraguay.




Drought tolerance of the transgenic plants
JIRCAS
Wheat (CIMMYT)
Rice (JIRCAS)
Groundnut (ICRISAT)
Soybean (Embrapa)
14


Galactinol synthase can improve dehydration tolerance in Arabidopsis
JIRCAS
Galactinol accumulates in seeds and stressed plants.
Raffinose family oligosaccharides (RFOs) are synthesized from galactinol, and function for stress tolerance.
Overexpression of galactinol synthase can improve dehydration tolerance of Arabidopsis.
Galactinol Synthase (AtGolS2)
UDP-Galactose
myo-Inositol
Galactinol
Sucrose
Raffinose
Stachiose
Control
AtGolS2 OE
16

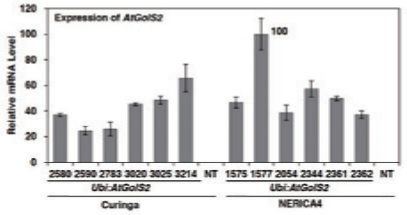

Genetic modification (Biotech)
JIRCAS
Molecular networks in abiotic stress responses
Drought
Low temperature
Drought & high salinity
Low temperature
Plant Cell
Signal Perception
NCED
Signal Transduction
ABA
ABA / AREB, DREB independent
Gene Expression
LEA, GolS etc.
Stress Response and Tolerance
High salinity
13


Development of drought-tolerant crops for developing countries (Japan MAFF Project)
JIRCAS
Making of constructs
32 constructs
Expression and functional analysis of stress-related genes
Gene expression analysis
Microarray analysis
Expression of transgene
IRRI
CIAT
CIMMYT
Transformation of rice and wheat
Evaluation in confined field (CIMMYT)
Evaluation in confined field (IRRI)
Evaluation in confined field (CIAT)
Evaluation of drought tolerance
Candidate lines were selected
MAFF project
Development of drought-tolerant crops for developing countries (GM Drought Tolerance)
15

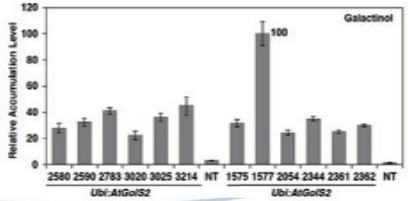
Ectopic overexpression of *AtGolS2* gene confers higher galactinol accumulation on transgenic Curinga and NERICA4 rice

Selvaraj et al. (2017) Plant Biotech J

Expression of *AtGolS2* in Ubi:AtGolS2 transgenic rice events

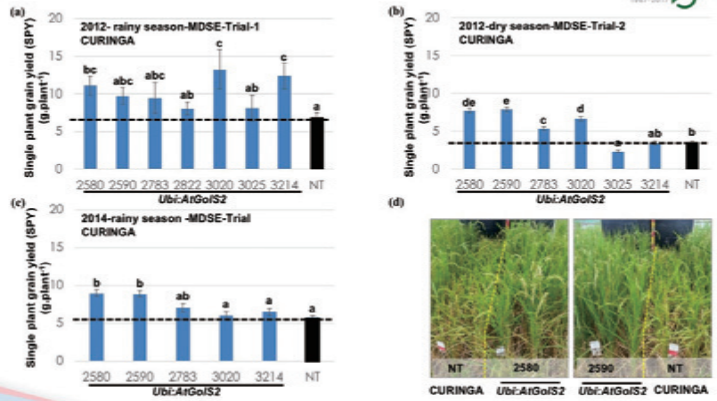


Accumulation of galactinol in Ubi:AtGolS2 transgenic rice events



17

Ubi:AtGolS2 improves rice grain yield in Managed Drought Stress Environment (MDSE) over the growing seasons – Rainout shelter experiments, CIAT, Palmira



Selvaraj et al. (2017) Plant Biotech J

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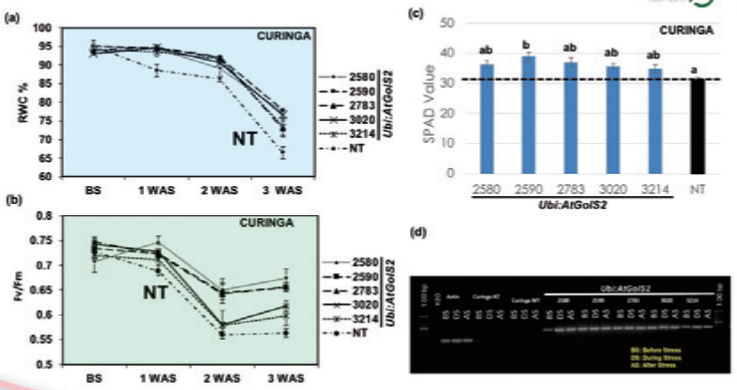
Variation in physiological parameters among the Curinga Ubi:AtGolS2 transgenic lines in 2014-rainy-MDSE-Trial



Selvaraj et al. (2017) Plant Biotech J

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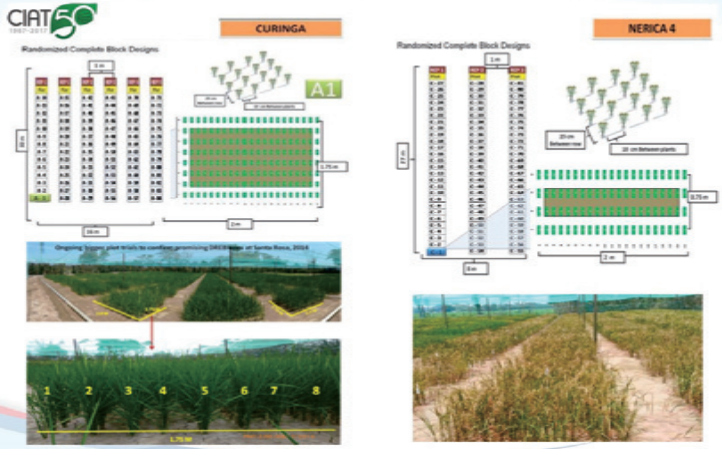
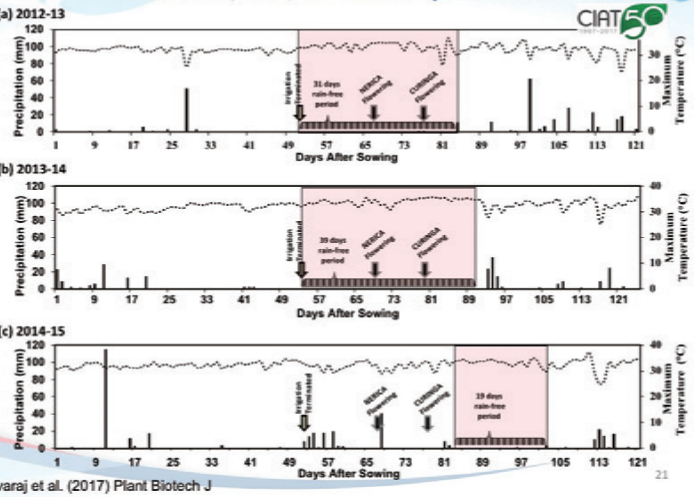
Field evaluation of transgenic rices at Santa Rosa Station of CIAT in Colombia



20

Rainfall and temperature pattern during crop period in upland confined rainfed field trial, CIAT, Santa Rosa upland station



Selvaraj et al. (2017) Plant Biotech J

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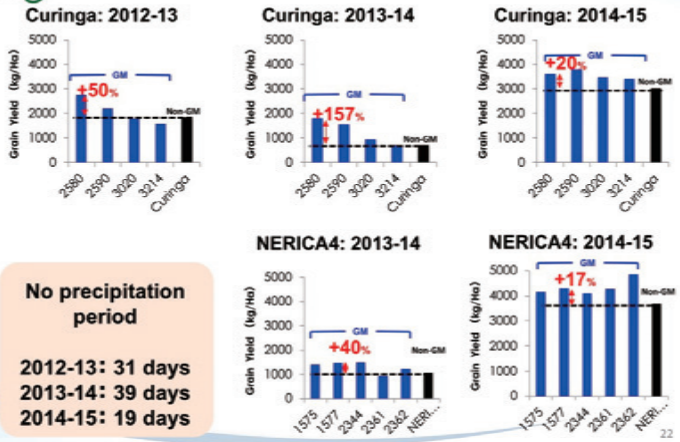
Ubi:AtGolS2 improves Curinga and NERICA4 grain yield in Target Environment

Selvaraj et al. (2017) Plant Biotech J



No precipitation period

2012-13: 31 days
2013-14: 39 days
2014-15: 19 days

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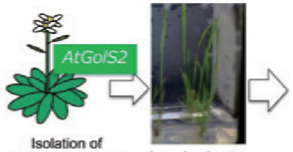
Successful demonstration cultivation of rice resistant to drought using biotechnology
- A big step toward practical application of dream crops –

- Crops with high productivity are required even under drought conditions.
- Succeeded in developing drought-tolerant rice utilizing dehydration-resistance gene *AtGolS2* of *Arabidopsis*.
- Demonstrated to show higher yield than original varieties under different conditions of drought.
- In the future, we will conduct cultivation tests in Africa etc., aiming for stable increase under drought conditions.

Introduced in NHK National News (News Check 11 April 4, 2017)



Isolation of Arabidopsis gene enhancing power to withstand dehydation

Introduction to rice varieties Curinga and NERICA 4

Improvement of yield in field under drought condition

Collaborating with RIKEN, CIAT, and Tsukuba Univ

Published in "Plant Biotechnology Journal" and press release

Selvaraj et al. (2017) Plant Biotech J

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SATREPS Project (2010-2015)

Development of Genetic Engineering Technology for Crops with Stress Tolerance against Degradation of Global Environment

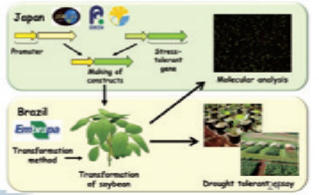
[Overall Goal]
Development of soybean varieties adapted to environmental stresses, aimed at contributing to the stabilization of soybean production in Brazil

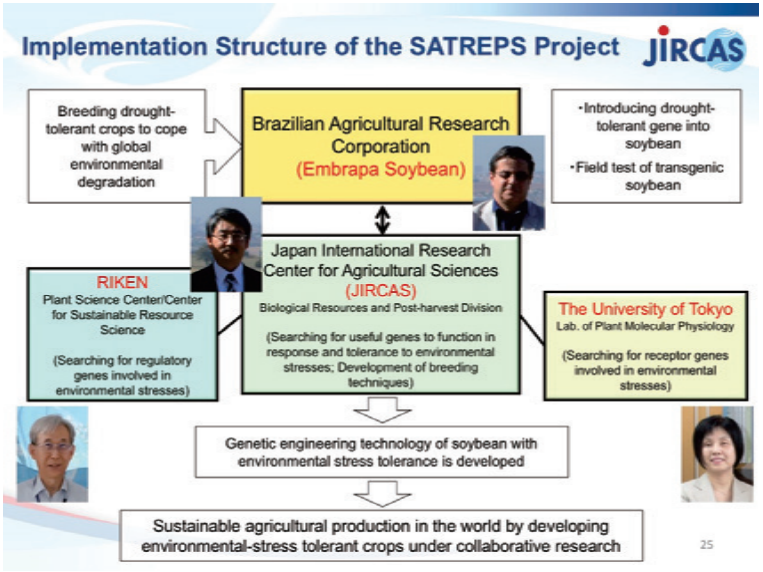
[Objective]
To develop genetic engineering technology for soybeans with environmental stress tolerance

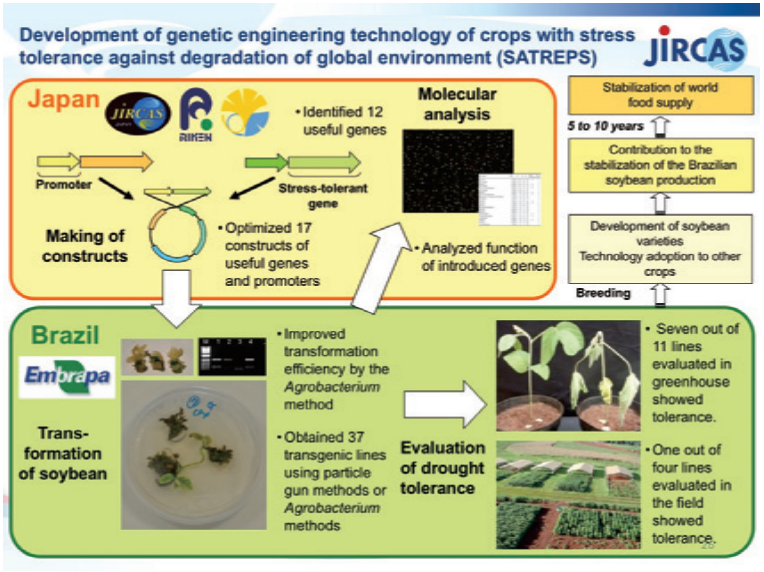
[Outputs]
1. Useful genes related to environmental stress tolerance were identified.
2. Stress-responsive promoters were isolated and their combinations with useful genes were optimized.
3. Transgenic soybean lines containing constructs of promoters and useful genes were produced.
4. Transgenic soybean lines with environmental stress tolerance were selected.

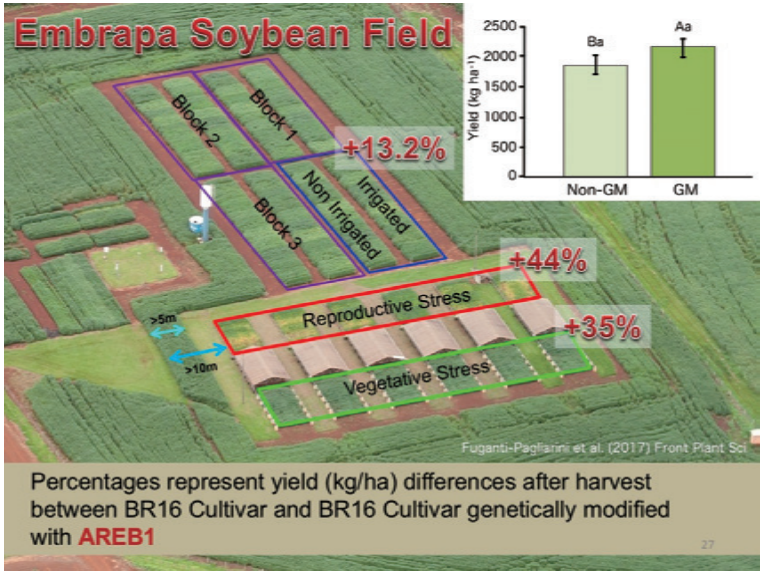
Drought damage in Brazil

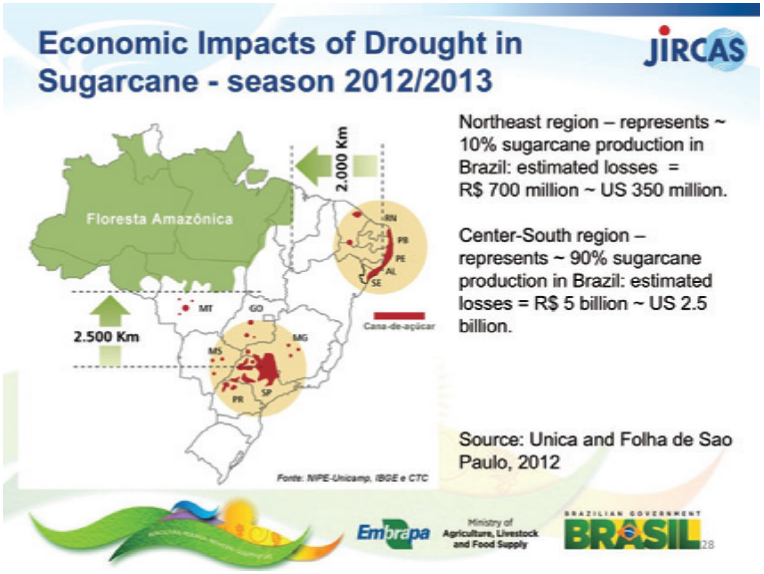
Field test of transgenic soybean in Brazil




Implementation Structure of the SATREPS Project
JIRCAS
Breeding drought-tolerant crops to cope with global environmental degradation
Brazilian Agricultural Research Corporation (Embrapa Soybean)
·Introducing drought-tolerant gene into soybean
·Field test of transgenic soybean
RIKEN
Plant Science Center/Center for Sustainable Resource Science
(Searching for regulatory genes involved in environmental stresses)
Japan International Research Center for Agricultural Sciences (JIRCAS)
Biological Resources and Post-harvest Division
(Searching for useful genes to function in response and tolerance to environmental stresses; Development of breeding techniques)
The University of Tokyo
Lab. of Plant Molecular Physiology
(Searching for receptor genes involved in environmental stresses)
Genetic engineering technology of soybean with environmental stress tolerance is developed
Sustainable agricultural production in the world by developing environmental-stress tolerant crops under collaborative research
25


Development of genetic engineering technology of crops with stress tolerance against degradation of global environment (SATREPS)
JIRCAS
Japan
RIKEN
·Identified 12 useful genes
Molecular analysis
Promoter
Stress-tolerant gene
Making of constructs
·Optimized 17 constructs of useful genes and promoters
·Analyzed function of introduced genes
Stabilization of world food supply
5 to 10 years
Contribution to the stabilization of the Brazilian soybean production
Development of soybean varieties
Technology adoption to other crops
Breeding
Brazil
Embrapa
Trans-formation of soybean
·Improved transformation efficiency by the Agrobacterium method
·Obtained 37 transgenic lines using particle gun methods or Agrobacterium methods
Evaluation of drought tolerance
·Seven out of 11 lines evaluated in greenhouse showed tolerance.
·One out of four lines evaluated in the field showed tolerance.

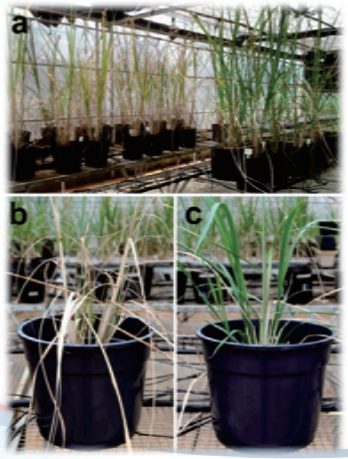

Embrapa Soybean Field
Block 1
Block 2
Block 3
+13.2%
Irrigated
Non Irrigated
+44%
+35%
Reproductive Stress
Vegetative Stress
>5m
>10m
Yield (kg ha⁻¹)
2500
2000
1500
1000
500
0
Ba
Aa
Non-GM
GM
Fuganti-Pagliarini et al. (2017) Front Plant Sci
Percentages represent yield (kg/ha) differences after harvest between BR16 Cultivar and BR16 Cultivar genetically modified with AREB1
27


Economic Impacts of Drought in Sugarcane - season 2012/2013
JIRCAS
Floresta Amazônica
2.000 Km
2.500 Km
Cana-de-açúcar
Fonte: NIPE-Unicamp, IBGE e CTC
Northeast region – represents ~ 10% sugarcane production in Brazil: estimated losses = R$ 700 million ~ US 350 million.
Center-South region – represents ~ 90% sugarcane production in Brazil: estimated losses = R$ 5 billion ~ US 2.5 billion.
Source: Unica and Folha de Sao Paulo, 2012
Embrapa
Ministry of Agriculture, Livestock and Food Supply
BRAZILIAN GOVERNMENT
BRASIL
28

Behavior of sugarcane with drought-inducible expression of AtDREB2A CA during preliminary 'survival' drought tolerance tests

JIRCAS

Embrapa Agroenergia



Three-month-old plants submitted to 6 days of withholding water, control non-transgenic plants (left side) and transgenic events (right side)

One-month-old control non-transgenic plant (b) and transgenic event (24.2) (c) were grown in 8-liter plastic pots and submitted to water deficit by withholding irrigation for 21 days

Reis et al. (2014) Plant Sci

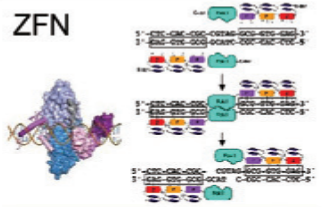
29

New breeding technology (NBT)

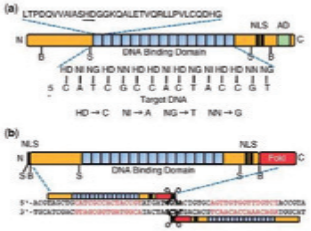
JIRCAS

Genome editing

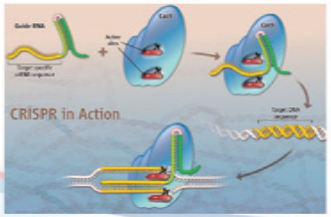
ZFN



TALEN



Cermak et al. (2010) Nucleic Acids Res.

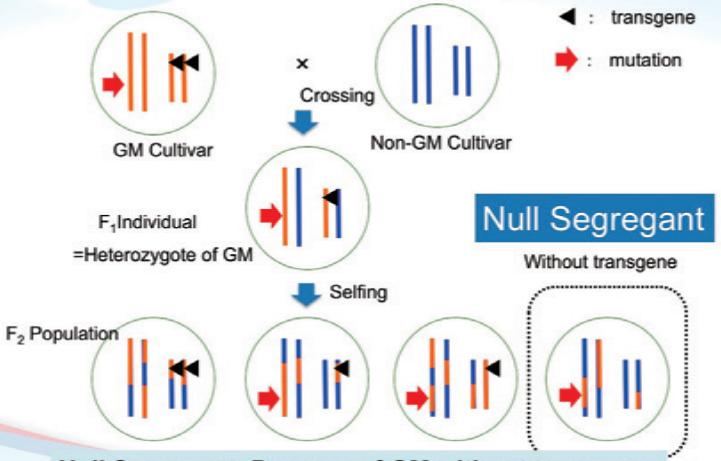


CRIPR/Cas9

30

Concept of "Null Segregant"

JIRCAS



Null Segregant: Progeny of GM without transgene

31

Example of product-based evaluation

JIRCAS

- The progeny of derived from Seed Production Technology (SPT) process using GM maize (DP-32138-1) , which will be imported to Japan, is considered not subject to the Cartagena Law.
- The main reason is because the offspring of this GM maize is controlled not to contain transgene – "Null Segregant".

✓ Genome editing will be accepted by farmers/consumers as offspring of the GM is controlled not to contain transgene – "Null Segregant".

✓ If Null Segregant is treated as non-GM, GM's deregulation process can be expected to be unnecessary.

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Genome editing systems in rice cultivars and strategy for producing desired mutants with homozygous mutation

CRISPR/Cas9 is a novel tool for targeted mutagenesis. *Agrobacterium*-mediated methods using immature embryos successfully transformed a CRISPR/Cas9 system into five rice cultivars and subsequently induced mutation.

Table 1. Targeted mutagenesis in rice cultivars using the CRISPR/Cas9 system and the *Agrobacterium*-mediated transformation of immature embryos

Cultivar	Number of transgenic plants	Number of monoallelic mutants	Number of biallelic mutants	Total number of mutants
Nipponbare	106	26	64	90
Koshihikari	17	1	10	11
NERICA1	54	16	33	49
Curinga	272	137	80	217
IR64	5	4	1	5
Total	454	184	188	372

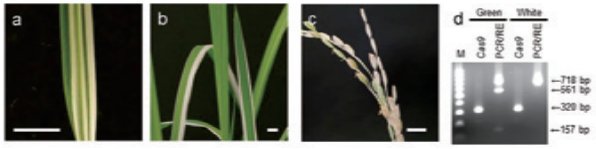


Fig. 1. Diagrammatic model showing the unpredicted segregation of a targeted mutation

Ishizaki T (2016) Mol. Breeding

Virus-induced down-regulation will accelerate the functional analysis of stress-related gene

ALSV-induced down-regulation of *GmERA1* genes enhances the stomatal response to abscisic acid and drought resistance in soybean.

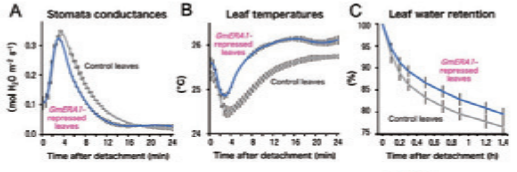
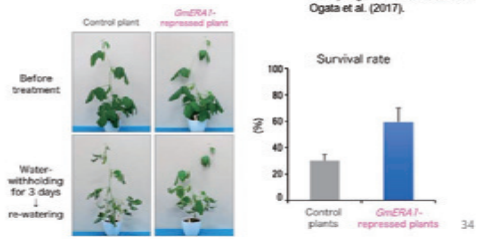


Fig. 1. Improved drought stress responses in GmERA1-repressed soybean leaves
Leaf detachment-induced response of stomatal conductance (A), leaf temperature (B), and water retention (C) in soybean leaves infected with GmERA1-recombinant virus were measured. Data are means ± SE (n = 3 to 6). Figures are modified from Ogata et al. (2017).

Fig. 2. Improved drought tolerance in GmERA1-repressed soybean plants
The soybean plants infected with GmERA1-recombinant virus were withheld water for three days before re-watering (left panels). Survival rate of the plants were recorded after re-watering (right panel). Data are means ± SE (n = 3 to 6). Figures are modified from Ogata et al. (2017).



Conclusion

1. To ensure food and nutrition security, we will endeavor to develop technologies and crops with high productivity and adaptability to adverse environments and changing climate conditions.
2. MAS : We have developed near-isogenic lines (NILs) with stress tolerance of crops such as soybean and rice using MAS.
3. Biotech: We have shown that overexpression of genes encoding stress-related transcription factors (e.g., DREB, AREB) and enzymes (e.g., galactinol synthase) improved drought tolerance in transgenic crops such as soybean and rice.
4. NBT: We are challenging to generate stress-tolerant crops utilizing NBT such as genome editing.
5. We hope these technologies and materials could contribute to achieving food and nutrition security in developing regions.

Important activity for researchers:
Public relations and two-way communication
Example: Civic participation type of two-way communication

Acknowledgement

MAS

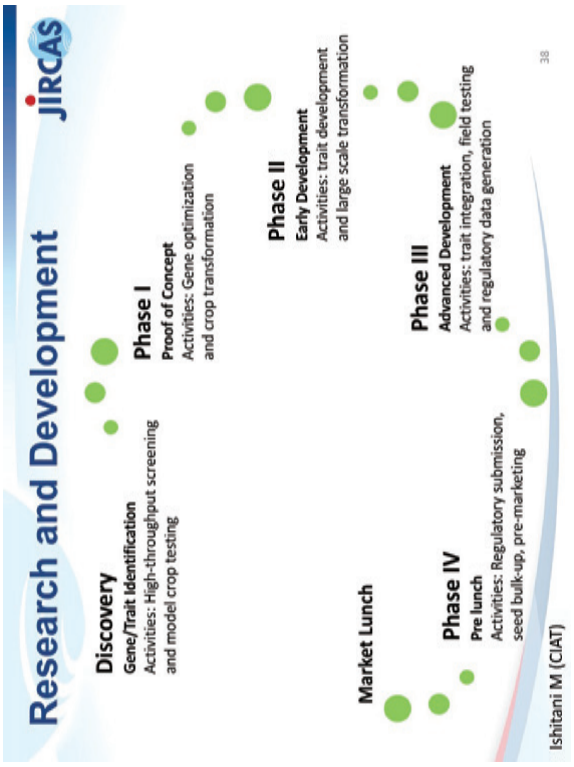
N Rice: Obara M, Fukuta Y (JIRCAS), Ishimaru T (JIRCAS/NARO), Kobayashi N, Fujita D (NARO) et al.
P Rice: Wissuwa M, Juan P-T (JIRCAS), Gamuyao R, Hyoun CJ, Catausan S, Dalid C, Slamet-Loedin I, Heuer S. (IRRI)、Pesaresi P (Univ of Milano)、Tecson-Mendoza EM (Univ of Philippines, Los Baño) et al.
SATREPS Madagascar: Tsujimoo Y, Wissuwa M, Yokoyama S (JIRCAS), Moritsuka N (Univ of Kyoto)、Razafimbelo T (LRI, Univ of Antananarivo)、Ramanantsoanirina A, Raymond R, Abel-Ratovo H (FOFIFA) et al.
EMF Rice: Ishimaru T (JIRCAS/NARO), Hirabayashi H, Kobayashi N, Fujita D (NARO) , Sasaki K (Univ of Tokyo), Gannaban RB, Simon EV, Lumanglas PD, Jagadish KSV (IRRI), Miras MA, Mendioro MS (U o Philippines, Los Baño) et al.
NaCl Soybean: Xu D, Shono M, Suenaga K, Do TD, Chen H, Vu HTT, Hamwieh A (JIRCAS), Yamada T (Hokkaido Univ), Sato T (Tohoku Univ), Yan Y, Cong H (Xinjiang Academy of Agricultural Sciences) et al.
Rust Soybean: Yamanaka N (JIRCAS), Shimakawa MU, Espinola C, Ohyama I (Nikkei-Cetapar, Paraguay)

Biotech

Rice: Selvaraj MG, Ishitani M (CIAT), Shinozaki K (RIKEN), Kusano M (Univ of Tsukuba), Ishizaki T (JIRCAS) et al.
Soybean: Nepomuceno AL (Embrapa Soybean), Yamaguchi-Shinozaki K (Univ of Tokyo) , Kanamori N (JIRCAS) et al.
Sugarcane: Molinari HBC (Embrapa Agroenergy) , Yamaguchi-Shinozaki K (Univ of Tokyo) et al.

NBT

CRISPR/Cas9 Rice: Ishizaki T (JIRCAS)
VIGS Soybean : Ogata T, Nagatoshi Y, , Fujita Y (JIRCAS) Yamagishi N, Yoshikawa N (Iwate Univ)


Research and Development
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Discovery
Gene/Trait Identification
Activities: High-throughput screening and model crop testing
Phase I
Proof of Concept
Activities: Gene optimization and crop transformation
Phase II
Early Development
Activities: trait development and large scale transformation
Phase III
Advanced Development
Activities: trait integration, field testing and regulatory data generation
Market Lunch
Phase IV
Pre lunch
Activities: Regulatory submission, seed bulk-up, pre-marketing
Ishitani M (CIAT)
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Development of biotechnologies and biotech crops for stable food production under adverse environments and changing climate conditions

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