Advancement of Asian Cassava Molecular Breeding towards SDGs

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Abstract: Cassava is an important tropical starch crop that provides food security, income generation and industrial application in tropical countries. We have developed cassava research platform towards advancing molecular breeding and understanding biological phenomena by the international collaborations between Japan, Vietnam, Thailand, and Colombia. The platform provides: a) 20,000 full-length cDNA resources, b) Omic analyses, c) integrative database, d) transformation system and e) CRISPR/Cas9-mediated mutagenesis. We optimized the system for induction of friable embryonic calli in cassava. Using the improved protocol, we have produced several transgenic cassava plants to improve cassava biomass quantitatively and qualitatively. We also have analyzed the molecular mechanisms of various biological phenomena, such as tuberization, disease resistance, drought and high-salinity stress response by integrative Omic analyses. Furthermore, we have analyzed the effects of environmental factors on the developmental phenotypes in the fields of Vietnam and Cambodia. RIKEN group has educated young Asian researchers cutting-edge plant science. We hope that our approach using the cutting-edge research platform and ICT will advance the molecular breeding of useful cassava plants, such as high-yield, increased stress tolerance and beneficial modifications of starch quality and contribute to solve the SDGs.

Keywords: Biomass, Cassava, Molecular Breeding, Southeast Asia, Starch, Stress Tolerance

Introduction

Cassava (*Manihot esculenta* Crantz) is a tropical crop and its tuberous roots are a major source of starch for more than a half billion people in Africa, Latin America, and Asia. Recently, the demand of cassava has been increased as a cash crop. Cassava plants are not only widely used as animal foods and industrial materials such as paper, textile, glue, etc, but also the cassava starch is further processed into a chemical modified starch and sweeter through saccharification process and fuel ethanol through fermentation process in addition to saccharification (*Fig. 1*).

As cassava demand increases, global climate changes have highlighted the importance of cassava studies on plant responses to various biotic and abiotic stresses. Conventional breeding efforts have tried to address many

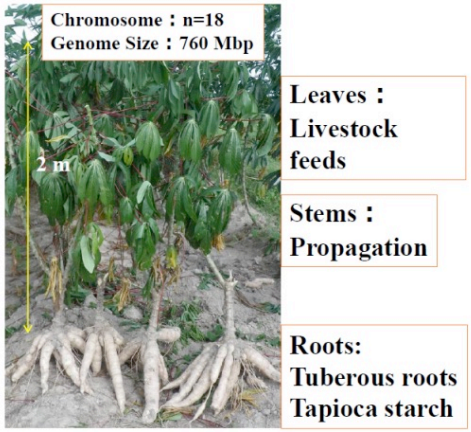

Figure 1. Cassava is an important tropical crop without waste.
After cutting, cassava plants grow up to about 2 m in 1 year. The number of chromosome and estimated genome size are n=18 and 760 Mbp, respectively. Cassava has good characters; 1) leaves are used as livestock feeds and called "Tropical Alfalfa", 2) stems are used for propagation, 3) cassava plants can produce tuberous roots, in which 20~30% starch per fresh weight accumulates. The starch is available for foods, feeds and industrial materials.

of the constraints facing cassava productivity, such as biotic and abiotic stresses, and starch quality. Although cassava exhibits higher tolerance to drought than other crops, it would still be expected to benefit from the efforts aimed at increased tolerance to drought and other stresses. In Southeast Asia, cassava has been distressed by various pest and disease problems. The analysts reported that there was a reduction in Thai cassava output by at least 30% in 2010 by mealybugs. Recently, Sri Lankan Cassava Mosaic Virus (SLCMV) has been found in Cambodia in 2016 and in Vietnam in 2018 [1, 2]. Among them, cassava mosaic disease (CMD) caused by CMV has caused serious and widespread damages, limiting production of the crop in Africa. CMD outbreak is on-going in Southeast Asia. On the other hand, applications of cassava starch (also known as tapioca) are found in the textile and pharmaceutical industry and food manufacturing [3]. The starch is composed of two molecules; amylopectin and amylose. The modifications of ratio and structure of these molecules improve the quality of cassava starch. The amylose-free cassava starch has been bred to show a higher swelling power, clearer pastes and a better freeze-thaw stability, of which properties are highly desirable in many food applications in comparison to starch from wild-type [3]. However, the breeding progress has been slow due to cassava's long generation time and complex genetic makeup, which makes it difficult to breed efficiently. Therefore, to overcome these obstacles, effective molecular breeding strategies are necessary to develop the useful cassava plants, such as high-yield and high-value starch under the stress conditions by the use of advanced technologies. Towards such Sustainable Development Goals (SDGs), we have advanced international cassava collaborative research since about 10 years ago in collaboration with the research groups of Thailand, Vietnam and Cambodia.

In this conference, we report our international cassava collaborative research including transformation and genome editing technology applications.

1. Materials and Methods

Cassava Cultivars: The African cassava cultivar "60444 (*Manihot esculenta* Crantz)" was obtained from the International Institute of Tropical Agriculture (IITA, Nigeria) *in vitro* cassava germplasm collections.

Plant preparation: Approximately 2 to 3 cm stems from cassava plantlets were transplanted to a plastic pot with 100 mL of Murashige & Skoog (MS) media (pH5.8) containing 20 g/L sucrose, 4.4 g/L MS salts containing vitamins (Duchefa), 2 μM $CuSO_4$ (Wako), and 3.0 g/L gelrite (Wako) [4]. The plantlets were maintained under a 16-h illumination of 100 μmol photons $m^{-2}sec^{-1}$ at 28ºC.

Vector construction: Oligonucleotide pairs for the target sequences (5'-AAACGGAGCTCACACTTGAGCATC-3' and 5'-GAGGGATGCTCAAGTGTGAGCTCC-3') of granule-bound starch synthase I (GBSS1) from cassava were annealed, and the resulting fragments cloned into the *Bbs*I site of the sgRNA cloning vector p2x35SgRNA between the two *I-Sce*I sites of the vector. To complete the all-in-one binary vector harboring sgRNA, Cas9 and an HPT expression construct, pZH_gYSA_PcUbiFFCas9 was used. The p2x35SgRNA was excised at the *I-Sce*I sites and the resulting fragment was ligated into pZH_gYSA_PcUbiFFCas9 [5].

Cassava transformation: *Agrobacterium*-mediated transformation was performed using friable embryogenic calli (FEC) from the 60444 cultivar essentially as reported previously (*Fig. 2*) [6].

2. Results and Discussion:

To apply on the cassava plant science using current advanced technologies, the activities supported by Ministry of Education, Culture, Sports, Science and Technology (MEXT) and JST e-ASIA Projects has advanced two international cassava collaborative projects:(1) "Towards food security in Asia and Africa by development and application of advanced molecular breeding technologies for the tropical crop, cassava" (2009–2012, with CIAT, Mahidol University, and AGI, supported by Strategic Funds for the Promotion of Science and Technology, MEXT) and (2) "Advancement of Asian cassava molecular breeding by cutting-edge technologies" (2012–2015, with AGI and Mahidol University, e-ASIA project supported by JST) [7, 8]. RIKEN group has developed an integrative and functional genomics platform necessary for the advancement of the cassava molecular breeding (*Fig. 3*). The oligo DNA microarray containing about 30,000 cassava genes, heavy ion beam-mediated mutagenesis, and transformation technology of these platform have been used for identification of useful gene candidates and the evaluation of gene function (*Fig. 3*) [9-11].

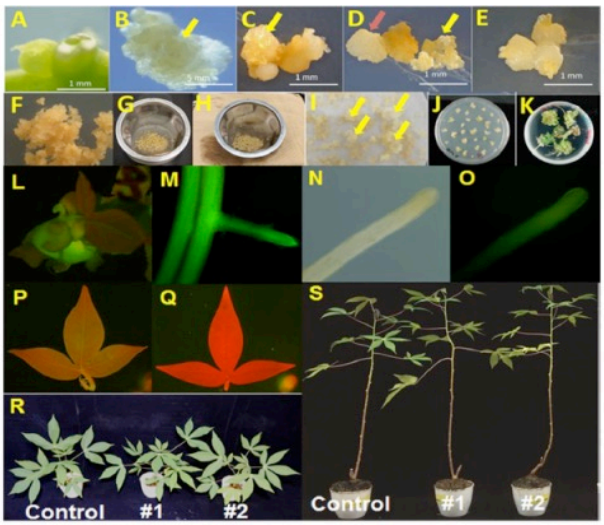

Figure 2. FEC induction and genetic transformation in the cassava cultivar 60444'.

(A) Axillary bud formed in stem cuttings cultured on CAM, (B) SE produced on CIM, (C) Somatic embryo (SE) produced on DKW, (D) Friable Embryogenic callus (FEC, yellow arrow) and non-FEC (red arrow) produced on CIM-½ NPK, (E) non-FEC cultured on FIM for additional two weeks after transferring non-FEC in Fig 2D to fresh FIM, (F) FEC cultured on FIM for additional 4 weeks after transferring FEC in Fig 1D to fresh FIM, (G) Collection of FEC in a tea filter (30 mesh) and soaking the collected FEC in an Agrobacterium (EHA105) suspension for 2 min, (H) removal of excess Agrobacterium suspension by blotting on distilled Kimtowel, (I) multiplying FEC (shown in yellow arrows) on FIM amended with 20 mg/L of hygromycin, (J) induction of cotyledons from FEC on MSN amended with hygromycin, (K) induction of shoots on CEM from transferred cotyledons , (L) GFP fluorescence in transgenic in vitro plantlet of cassava, (M) GFP fluorescence in root tissue of a transgenic plantlet of cassava, (N) observation of root from non-transgenic in vitro plant using light microscope, (O) observation of root from non-transgenic in vitro plant using fluorescence microscope, (P) GFP fluorescence in leaves of transgenic plant, (Q) absence of GFP fluorescence in a non-transgenic cassava plant, and phenotype of transgenic (#1 and #2) and non-transgenic (control) cassava cultured for 2 (R) and 4 (S) months at 28°C in greenhouse after cutting.

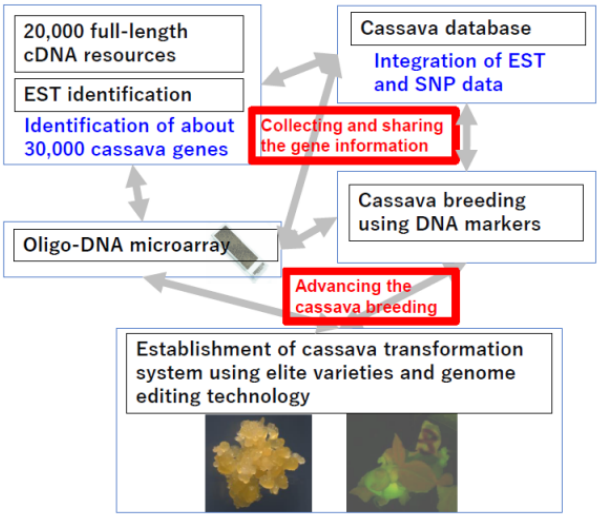

Figure 3. International Cassava Collaborative Research between Japan and Southeast Asia towards Advancement of Molecular Breeding.

The collaborative projects have also provided various opportunities for education of cutting-edge and global plant science to Asian young researchers, strengthened our cassava research network, and contributed to the advancement of cassava molecular breeding toward resolution of the problems [12]. RIKEN group has joined the International Laboratory for Cassava Molecular Breeding (ILCMB; established between AGI, Vietnam Academy of Agricultural Sciences (VAAS), and CIAT) as a core group since 2012 to advance the cassava molecular breeding.

RIKEN group has applied the genome editing technology such as CRISPR/Cas9 system to cassava in collaboration with NARO group in the program of Japan Society for the Promotion of Science (JSPS) for Advancing Strategic International Networks to Accelerate the Circulation of Talented Researchers (2016-2018). CRISPR/Cas9 technology has emerged as a powerful technology for genome editing and is now widely used for not only animal research but also plant studies to analyze the gene function. We attempted the targeted genome editing of cassava *granule bound starch synthase I (GBSSI)* gene to produce amylose-free cassava and confirmed the detection of genome editing on *GBSS1* gene and the loss of amylose as expected (*Fig. 4*). These results demonstrate that the CRISPR/Cas9 technology is effective for improving the cassava starch traits.

SATREPS cassava project (2016-2020; supported by JST/JICA) has been ongoing as an international collaboration among research groups from Japan, Vietnam, Cambodia, and Thailand towards establishment of the management system for healthy cassava seeds in addition to development of new cassava breeding technologies to shorten the breeding cycle [13]. Several technologies and research products developed in the previous research have been applied to the SATREPS project

[13].

Figure 4. Gene-editing by CRISPR-Cas9 of cassava GBSS1 gene involved in amylose biosynthesis of starch.
(A) Representative sequences of mutant alleles identified in transgenic plants of cassava GBSS1 gene. The sequence of wild-type (WT) is shown at the top with the protospacer adjacent motif (PAM) sequence highlighted in gray, and the target sequence in underline. Dashes, deleted bases. The net change in length is noted to the right of each sequence. The number of clones representing each mutant allele is shown in the column on the right. (B) Iodine staining of leaf starch from mutant line (GBSS1-6) and WT. Leaf from WT was stained to black, whereas leaf from the GBSS1-6 was colored to brown due to the loss of the amylose.

3. Conclusions and Future Perspectives:

The global research network is so important for advancing cassava molecular breeding and provides cassava researchers with the useful tools, knowledge and good opportunities each other. The goal of ILCMB is to provide genetic solutions to overcome the challenges highly relevant to cassava producers as well as to offer opportunities to producers and end users such as commercially valued traits. We are also trying to exploit ICT to cassava cultivation in the fields of Vietnam and Cambodia in the SATREPS program. It is limited to utilize many knowledges to problems that occur in natural environments or agricultural fields at present, although cutting-age technologies give many knowledges to our understanding of physiological manner. In particular, understanding of the molecular mechanisms of flowering and tuberous root formation in cassava are the most important research themes (*Fig. 5*). Our approach using the cutting-edge research platform and ICT will advance the molecular breeding of useful cassava plants, such as shortening of breeding cycle, high-yield, increased stress tolerance and beneficial modifications of starch quality, and contribute to solve the SDGs.

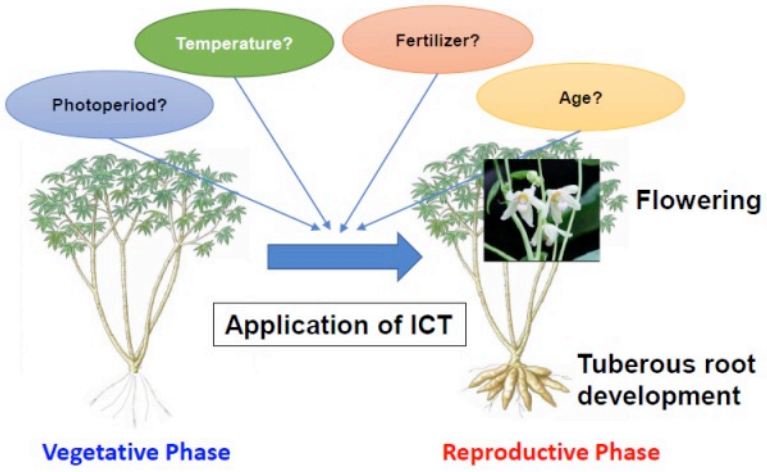

Figure 5. ICT application towards improvement of important agronomic traits, such as flowering and tuberous root developments in Southeast Asia.

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References:

[1] Hua-Ling Wang, Xi-Yun Cui, Xiao-Wei Wang, Liu Shu-Sheng, Zenghui Zhang, Xueping Zhou, “First Report of Sri Lankan cassava mosaic virus Infecting Cassava in Cambodia”, Plant Disease, 2018, vol. 100, pp. 1029.
[2] Ayaka Uke, Trinh Xuan Hoat, Mai Van Quan, Nguyen Van Liem, Masashi Ugaki, Keiko T. Natsuaki, “First Report of Sri Lankan cassava mosaic virus Infecting Cassava in Vietnam”, Plant Disease, 2018, accepted.
[3] Steve Jobling, “Improving starch for food and industrial applications”, Curr Opin Plant Biol, 2004, vol. 7, pp. 210-218.
[4] Toshio Murashige, Folke Skoog, “A revised medium for rapid growth and bio-assays with tobacco tissue cultures”, Physiol Plant, 1962, vol. 15, pp. 473-497.
[5] Masafumi Mikami, Seiichi Toki, Masaki Endo, “Comparison of CRISPR/Cas9 expression constructs for efficient targeted mutagenesis in rice”, Plant Mol Biol, 2015, vol. 88, pp. 561-572.
[6] Yoshinori Utsumi, Chikako Utsumi, Maho Tanaka, Vu The Ha, Akihiro Matsui, Satoshi Takahashi, Motoaki Seki, “Formation of friable embryogenic callus in cassava is enhanced under conditions of reduced nitrate, potassium and phosphate”, PLoS ONE, 2017, vol. 12, e0180736.
[7] Tetsuya Sakurai, Germán Plata, Fausto Rodríguez-Zapata, Motoaki Seki, Andrés Salcedo, Atsushi Toyoda, Atsushi Ishiwata, Joe Tohme, Yoshiyuki Sakaki, Kazuo Shinozaki, Manabu Ishitani, “Sequencing analysis of 20.000 full-length cDNA clones from cassava reveals lineage specific expansions in gene families related to stress response”, BMC Plant Biol, 2007, vol. 7, 66.
[8] Tetsuya Sakurai, Keiichi Mochida, Takuhiro Yoshida, Kenji Akiyama, Manabu Ishitani, Motoaki Seki, Kazuo Shinozaki, “Genome-wide discovery and information resource development of DNA polymorphisms in cassava”, PLos One, 2013, vol. 8, e74056.
[9] Yoshinori Utsumi, Maho Tanaka, Taeko Morosawa, Atsushi Kurotani, Takuhiro Yoshida, Keiichi Mochida, Akihiro Matsui, Yoshimi Umemura, Manabu Ishitani, Kazuo Shinozaki, Tetsuya Sakurai, Motoaki Seki, “Transcriptome analysis using a high-density oligomicroarray under drought stress in various genotypes of cassava: An important tropical crop”, DNA Res, 2012, vol. 19, pp. 335-345.
[10] Punchapat Sojikul, Treenut Saithong, Saowalak Kalapanulak, Nuttapat Pisuttinusart, Siripan Limsirichaikul, Maho Tanaka, Yoshinori Utsumi, Tetsuya Sakurai, Motoaki Seki, Jarunya Narangajavana, “Genome-wide analysis reveals phytohormone action during cassava storage root initiation”, Plant Mol Biol, 2015, vol. 88, pp. 531-543.
[11] Yoshinori Utsumi, Maho Tanaka, Atsushi Kurotani, Takuhiro Yoshida, Keiichi Mochida, Akihiro Matsui, Manabu Ishitani, Supajit Sraphet, Sukhuman Whankaew, Thipa Asvarak, Jarunya Narangajavana, Kanokporn Triwitayakorn, Tetsuya Sakurai, Motoaki Seki, “Cassava (*Manihot esculenta*) transcriptome analysis in response to infection by the fungus *Colletotrichum gloeosporioides* using an oligonucleotide–DNA microarray”, J Plant Res, 2016, vol. 129, pp. 711-726.
[12] Yoshinori Utsumi, Tetsuya Sakurai, Yoshimi Umemura, Sarah Ayling, Manabu Ishitani, Jarunya Narangajavana, Punchapat Sojikul, Kanokporn Triwitayakorn, Minami Matsui, Ri-ichiroh Manabe, Kazuo Shinozaki, Motoaki Seki, “RIKEN Cassava Initiative: Establishment of a cassava functional genomics platform”, Tropical Plant Biol, 2012, vol. 5, pp. 110-116.
[13] Hiroki Tokunaga, Tamon Baba, Manabu Ishitani, Kasumi Ito, Ok-Kyung Kim, Ham Huy Le, Hoang Khac Le, Kensaku Maejima, Shigeto Namba, Keiko T. Natsuaki, Dong Van Nguyen, Hy Huu Nguyen, Nien Chau Nguyen, Vu Anh Nguyen, Hisako Nomura, Motoaki Seki, Pao Srean, Hirotaka Tanaka, Bunna Touch, Hoat Xuan Trinh , Masashi Ugaki, Ayaka Uke, Yoshinori Utsumi, Prapit Wongtiem, Keiji Takasu, “Sustainable Management of Invasive Pests of Cassava in Vietnam, Cambodia and Thailand. In “Crop Production under Stressful Conditions: Application of Cutting-edge Science and Technology in Developing Countries (Edited by Drs. Makie Kokubun and Shuichi Asanuma)”, 2018, Springer, pp. 131-157.