**Agrobacterium**-mediated cassava transformation for the Asian elite variety KU50

Yoshinori Utsumi1 · Chikako Utsumi1 · Maho Tanaka1,2 · Yoshie Okamoto1 · Satoshi Takahashi1 · Tong Thi Huong3 · Anh Vu Nguyen3 · Nguyen Van Dong3 · Hiroki Tokunaga1 · Nigel Taylor4 · Motoaki Seki1,2,5

Received: 15 July 2021 / Accepted: 28 October 2021 / Published online: 26 November 2021
© The Author(s), under exclusive licence to Springer Nature B.V. 2021

**Abstract**

**Key message** Cassava genetic transformation has mostly been reported for African cassava varieties, but not for Asian varieties. This is the first report of cassava transformation in Asian elite varieties using friable embryogenic calli.

**Abstract** *Agrobacterium*-mediated cassava transformation via friable embryogenic calli (FEC) has enabled the robust production of transgenic cassava. So far, mostly the model cassava variety 60444 and African varieties have been transformed because of their good production and regeneration from embryogenic tissues. It is important to develop transformation methods for elite Asian cassava varieties to meet the changing needs in one of the world’s major cassava production areas. However, a suitable transformation method for the Asian elite variety Kasetsart 50 (KU50) has not been developed. Here, we report a transformation method for KU50, the cultivar with the highest planting area in Thailand and Vietnam. In cassava transformation, the preparation of FEC as the target tissue for transgene integration is a key step. FEC induction from KU50 was improved by using media with reduced nutrients and excess vitamin B1, and somatic embryo and plant regeneration optimized by manipulation of naphthalene acetic acid (NAA), and benzylamino purine (BAP). The transformation efficiency for KU50 was 22%, approximately half that of 60444 at 45%. Transcriptome analysis indicated that the expression of genes related to cell-wall loosening was upregulated in FEC from KU50 compared with 60444, indicating that cell-wall production and assembly were disproportionate in the Asian variety. The transformation system for KU50 reported here will contribute to the molecular breeding of cassava plants for Asian farmers using transgenic and genome-editing technologies.

**Keywords** Transformation · Cassava · KU50 · Asian elite variety · FEC

**Introduction**

Cassava (*Manihot esculenta* Crantz) is grown across the tropics and sub-tropical regions for the production of its starchy tuberous roots. It provides an important source of daily calories for hundreds of millions of people and plays a critical role in global food security and rural economies (Balat and Balat 2009). The acceleration of cassava breeding can help meet the demands of the increasing population and evolving market (Malik et al. 2020). However, conventional breeding based on sexual hybridization in cassava is challenging because of its high degree of genetic heterozygosity, inbreeding depression, asynchronous flowering, self-incompatibility, and low fruit set in some varieties (Ceballos et al. 2004; Liu et al. 2011). For semi-woody vegetative propagated species such as cassava, genetic transformation and genome editing can provide fundamental insights into plant biology along with methods for rapid crop improvement.
through integration of novel traits without altering the genetic background of elite farmer-preferred parent material (Altpetera et al. 2016). Recently, genome editing by CRISPR-Cas9 technology has been adopted (Hummel et al. 2018; Odipio et al. 2017; Seki et al. 2018). For both gene editing and conventional transformation, the preferred methods involve *Agrobacterium*-mediated delivery of molecular sequences into disorganized embryogenic calli, with subsequent regeneration of somatic embryos and their germination to produce plants.

The induction of friable embryogenic calli (FEC) is a key step for producing target tissues for cassava transformation and has been established as a reproducible method for cassava genetic transformation (Chavarriaga-Aguirre et al. 2016). FEC are generated from somatic embryos induced on media supplemented with the potent auxin picloram (Taylor et al. 1996, 2012; Bull et al. 2009). Improvement in FEC induction was achieved by adding tyrosine and moxolactam (Chauhan et al. 2015; Nyaboga et al. 2015), and the optimization of nutrient conditions in the culture media (Utsumi et al. 2017). Despite these advances, the production of transgenic cassava is limited to a relatively small range of varieties, mostly of African origin.

In this study, we report the establishment of *Agrobacterium*-mediated transformation of the Asian elite cassava variety, Kasetsart 50 (KU50). Currently, KU50 is cultivated in Thailand, Vietnam, Indonesia, Cambodia, Myanmar, and the Philippines. This variety has been cultivated in more than one million hectares of Thailand and Vietnam alone (Malik et al. 2020).

**Materials and methods**

**Plant materials and growth conditions**

Cassava cultivars Kasetsart 50 (KU50) and 60444 were obtained from the in vitro germplasm collection of the International Center for Tropical Agriculture (CIAT), Cali, Colombia. In vitro plantlets were maintained by sub-culturing microcuttings every 4–8 weeks on Murashige and Skoog basal salts (Murashige and Skoog 1962) (MS) supplemented with 20 g/L sucrose, 2 µM CuSO₄, MS vitamins, and 3 g/L geltrite. The media was adjusted to pH 5.8 using 1 M KOH before autoclaving for 20 min at 121 °C (Supplementary Table S1). Plantlets were cultured in plastic vessels containing 100 mL of media and incubated at 28 °C under a 16 h light/8 h dark photoperiod at 40 μmol m⁻² s⁻¹ intensity.

Axillary buds (AB) produced from nodal stems of in vitro plantlets of KU50 and 60444 were used as explants for the induction of organized embryogenic calli (OEC). To induce the formation of the AB, all leaves were removed from the in vitro plantlets (8–10 cm in size), and 20–30 mm stem sections carrying single nodes were excised. The single-nodal-stem fragments were placed horizontally on cassava axillary medium (CAM) consisting of MS medium supplemented with 10 mg/L BAP (Bull et al. 2009) and incubated for 4–7 days at 28 °C in the dark. Swollen AB were excised from the stem sections using a sterile syringe and incubated on callus induction medium (CIM) consisting of MS medium supplemented with 12 mg/L picloram for 2 weeks at 28 °C in the dark. The number of AB that formed the OEC was counted at two weeks after culturing on CIM. When multiple OEC were observed from one axillary bud, the number of OEC induced was counted as one. The OEC produced were excised and subcultured onto media containing Driver and Kuniyuki Walnut basal salts (Driver and Kuniyuki 1984), supplemented with 20 g/L sucrose, MS vitamins, and 12 mg/L picloram (Chauhan et al. 2015). After culturing for 2 weeks at 28 °C in the dark, OECs were transferred to FEC induction media (FIM) consisting of Gresshoff and Doy basal salts (Gresshoff and Doy 1974) (GD), supplemented with MS vitamins and reduced nutrients, excess vitamin B1, and 12 mg/L picloram as described by Utsumi et al. (2017) (Supplementary Table 1). The OEC were cultured on FIM for 2–3 weeks at 28 °C in the dark. Fine FEC were isolated from non-embryogenic tissues using fine tweezers and transferred to fresh FIM. The isolated FEC were stringently selected in this manner every 2–3 weeks for a maximum of six months to produce homogenous colonies of FEC. The number of ABs that formed the FEC was estimated at six months after culturing on FIM. When multiple FEC were induced from OEC induced from one bud, then the number of FEC induced was counted as one.

**Agrobacterium-mediated transformation and plant regeneration**

The pMDC111 binary plasmid vector (Curtis and Grossniklaus 2003) was used to transform KU50 and 60444 FEC tissues. The mGFP6 reporter gene (Born and Pfeifer 2019) in pMDC111 was under the control of an enhanced 35S promoter. A DNA sequence of the enhanced 35S promoter was obtained by PCR using the pCAMBIA1300 as the template using the primer pair: (Forward: 5′-GGGGCAAGTTTGTACAAAGGCAGGCTATGGTGAGCCGACGTA-3′) and (Reverse 5′-GGGGACCATTTCCTAGAACAAAGAGCTGGGTGGAGATAGATTGTAGAGA-3′). A DNA fragment of the enhanced 35S promoter was cloned into the pDONR/Zero vector using the BP cloning reaction (ThermoFisher). A DNA fragment in pDONR/Zero was ligated into the pMDC111 vector by the LR cloning reaction (Thermo Fisher). The pMDC111 vector with enhanced 35S promoter:mGFP6 was electroproporated into *Agrobacterium tumefaciens* strain LBA4404 (Hood et al. 1986),
and transformation of FEC was performed as described by Utsumi et al. (2017).

After co-culture, inoculated FEC were subcultured onto FIM containing 200 mg/L carbenicillin and 5 mg/L hygromycin, and cultured for one week at 28 °C under continuous dark conditions. Tissues were then subcultured on FIM containing 10 mg/L hygromycin and 200 mg/L carbenicillin, and cultured for one week. A third selection was subsequently performed on FIM supplemented with 20 mg/L hygromycin and 200 mg/L carbenicillin for one week. Regeneration of cotyledon-stage embryos was achieved by transferring FEC colonies to SE emerging medium (MSN) consisting of MS salts supplemented with 1 mg/L NAA, 100 mg/L carbenicillin and 20 mg/L hygromycin, and cultured for a maximum of four months. Cotyledon-stage embryos were selected and subcultured onto cassava elongation medium (CEM) composed of MS medium amended with 0.4 mg/L BAP, 100 mg/L carbenicillin, and 20 mg/L hygromycin. Embryos were cultured on CEM until the shoots and roots were produced. Regenerated plantlets were maintained on MS media supplemented with 50 mg/L carbenicillin and 5 mg/L hygromycin.

**Establishment and growth of plants in the greenhouse**

In vitro plants were transferred to a greenhouse. One stem cut per regenerated plant line was grown in 100 mL of MS medium solidified with 0.5% (w/v) agarose in a plastic vessel for two months at 28 °C. Subsequently, the medium was then carefully removed to prevent damage to the roots, and plants were cultivated for approximately 2 weeks in plastic vessels containing distilled water at 28 °C under cool-white fluorescent light at 40 μmol m⁻² s⁻¹ intensity. Thereafter, plantlets were transferred to soil consisting of a mixture of black soil, coconut peat, and vermiculite at a ratio of 2:1:2 in a pot. This was maintained within a plastic tray covered by plastic wrap for 21 days in a greenhouse maintained at 28 °C under natural light in Yokohama, Japan, and supplemental lighting to an approximate light/dark cycle of 12/12 h. The plastic wrap was removed gradually and plants were grown on an open bench in a greenhouse maintained at 28 °C under natural light in Yokohama, Japan, and supplemental lighting with an approximate light/dark cycle of 12 h/12 h.

**Microscopic observation**

GFP fluorescence was observed on the first to third leaves from the top of plantlets maintained in MS media and on the roots of plants grown in soil. Imaging was performed using an M165 FC fluorescence microscope (Leica, Wetzlar, Germany) equipped with a cooled CCD camera VB-7010 (Keyence, Osaka, Japan), 10× eyepiece, plan apo 2× Corr. objective or objective planapo 0.63× M series (Leica, Wetzlar, Germany). Images were taken using illumination with an excitation laser line of 486 nm and an emission filter of 518 nm.

**Detection of GFP and HPT gene**

The first or second youngest healthy leaves of in vitro plants were used for genomic DNA preparation. Leaf samples were disrupted using ShakeMaster and zirconia beads (Hirata Corporation), and genomic DNA was extracted according to the protocol of the Wizard Magnetic 96 DNA Plant System (Promega) using BIOMEK (Beckman Coulter). Genomic DNA was stored at −80 °C pending further use.

The presence of gfp and hpt transgenes in transgenic cassava lines was confirmed by PCR analysis using KOD-FX Neo (Toyobo) and gene-specific primers. The thermocycler parameters used for amplification of gfp and hpt were 95 °C for 5 min; 35 cycles of 98 °C for 10 s, 68 °C for 30 s, and 68 °C for 30 s; and a final 5-min extension at 68 °C. PCR products were analyzed by gel electrophoresis on a 1% (w/v) agarose gel stained with ethidium bromide. The primers used for PCR were as follows: GFP, 5‘- CACTGGAGTTGTCCCCAATTCTTGTTG-3‘ (forward) and 5‘-CATGCCATGTGAATCCCAGCA-3‘ (reverse); HPT, 5‘-TCACCGCGACGTCTGTCGAG-3‘ (forward) and 5‘-GCTCCATAAACGCAACCAC-3‘ (reverse).

**Total RNA extraction from FEC samples**

Total RNA was extracted from the homogenous FEC tissues of KU50 and 60444. Total RNA was extracted from at least 100 mg of fresh weight (FW) per biological replicate as described by Utsumi et al. (2017) and then stored at −80 °C until use.

**Oligo-microarray analysis of gene expression**

Total RNA was used to evaluate gene expression levels with a cassava DNA oligo-microarray comprising more than 30,000 probes, as described by Utsumi et al. (2016). Gene expression data represent four independent biological replicates per FEC from KU50 and 60444. Eight microarray data sets were analyzed using GeneSpring GX (Agilent Technologies, USA). Specifically, the data were analyzed using Student’s t-test with a Benjamini-Hochberg (BH) false discovery rate (FDR), to identify differentially expressed genes (DEGs) between two data sets. The oligo-DNA microarray data were deposited in the NCBI Gene Expression Omnibus (GEO) database and are accessible via the GEO Series accession number GSE169685.
Southern blot analysis to determine T-DNA copy number

Genomic DNA was prepared using the CTAB method (Doyle and Doyle 1987). Homogenized leaf samples were prepared by cryogenically grinding the tissue in a multibead shaker after chilling in liquid nitrogen. One gram of homogenized tissue was incubated in 6 ml of 2×CTAB (2% CTAB, 100 mM Tris–HCl [pH 8.0], 1.4 M NaCl, 20 mM EDTA) at 55 °C for 60 min. After centrifuging the homogenate for 20 min at 2800 × g, the supernatant was transferred to a new tube and gently mixed with 1 volume of chloroform/isoamyl alcohol (24:1) for 20 min at room temperature, followed by centrifugation for 20 min at 2800 × g to separate the phases. The aqueous upper phase was collected by centrifugation for 30 min at 2,800 × g. The DNA was precipitated by adding 1 volume of CTAB precipitation buffer (1% CTAB, 50 mM Tris–HCl [pH 8.0], 0.7 M NaCl) and 1 volume of chloroform/isoamyl alcohol (24:1), and incubated for 10 min at room temperature. The aqueous upper phase was collected by centrifugation for 30 min at 2,800 × g. The DNA was precipitated by adding 1 volume of CTAB precipitation buffer (1% CTAB, 50 mM Tris–HCl [pH 8.0], 10 mM EDTA) to the aqueous upper phase collected, and incubated at room temperature overnight. The DNA was collected by centrifugation at 5,000 × g for 10 min and dissolved in 100 µL of TE buffer (10 mM Tris–HCl [pH 8.0], 1 mM EDTA). Three microliters of RNase solution A (10 mg/mL) was added to the DNA solution and incubated at 37 °C for 30 min. Southern blot analysis was performed according to the standard protocol (Southern 2006). Five micrograms of genomic DNA were incubated for 20 h with 30 units of HindIII, which cuts the T-DNA once and subjected to electrophoresis on a 0.8% agarose gel. The agarose gel was soaked in depurination solution (0.2 N HCl) for 10 min at room temperature and denatured in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 30 min. The resultant agarose gel was then soaked in neutralization solution (1.5 M NaCl, 0.5 M Tris–HCl [pH 7.5]) for 30 min. Thereafter, the fractionated DNA in agarose gel was transferred to a positively charged nylon membrane using capillary blotting in 20×SSC (3 M NaCl in 0.3 M sodium citrate [pH 7.0]). The hybridization probe for hpt of T-DNA was DIG-dUTP-labeled using a PCR DIG probe synthesis kit (Roche Diagnostics GmbH, Germany), and used for the hybridization experiment according to the manufacturer’s instructions.

Statistical analyses

All data, except for transcriptome data, is represented as mean ± SD from at least three biological experiments. Statistical analysis of means was performed by analysis of variance using StatPlus 5 pro (AnalystSoft Inc. USA). Data for the optimization experiments were subjected to a one-way ANOVA, and differences among means were analyzed by Scheffe’s method at a 95% confidence level (p ≤ 0.05) with StatPlus 5 pro (AnalystSoft Inc. USA) (Bother 1967).

Results

Optimization of OEC and FEC induction, and shoot regeneration in KU50

We compared the induction efficiency for OEC and FEC from KU50 and 60444 determined per the number of starting axillary bud explants as described by Utsumi et al. (2017) (Fig. 1). OEC were induced from AB of KU50 within 2 weeks of culture on MS medium supplemented with 12 mg/L picloram (CIM). The morphology of the OEC produced from KU50 (Fig. 2c) was similar to that observed in 60444 (Fig. 2a). The OEC induction efficiency from AB after 2 weeks on CIM ranged from 47–67% with an average of 60% in KU50, which was slightly lower than that of 60444, which averaged 71% (Table 1). The efficiency of FEC induction from OEC was assessed after 6 months of culture on FIM. FEC induction was less efficient at 12% in KU50 than at 50% for 60444 (Table 1). The morphology of FEC generated from KU50 was similar to that produced by 60444 (Fig. 2b, d), but took at least 3 months to produce, compared to one month from 60444.

Although the basic procedures of cassava transformation employed in the present study were similar to those previously reported, optimization of regeneration conditions was found to be necessary for KU50. We investigated the optimal concentrations of NAA and BAP necessary for stimulating regeneration of cotyledon-stage embryos from FEC, and for the germination of mature embryos. Six colonies of FEC from KU50 were cultivated on MS media supplemented with various concentrations of NAA, and subcultured every two weeks as shown in Fig. 1a. Cotyledon-stage embryos were formed (Fig. 1b), with an optimal concentration of NAA (1.0 mg/L (Fig. 1c). The cotyledon-stage embryos produced were incubated on MS media supplemented with various concentrations of BAP, and evaluated for shoot regeneration efficiency over 2 months (Fig. 1d, e). The optimal concentration of BAP was found to be 0.4 mg/L (Fig. 1f), which is similar to that reported for 60444 (Bull et al. 2009; Taylor et al. 2012).

Agrobacterium-mediated transformation for KU50

Agrobacterium-mediated transformation of KU50 followed the process reported for 60444 (Utsumi et al. 2017). To perform the transformation experiments, approximately 100 AB were prepared from in vitro plants. Stems were placed on MS medium containing 10 mg/L BAP to induce swelling
of the AB, followed by excision and culture on MS basal medium containing picloram. (Utsumi et al. 2017). OEC production occurred at approximately 74% and 64% of the total number of starting AB for 60444 and KU50, respectively (Table 1). OEC produced from AB were incubated on DKW medium with 12 mg/L picloram for 2 weeks. In this step, no significant differences in growth and OEC formation were observed between 60444 and KU50. OEC (2–3 mm in diameter) were manipulated under a microscope using fine tweezers with OEC from one bud arranged as one group on the FIM containing 12 mg/L picloram to assess the efficiency of FEC induction.

FEC can be generated effectively by culturing primary OEC on FIM containing 12 mg/L picloram. The first observation of FEC after culturing on FIM media with picloram was at 4 weeks and 12 weeks in 60444 and KU50, respectively (Table 1). OEC produced from AB were incubated on FIM for 9 weeks, whereas approximately 5.4 g of FEC was generated from 100 AB in 60444, over the same period. The type and appearance of FEC generated from KU50 were similar to that generated from 60444. However, the lower frequency of production, longer culture period required, and lesser amount of FEC produced indicated a lower potential for FEC production in KU50 compared to the highly amenable variety 60444 (Table 1).

Cassava transformation was carried out using the pMDC111 vector, which carries the visual marker including the mgfp6 gene. FEC were collected and co-cultured with Agrobacterium suspension (OD600 = 0.1). The OD of Agrobacterium suspension neither affected the copy number of T-DNA in transgenic plants nor the transformation process (Taylor et al. 2012). To prevent the re-growth of Agrobacterium, careful manipulation of the washing process was required. The transformed FEC were evenly spread over the FIM with hygromycin and carbenicillin. In KU50, the selection of FIM with hygromycin for 3 weeks was not sufficient for the full selection of the transgenic
Fig. 2 Agrobacterium-mediated transformation of KU50. a Globular and torpedo-stage embryos (arrowed) developing from FEC of 60444. b FEC from 60444 induced on FIM media. c Globular and torpedo-stage embryos (arrowed) developing from FEC of KU50. d FEC from KU50 induced on FIM media. e GFP expression observable from regenerating transformed KU50 plantlet. f GFP expression observation in roots of transformed KU50 plantlet. g No GFP fluorescence observable from non-transgenic KU50 plantlet. h No GFP fluorescence in root of non-transgenic KU50 plantlet. i Transgenic (#7 and #18) and non-transgenic (control) KU50 plants growing on soil for 2 months at 28 °C in the greenhouse. j GFP fluorescence in the leaf (shown by the arrow in Fig. 1i) of transgenic plant #18 k No GFP fluorescence seen in the leaf of non-transgenic (control) plant. l GFP fluorescence observed in the root of transgenic plant #18. m No GFP fluorescence in the root of non-transgenic (control) plant. Bars represent 5 mm. n PCR confirmation for transgenic nature of mgfp6 gene in genomic DNA extracted from transformed KU50 plants. Lane M: DNA molecular marker; Lane P: pMDC111 plasmid DNA; Lanes 1 to 3: GFP transgenic lines #7, #8 and #18 from KU50; Lane 4: non-transgenic KU50; Lane 5 to 7: GFP transgenic lines #2, #3 and #6 from 60444; Lane 8: non-transgenic 60444. o PCR confirmation of the integration of hpt gene in genomic DNA from transgenic plants. Lane M: DNA molecular marker; Lane P: pMDC111 plasmid DNA; Lanes 1 to 3: GFP transgenic lines #7, #8 and #18 from KU50; Lane 4: non-transgenic KU50; Lane 5 to 7: GFP transgenic lines #2, #3 and #6 from 60444; Lane 8: non-transgenic 60444. p Southern blot of genomic DNA from cassava plants. Lane M: DIG-labeled DNA molecular marker (the size of molecular markers is 23,130 bp, 9,416 bp, 6,557 bp, 4,361 bp, 2,322 bp, 2,027 bp from the upper); Lane 1 to 3: GFP transgenic lines #2, #3 and #6 from 60444; Lane 4: non-transgenic 60444; Lane 5 to 8: GFP transgenic lines #7, #8, #18 and #19 of KU50; Lane 9: non-transgenic KU50; Lane P: pMDC111 plasmid DNA as positive control. Genomic DNA from transgenic and non-transgenic cassava was digested with HindIII.
calli. Therefore, FEC was spread evenly over MSN medium containing hygromycin and NAA and subcultured every 21 days to induce the formation of cotyledon-stage embryos. Table 2 shows the efficiency of cotyledon-stage embryo production and plantlet formation per 1 g of FW of starting FEC (normalized for the amount of starting tissues used for Agrobacterium co-cultivation). Cotyledon-stage embryos appeared approximately one month after culturing on MSN with hygromycin and continued over 3 months. Notably, 58 and 151 cotyledon-stage embryos were generated for 60444 and KU50, respectively. Cotyledon-stage embryos were transferred to a CEM containing hygromycin and BAP for the induction of shoots. Shoot formation was observed within one month, with plantlets forming from 22% of the cotyledon-stage embryos for KU50 (Table 2).

In this study, mGFP6 was used as the selection marker because it generates a stronger fluorescence signal due to substitutions of ten amino acids from the soluble-modified GFP (smGFP) (Born and Pfeifer 2019). GFP fluorescence was observed in the process of selection on FIM and the plantlet-inducing stage on CEM (Fig. 2e and f). However, it was difficult to select GFP-expressing tissues from transgenic FEC due to its autofluorescence at an early stage, after co-culture with Agrobacterium. Therefore, we evaluated the transformation efficiency by confirming GFP fluorescence in the leaf veins and stems of regenerated plants (Table 2). The transformation efficiency (calculated as the number of GFP-positive plantlets per number of cotyledon-stage embryos regenerated from 1 g of FEC) was 45% and 22% for 60444 and KU50, respectively (Table 2). In addition to GFP expression, the transgenic nature of regenerated plants was confirmed by PCR, and Southern blotting analyses (Fig. 2n, o, p). Southern blotting confirmed the integration of single copies of T-DNA in 60444 and KU50 transgenic plant lines.

Acclimatizing in vitro cassava plantlets can result in high mortality, often due to damage to fragile roots. Therefore, we reduced the agar concentration in MS micropropagation media from 2.0% to 0.5% (w/v) prior to transplantation. Using this method, plants with well-developed root systems were transferred from the culture vessel to the soil pot without root damage and kept under high humidity. GFP signals were confirmed in plants growing in the soil. GFP fluorescence was observed in the leaf veins and roots of the transgenic lines (Figs. 2) and 1), with no such signals observed in non-transgenic control plants (Figs. 2g and h).

**Lower FEC induction efficiency in KU50 might be due to excess cell-wall loosening and increased stress response compared with 60444**

To compare the physiological status of FEC produced by KU50 and 60444, gene expression analysis was performed using oligo-microarray, and total RNA extracted from these tissues. Using the criteria of FDR ≤ 0.00005 by the BH method and a two-fold change in expression, 2,213 DEGs were identified by comparing 60444 and KU50. Among these, 1,287 genes were upregulated and 926 genes were downregulated in KU50 compared with 60444 (Supplementary Table S2). The DEGs annotated based on the Arabidopsis thaliana genome sequence were functionally classified using agriGO. The GO terms of “cellular component” (Supplementary Fig. S1 legend), “molecular function” and “biological process” were significantly enriched (Supplementary Figure S1-5 and Supplementary Table S3-6).

In the “cellular component,” the genes with GO terms including “GO:0005618 Cell wall,” “GO:0044459 Plasma membrane part,” and “GO:0005576 Extracellular region” were enriched among the upregulated genes in FEC from KU50 (Supplementary Fig. S1 and Supplementary Table S3). The following cell-wall modification-related genes exist: pectinesterase (PE), which is involved in the dimethyl esterification of pectins (Willats et al. 2001), polygalacturonase (Park et al. 2015) which modifies the cell-wall structure, xyloglucan endotransglucosylase (XTH), which encodes a cell-wall-modifying enzyme and expansin, which plays an important role in plant cell growth where cell-wall loosening occurs (Li et al. 2002). Within “molecular function,” the genes with GO terms including “GO:0035251 UDP-glucosyltransferase activity” were upregulated in FEC from KU50 (Supplementary Fig. S2). This suggests that increased stress response through up-regulation of several UDP-glucosyltransferases (Rehman et al. 2018) might occur in FEC from KU50 (Supplementary Table S4). The GO terms enriched in the upregulated genes in KU50 were “response to abscisic acid” (GO:0009737), “response to osmotic stress” (GO:0006970), “response to oxidative stress” (GO:0006979), and “response to wounding” (GO:0009611) (Supplementary Fig. S3) in the “biological process”. The expression of the following genes was increased in KU50 compared with 60444: P5CS2 (AT2G17840) which is involved in proline biosynthesis (Funck et al. 2020), ADC2 (AT4G34710), involved in the first step of polyamine biosynthesis (Urano et al. 2004), ZAT10 (AT1G27730) involved in photooxidative stress response, ERD7 (AT2G17840) involved in remodeling cell membrane lipid composition during cold stress (Barajas-Lopez et al. 2020), chitinase A (AT5G24090) whose expression is induced by various abiotic and biotic stress conditions (Takenaka et al. 2009), SAG14 (AT5G20230) which encodes a glycosylphosphatidylinositol-anchored protein that regulates lignin biosynthesis (Ji et al. 2015), and CYP94B1 (AT5G63450) which is involved in apoplastic barrier formation through suberin biosynthesis (Krishnamurthy et al. 2020) (Supplementary Table S5).
We also compared the genes upregulated in KU50 FEC with genes upregulated or downregulated in 60444 FEC, in comparison with the genes upregulated in 60444 grown under FIM (Utsumi et al. 2017), and DEGs in transition from somatic embryos to FEC (Ma et al. 2015) (Table 3). The expression of several upregulated genes in KU50 FEC included PPCK1 and PPCK2, which play a key role in the control of plant metabolism by phosphorylation of phosphoenolpyruvate carboxylase, XTH16 (AT3G23730), which is involved in loosening of the cell-wall structure, and non-specific phospholipase C4 (NPC4) (AT3G03530) which is important for the supply of inorganic phosphate. Finally, diacylglycerol from membrane-localized phospholipids (Peters et al. 2010) was also upregulated in 60444 grown on CIM with reduced nutrients, and excess vitamin B1 (Utsumi et al. 2017) (Table 3). Several upregulated genes in KU50 FEC, such as acetyl-CoA synthetase, were also upregulated in fresh 60444 FEC compared with SE (Ma et al. 2015). There were no differences in KU50 FEC with the genes involved in callus induction or repression (Ikeuchi et al. 2013) (Supplementary Table S7), and the DEGs involved in the cell cycle-related processes (Supplementary Table S8) were not observed.

Discussion

In this study, we developed a system for Agrobacterium-mediated transformation of the Asian elite variety KU50. The use of picloram and limited nutrient media in addition to excess vitamin B1 resulted in increased induction of FEC, while optimization of BAP and NAA improved somatic embryo regeneration from FEC, and germination to produce transgenic plantlets. This is the first report on the establishment of an Agrobacterium-mediated cassava transformation system via FEC for KU50.

Many factors affect the efficiency of genetic transformation in cassava. Efficient induction of FEC is crucial as it acts as an effective target tissue for transgene integration (Bull et al. 2011; Chavarriaga-Aguirre et al. 2016; Taylor et al. 2012). Successful induction of SE, the first step in FEC production, in Asian cassava varieties has been reported (Ntui et al. 2015; Saelim et al. 2009), not to be a limiting factor for the transformation of KU50. The FEC of KU50 was induced...
during the extended culture on FIM. However, the induction efficiency of FEC was five times lower in KU50 than in the model variety 60444 (Table 1), indicating that the capacity of KU50 to induce FEC was lower than that of model cultivar 60444. As a result, the DEGs between KU50 and 60444, the GO term of the category of “cell wall” was enriched in

### Table 2 Transformation efficiency of 60444 and KU50

|         | 60444 |          |         | KU50 |          |         |
|---------|-------|----------|---------|------|----------|---------|
|         | No. of cotyledon-stage embryo<sup>a</sup> | No. of GFP-positive plantlets<sup>b</sup> | Transformation efficiency (%) | No. of cotyledon-stage embryo<sup>a</sup> | No. of GFP-positive plantlets<sup>b</sup> | Transformation efficiency (%) |
| exp1    | 45    | 19       | 42      | 232  | 60       | 26      |
| exp2    | 54    | 30       | 57      | 121  | 32       | 26      |
| exp3    | 77    | 38       | 49      | 136  | 16       | 12      |
| exp4    | 54    | 13       | 24      | 107  | 41       | 38      |
| exp5    | 59    | 30       | 51      | 159  | 15       | 9       |
| Average | 58    | 26       | 45      | 151  | 33       | 22      |
| S.D     | 12    | 10       | 13      | 49   | 19       | 12      |

<sup>a</sup>No. of cotyledon-stage embryo was measured per 1 g of FEC

<sup>b</sup>No. of GFP-positive plantlets was measured per 1 g of FEC

### Table 3 Differentially expressed genes between KU50 FEC and 60444 FEC by the incubation on FIM

| ProbeName | q value by BH method | Fold change (KU50/60444)<sup>a</sup> | Encoded Proteins/other features<sup>b</sup> | Gene name | AGI code<sup>c</sup> | E-value | Cassava gene model<sup>d</sup> | Ref.<sup>e</sup> |
|-----------|----------------------|------------------------------------|------------------------------------------|------------|----------------------|--------|-------------------------------|--------------|
| RknMes02_053373 | 7.90E-08 | 7.0                                  | non-specific phospholipase C4             | NPC4       | AT3G03530.1          | 0      | Manes.09G131600               | (1)          |
| RknMes02_027216 | 1.30E-05 | 2.4                                  | phosphoenolpyruvate carboxylase kinase 2 | PPCK2      | AT3G04530.1          | 0      | Manes.08G138300               | (1)          |
| RknMes02_025148 | 2.30E-05 | 2.4                                  | Plant invertase/pectin methylesterase inhibitor superfamily | AT4G02320.1 | 0                    | Manes.01G221700 | (2) |
| RknMes02_039364 | 3.60E-06 | 2.1                                  | acyl-CoA sterol acyl transferase 1        | ASAT1      | AT3G51970.1          | 0      | Manes.10G134500               | (1)          |
| RknMes02_033632 | 1.30E-05 | 1.4                                  | xyloglucan endotransglucosylase/hydrolase 16 | XTH16      | AT3G23730.1          | 0      | Manes.05G199600               | (1)          |
| RknMes02_048202 | 9.10E-06 | 1.4                                  | Auxin-responsive GH3 family protein       | GH3.17     | AT1G28130.1          | 0      | Manes.08G100100               | (2)          |
| RknMes02_014383 | 2.60E-06 | 1.1                                  | glutamate decarboxylase 4                | GAD4       | AT2G02010.1          | 1.00E-38 | Manes.17G091400               | (2)          |
| RknMes02_022298 | 5.50E-07 | 1.1                                  | phosphoenolpyruvate carboxylase kinase 1 | PPCK1      | AT1G08650.1          | 8.00E-39 | Manes.03G164500               | (1)          |
| RknMes02_048606 | 1.20E-04 | -1.6                                 | Leucine-rich repeat (LRR) family protein  | AT4G06744.1 | 0                    | Manes.18G040900 | (1) |

<sup>a</sup>The values represent Log<sub>2</sub> (KU50 FEC on FIM)—Log<sub>2</sub> (60444 FEC on FIM)

<sup>b</sup>Encoded proteins/other features indicate the putative functions of the gene products that are expected from sequence similarity. The information for the NCBI protein reference sequence with the highest sequence similarity with the probes is shown

<sup>c</sup>The information for AGI locus ID with the highest sequence similarity with the probe is shown

<sup>d</sup>The information for cassava gene code with the highest sequence similarity with the probe is shown

<sup>e</sup>The genes have been reported in previous studies: (1) Utsumi et al. (2017), (2) Ma et al. (2015)
the “cellular component”, of which category was associated with cell reconstruction in FEC formation. Among the genes categorized into “cell wall,” 19 genes were significantly upregulated in KU50 and 7 genes were significantly downregulated in KU50 (Supplementary Table S3). Among them, genes such as pectinesterase (Manes.11G086400.1), xyloglucan endotransglycosylase (XTH) (Manes.14G152200.1), alpha-xylosidase 1 (Manes.14G101700.1), and pectin acetyltransferase (Manes.17G001900.1) were upregulated. Genes such as pectin methyltransferase (Manes.16G089100.1) and other types of XTH (Manes.01G262300.1) were downregulated. XTH catalyzes the endo-cleavage of xyloglucan (the donor substrate) and seems to mediate post-synthetic remodeling of the cellulose-xyloglucan network during plant growth (Carpita and Gibeaut 1993; Rose et al. 2002). Xylosidase cleaves xylosyl residues from the non-reducing end of xyloglucan, and xyloglucan oligosaccharides contribute to maintaining the mechanical integrity of the cell wall in the growing tissues (Shigeyama et al. 2016). Pectin acetyltransferase modulates the degree of pectin acetylation, which affects pectin solubility as a crucial structural factor (Liners et al. 1994). Pectin esterase catalyzes the de-esterification of pectin into pectate and methanol. The enzymatic de-esterification of pectin results in low methoxylated pectin, which forms a strong gel in the presence of calcium ions (Micheli 2001). Pectin methyltransferase catalyzes the demethoxylation of pectin. Through modification of the number and distribution of methyl esters on the pectin backbone, pectin methyltransferase affects the susceptibility of pectin for subsequent enzymatic conversion, and finally functionality in the cell wall (Jolie et al. 2010). Previous studies using 60444 showed that the expression of pectin esterase precursors (Manes.01G221700.1, Manes.05G047700.1, Manes.09G173600.1, and Manes.11G012600.1) genes was downregulated dramatically, and the expression of pectin acetyltransferase (Manes.06G148800.1, Manes.17G001900.1) genes were upregulated in fresh FEC during the transition from SEs to FFEC (Ma et al. 2015). Structural and physicochemical changes in xyloglucan and pectin in the cell wall are critical for callus formation (Ikeuchi et al., 2013). The high capacity of 60444 to induce FEC might be due to the production and assembly of production and assembly. Cassava transformation has been reported in most cases using FEC induced by culturing on GD media containing picloram (Bull et al. 2009; Taylor et al. 2012; Utsumi et al. 2017), with FEC formation observed after 2 months of incubation on media with picloram. In KU50, it takes at least four months of cultivation on FIM media to induce FEC. Further technical improvement is therefore necessary to shorten production time and reduce the potential frequency of somaclonal variation resulting from longer culture durations.

The transformation efficiencies of 60444 and KU50 were 45% and 22%, respectively. An average of 26 transgenic lines per gram of FEC from 60444 was obtained, whereas 33 transgenic lines per gram of FEC from KU50 were obtained within 12 months after Agrobacterium inoculation (Table 2). This data aligns with previous reports, where the number of transgenic lines obtained with one gram of FEC was in the range of 22–50 plants (Chetty et al. 2013; Nyaboga et al. 2015; Taylor et al. 2012). The addition of silver nitrate (AgNO₃) was effective for shoot organogenesis from somatic cotyledons (Zhang et al. 2001) and inhibition of A. tumefaciens growth during selection (Gyves et al. 2010).

The development of a genetic transformation method for KU50 described here will contribute to the improvement of key characteristics in this very important Asian cassava cultivar. The use of novel cutting-edge technologies, such as CRISPR-Cas9, can now be applied and contribute to molecular breeding (Altpetera et al. 2016). For example, the introduction of the flowering locus T gene has been demonstrated to be successful in shortening the breeding time, generating transgene-free progenies, and producing amylose-free cassava starch, which is a useful trait for the food and industrial applications of cassava (Bull et al. 2018; Malik et al. 2020). This approach is now feasible for KU50. Such approaches will contribute to expanding cassava diversity toward food security, commodity diversity, and sustainability for global demand.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11103-021-01212-1.

Acknowledgements This work was supported by the following funding: Strategic Funds for the Promotion of Science and Technology, Japan Society for the Promotion of Science (JSPS) Program for Advancing Strategic International Networks to Accelerate the Circulation of Talented Researchers, EIG CONCERT-Japan 4th Call under the Strategic International Research Cooperative Program of the Japan Science and Technology Agency (JST, JPMJSC16C4), the Science and Technology Research Partnership for Sustainable Development (SATREPS) in collaboration with the Japan Science and Technology Agency (JST, JMPJSA1508) and the Japan International Cooperation Agency (JICA), and the grant from RIKEN Center for Sustainable Resource Science (CSRS). We would like to thank Editage (www.editage.com) for English language editing.

Authors Contributions YU and MS supervised the experiments, and YU, MS, and NJT wrote the manuscript. YU, CU, and MT performed the experiments and analyzed the data. ANV, NVD, and HT provided constructive comments. YU, MT, and ST performed transcriptome analysis. CU, YO, MT, and TTH managed the plants. The NJT provided critical suggestions on methodologies.

Data Availability The novel data generated in this study, including microarray data, has been deposited in the National Center for Biotechnology Information under the accession number GSE169685.
References

Altpeter F, Springer NM, Bartley LE, Blechle AE, Brutnell TP, Citovsky V, Conrad LJ, Gelvin SB, Jackson DP, Kausch AP, Lemieux PG, Medford JJ, Orozco-Cárdenas ML, Tricoli DM, Van Eck J, Voytas DF, Walbot V, Wang K, Zhang ZJ, Stewart CN (2016) Advancing crop transformation in the era of genome editing. Plant Cell 28:1510–1520

Balat M, Balat H (2009) Recent trends in global production and utilization of biofuel ethanol. Appl Energy 86:2273–2282

Barajas-Lopez JD, Tiwari A, Zarza X, Shaw MW, Pascual J, Punkkinen M, Bakowska JC, Munnik T, Fujii H (2020) Early response to dehydration 7 remodels cell membrane lipid composition during cold stress in arabidopsis. Plant Cell Physiol 62:139

Bother R (1967) On sharpening Scheffe bounds. J Roy Stat Soc 29:110–114

Borm J, Pfeifer F (2019) Improved GFP variants to study gene expression in Haloarchaea. Front Microbiol 10:1200

Bull SE, Ndunguru J, Gruissem W, Beeching JR, Vanderschuren H (2010) Cassava breeding and opine synthetic loci in tumors incited by Agrobacterium tumefaciens A281 on Soybean and Alfalfa plants. J Bacteriol 168:1283–1290

Hummel AW, Chauhan RD, Cermak Mam, Vijayaraghavan A, Boyher A, Starker CG, Bart R, Voytas DF, Taylor NJ (2018) Allele exchange at the EPSPS locus confers glyphosate tolerance in cassava. Plant Biotechnol J 16:1275–1282

Ikuchi M, Sugimoto K, Ishane A (2013) Plant callus: mechanisms of induction and repression. Plant Cell 25:3159–3173

Ji H, Wang Y, Cloix C, Li K, Jenkins GI, Wang S, Zhang Z, Shi Y, Yang S, Li X (2015) The Arabidopsis RCC1 family protein TCF1 regulates freezing tolerance and cold acclimation through modulating lignin biosynthesis. PLoS Genet 11:e1005471

Jolie RP, Duvetter T, Loey AMV, Hendrickx ME (2010) Pectin methylesterase and its proteinase inhibitor: a review. Carbohydr Res 345:2583–2595

Krishnamurthy P, Vishal B, Ho WJ, Lok FC, Lee FSM, Kumar PP (2020) Regulation of a Cytochrome P450 Gene CYP94B1 by WRKY33 transcription factor controls apoplastic barrier formation in roots to confer salt tolerance. Plant Physiol 184:2199–2215

Li Y, Darley CP, Ongaro V, Fleming A, Schipper O, Baldauf SL, McQueen-Mason SJ (2002) Plant expansins are a complex multigene family with an ancient evolutionary origin. Plant Physiol 128:854–864

Liners F, Gaspar T, Van Cutsem P (1994) Acetyl- and methyl-esterification of pectins of friable and compact sugar-beet calli: consequences for intercellular adhesion. Planta 192:545–556

Liu J, Zheng Q, Ma Q, Gadidasu KK, Zhang P (2011) Cassava genetic transformation and its application in breeding. J Integr Plant Biol 53:52–69

Ma Q, Zhou W, Zhang P (2015) Transition from somatic embryo to friable embryogenic callus in cassava: dynamic changes in cellular structure, physiological status, and gene expression profiles. Front Plant Sci 6:824

Malik AI, Kongsil P, Nguyen VA, Ou W, Sholihpin SP, Sheena ML, Becerra Lopez-Lavalle LA, Utsumi Y, Lu C, Kittipadakul P, Nguyen HH, Ceballos H, Nguyen TH, Selvaraj Gomez M, Aiempaka P, Labarta R, Chen S, Amawan S, Sok S, Youabe L, Seki M, Tokunaga H, Wang W, Li K, Nguyen HA, Nguyen VN, Ham LH, Ishitani M (2020) Cassava breeding and agronomy in Asia: 50 years of history and future directions. Breed Sci 70:145–166

Micheli F (2001) Pectin methylestersases: cell wall enzymes with important roles in plant physiology. Trends Plant Sci 6:414–419

Murashige T, Skoog S (1962) A revised medium for rapid growth and bioassays with tabacco tissue cultures. Physiol Plantarum 15:473–497

Ntu NV, Kong K, Khan RS, Igawa T, Janavi GJ, Rabindran R, Naka- muru I, Mii M (2015) Resistance to Sri Lankan Cassava Mosaic Virus (SLCMV) in Genetically Engineered Cassava cv. KU50 through RNA Silencing. PLoS ONE 10:e0120551

Nyaboga EN, NJiru JM, Tripathi L (2015) Factors influencing somatic embryogenesis, regeneration, and Agrobacterium-mediated transformation of cassava (Manihot esculenta Crantz) cultivar TME14. Front Plant Sci 6:411

Odipio J, Ailicai T, Ingebrecht I, Nusinow DA, Bart R, Taylor NJ (2017) Efficient CRISPR/Cas9 genome editing of phytoene desaturase in cassava. Front Plant Sci 8:1780

Park J, Cui Y, Kang B-H (2015) AtPGL3 is an Arabidopsis BURP domain protein that is localized to the cell wall and promotes cell enlargement. Front Plant Sci 6:412

Peters C, Li M, Narasimhan R, Roth M, Welti R, Wang X (2010) Non-specific phospholipase C NPC4 promotes responses to abscisic acid and tolerance to hyperosmotic stress in Arabidopsis. Plant Cell 22:2642–2659

Rehman HM, Nawaz MA, Shah ZH, Ludwig-Müller J, Chung G, Ahmad MQ, Yang SH, Lee SI (2018) Comparative genomic and transcriptomic analyses of Family-1 UDP-glycosyltransferase in
three Brassica species and Arabidopsis indicates stress-responsive regulation. Sci Rep 8:1875

Rose JKC, Janet B, Fry SC, Nishitani K (2002) The XTH family of enzymes involved in xyloglucan endotransglucosylation and endo-hydrolysis: current perspectives and a new unifying nomenclature. Plant Cell Physiol 43:1421–1435

Saelim L, Phansiri S, Suksangpanomrung M, Netrphan S, Narangajavana J (2009) Evaluation of a morphological marker selection and excision system to generate marker-free transgenic cassava plants. Plant Cell Rep 28:445–455

Seki M, Tokunaga H, Utsumi C et al (2018) Advancement of Asian Cassava Molecular Breeding towards SDGs. Proceedings of the 18th Science Council of Asia (SCA) Conference Role of Science for Society: Strategies towards SDGs in Asia. http://www.scj.go.jp/en/scj/. Accessed Dec 2018.

Shigeyama T, Watanabe A, Tokuchi K, Toh S, Sakurai N, Shibuya N, Kawakami N (2016) α-Xylosidase plays essential roles in xyloglucan remodelling, maintenance of cell wall integrity, and seed germination in Arabidopsis thaliana. J Exp Bot 67:5615–5629

Southern ED (2006) Southern blotting. Nat Protoc 1:518–525

Takenaka Y, Nakano S, Tamo M, Sakuda S, Fukamizo T (2009) Chitinase gene expression in response to environmental stresses in Arabidopsis thaliana: chitinase inhibitor allosamidin enhances stress tolerance. Biosci Biotechnol Biochem 73:1066–1071

Taylor NJ, Edwards M, Kierman RJ, Davey CDM, Blakesley D, Henshaw GG (1996) Development of friable embryogenic callus and embryogenic suspension culture systems in cassava (Manihot esculenta Crantz). Nat Biotechnol 14:726–730

Taylor N, Gaitán-Solís E, Moll T, Trauterman B, Jones T, Pranjal A, Trembley C, Abernathy V, Corbin D, Faquet CM (2012) A high-throughput platform for the production and analysis of transgenic cassava (Manihot esculenta) plants. Tropical Plant Biol 5:127–139

Urano K, Yoshiba Y, Nanjo T, Ito T, Yamaguchi-Shinozaki K, Shinozaki K (2004) Arabidopsis stress-inducible gene for arginine decarboxylase AtADC2 is required for accumulation of putrescine in salt tolerance. Biochem Biophys Res Commun 313:369–375

Utsumi Y, Tanaka M, Kurotani A, Yoshida T, Mochida K, Matsui A, Ishitani M, Srithaph S, Whankaew S, Asvarak T, Narangajavana J, Triwitayakorn K, Sakurai T, Seki M (2016) Cassava (Manihot esculenta) transcriptome analysis in response to infection by the fungus Colletotrichum gloeosporioides using an oligonucleotide-DNA microarray. J Plant Res 129:711–726

Willats WG, Orfila C, Limberg G, Buchholt HC, van Alebeek GJ, Voragen AG, Marcus SE, Christensen TM, Mikkelens JD, Murray BS, Knox JP (2001) Modulation of the degree and pattern of methyl-esterification of pectic homogalacturonan in plant cell walls. Implications for pectin methyl esterase action, matrix properties, and cell adhesion. J Biol Chem 276:19404–19413

Zhang P, Phansiri S, Puonti-Kaerlas J (2001) Improvement of cassava shoot organogenesis by the use of silver nitrate in vitro. Plant Cell Tissue Organ Cult 67:47–54

Publisher’s Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.