Appendix

Appendix A Plant microtecnique (Johansen, 1940)

Reagent preparation

Fixation

FAA (Formalin, Acetic acid, and Alcohol) fixative contains:

- EtOH 95% 50 ml
- Acetic acid 5 ml
- Formalin (37% Formaldehyde) 10 ml
- H_2O 35 ml

Dehydration series

Incubate sample for 1 day, depending on toughness and size, in:

- EtOH 70%
- EtOH 85%
- EtOH 95%
- EtOH 100%

Stain

Hematoxylin contains:

- Hematoxylin 6 g
- Sodium iodate 0.6 g
- Aluminum sulfate 52.8 g
- Distilled water 690 ml

- Ethylene glycol 250 ml
- Glacial acetic acid 60 ml

Analysis methods

Determination of the developmental stage of the rhizome formation was done by using the paraffin embedded technique and a method described by Johansen(1940). Two rhizome and storage roots were sampling at two week. The rhizome and storage roots samples were stopped activity and fixed with FAA about 1 week. Then they were suctioned by vacuum pump at 6000 mm Hg for 1 hrs and left under vacuum condition for at least 24 hrs until bubble was not occur. After that the rhizome and storage roots samples were dehydrated for 24 hrs in a solution containing of tertiary butyl alcohol (TBA) and mixed with 5 series of 50, 70, 85, 95 and 100% alcohol respectively. TBA plus alcohol 100% erythosine was added as dye. Ease samples were then infiltrated 3 times with pure TBA for 12 hrs each then transferred into solution of pure TBA mixed with paraffin oil (1:1), and pure paraffin oil for each 12 hrs respectively. After that the samples were kept in paraplast solution in the oven (55 – 60 ^{o}C) around 2 – 3 months and embedded in paraplast. Thus, the paraplast samples were sectioned at thickness of 10 µm using rotary microtome, and affixed on the slide with 2% Hapt's adhesive. The slides of microtome section were studied and photographed under stereo microscopy.

<u>Appendix B</u> Total non-structural carbohydrate analysis (TNC) by Nelson method (Hodge and Hofreiter, 1962)

Sample extraction

1. Weight 0.05 g sample, add 40 ml of 0.2 N H_2SO_4 and cover flask with aluminum foil.
2. Heat at 100^oC for 1 hrs, after keep flask to cooling at room temperature.
3. Adjust pH the solution about 6.95 – 7.05, add deionized water to 100 ml.
4. Take solution to filter with Whatman No 5 or 42.
5. Keep the solution in plastic bottle for analysis.

Reagent preparation

1. Nelson's alkaline copper reagent

Prepare solution No.1 by dissolve 25 g of anhydrous sodium carbonate (Na_2CO_3) into 250 ml deionized water, after added 12 g of potassium sodium tartrate ($C_4H_4KNO_6.4H_2O$) and 40 ml of 10% copper sulfate (Make 4 g $CuSO_4.5H_2O$ dissolve into deionized water to 40 ml), add 16 g of sodium bicarbonate. The Solution No.2 by dissolve make 180 g of anhydrous sodium sulfate (Na_2SO_4) into 500 ml of deionized water. Mix solutions No.1 with No.2 adjust to 1 L, after about 1 week, take to filter and keep at 30 – 37^oC.

2. Arsenomolybdic acid reagent

Prepare solution No.3 by dissolve 50 g of ammonium molybdate ($(NH_4)_6AsO_{24}.4H_2O$) into 90 ml of deionized water and add 42 ml H_2SO_4. Solution No.4 prepared by dissolve 6 g of disodium hydrogen arsenate ($Na_2HAsO_4.7H_2O$) into 50 ml of deionized water. Then mix solution No.3 with No.4 and adjust to 1 L, keep at 30 – 37^oC.

Analysis method

1. Standard solution was made by dissolving D-glucose. Make standard solution concentration 0 – 1 ppm.

2. Add 1 ml of Nelson's alkaline copper reagent, mix well and cover flask with aluminum foil.

3. After take the flask in water bath at 100^{o}C for 20 min. keep flask to cooling at room temperature.

4. Add 1 ml of arsenomolybdic acid reagent, shaking vigorously and add deionized water to 25 ml with mix well, keep at room temperature for 30 min.

5. Determine the absorbance at 540 nm.

6. Calculation;

$$TNC = \frac{\text{(mg glucose equivalent)}}{\text{Weight of sample}} \times \text{(mg D-glucose/ mg dry weight)}$$

Appendix C Starch analysis by Anthrsone method (Herbert *et al.*, 1971)

***Sample extraction by 80% ethanol* (Ohyama *et al.*, 1986)**

1. Measure the weight of freeze dried powder samples (about 50 mg).
2. Put the power into 1.5 ml eppendorf tube.
3. Put 1 ml of 80% ethanol.
4. Heat at 60^{o}C for 15 min.
5. Mix well with a bortex tube mixer.
6. Keep refrigerator overnight.
7. Mix well with a bortex tube mixer.
8. Centrifuge at 10,000 rpm for 15 min.

9. Separate the supernatant to 5 ml glass bottle.
10. Put 1 ml of 80% ethanol.
11. Mix well with a bortex tube mixer.
12. Keep refrigerator overnight.
13. Mix well with a bortex tube mixer.
14. Centrifuge at 10,000 rpm for 15 min.
15. Separate the supernatant to 5 ml glass bottle.
16. Take a one cycle to the step 10-15.
17. Keep the precipitated pellet in freezer.

Extraction with 8.14 N of $HClO_4$

1. Precipitated fraction from 80% ethanol extraction is placed into a centrifuged tube, add 2.5 ml deionized water and cover with aluminum foil, heat by the water bath for 15 min.
2. Add 3.25 ml of 8.14 N $HClO_4$ by (mix 452 ml 60% $HClO_4$ with deionized water 48 ml).
3. Stir with glass rod or vortex for 5 min, and do it sometimes for 15 min.
4. Add 10 ml of deionized water.
5. Spin 10,000 rpm at room temperature for 10 min.
6. The supernatant is transferred to 50 ml volumetric flak.
7. Add 2.5 ml deionized water and repeat the protocol from 2 – 6.
8. Fill up with deionized water to final volume at 50 ml.

***Anthrone method* (Herbert *et al.*, 1971)**

Reagent preparation

1. Glucose standard reagent.

Prepare 1,000 mg/l of glucose standard by dissolve glucose 1 g in 1,000 ml of deionized water, dilute this into 0, 10 and 20 mg/l to make a standard curve.

2. Anthrone reagent

Dissolve 0.2 g of anthrone ($C_{14}H_{10}O$) in 100 ml of the concentrated H_2SO_4.

Analysis method

1. Place a test tube into an ice box, pipette 2.5 ml of sample solution into a test tube, then add 5 ml of anthrone reagent, shaking vigorously and heat by a water bath at 100^{o}C for 7.5 min.

2. Cooling a test tube in an ice box, and place in the room temperature before determine the absorbance at 630 nm.

3. Calculation;

The starch concentration (mg/g DW) = glucose concentration x 0.9

<u>Appendix D</u> Total soluble sugar analysis (TSS) by the phenol-sulfuric acid assay (Ohyama *et al.*, 1986)

Reagent preparation

1. 5% (v/v) of phenol (by dissolve 5.47 ml of phenol added 9.4.53 ml of deinoized water)

2. D-glucose standard is prepared by dissolve 0, 0.04 and 0.08 g of D-glucose (dextrose) in 100 ml of 80% ethanol.

Analysis method

1. Mix 50 µl sample from 3.2.3.3 with 1 ml deionized water and 1 ml 5% phenol in a test tube.

2. For standard reaction, put 1 ml of deionized water and 1 ml of 5% phenol in standard tubes.

3. Added concentrated 5 ml H_2SO_4 rapidly and directly to the solution surface both sample and standard tubes without allowing it to touch the sides of the tube and shake vigorously.

4. Leave the solutions undisturbed for 10 min before shaking vigorously again.

5. Cooling in a water bath at 30°C for 10 min.

6. Determine the absorbance at 485 nm.

<u>Appendix E</u> Reducing sugar analysis (RS)

***Sample extraction by 85% ethanol* (Yemm, 1935)**

1. Weight 0.20 g dried sample; add 20 ml of 85% ethanol and cover flask with aluminum foil.

2. Heat at 60°C for 2 hrs, after keep flask to cooling at room temperature.

3. Adjust with deionized water to 50 ml.

4. Take solution to filter with Whatman No 5 or 42.

5. Keep the solution in plastic bottle for analysis.

***Analysis method of RS by Nelson method* (Hodge and Hofreiter, 1962)**

- Standard solution was made by dissolving D-glucose. Make standard solution concentration 0 – 1 mg/l.

- Add 1 ml of Nelson's alkaline copper reagent, mix well and cover flask with aluminum foil.
- After take the flask in water bath at 100^{o}C for 20 min. keep flask to cooling at room temperature.
- Add 1 ml of arsenomolybdic acid reagent, shaking vigorously and add deionized water to 25 ml with mix well, keep at room temperature for 30 min.
- Determine the absorbance at 540 nm.
- Calculation;

$$RS = \frac{\text{(mg glucose equivalent)}}{\text{Weight of sample}} \times \text{(mg D-glucose/ mg dry weight)}$$

Appendix F Free sugar analysis (Ohyama *et al*., 1986)

The analysis of free sugar (Fructose, Glucose and Sucrose) used by Gas Liquid Chrsomatography.

Analytical method

1. Standard preparation;

Dissolve standard sugars which combined with 100 mg Rhamnose, 100 mg Fructose, 100 mg Glucose and 100 mg Sucrose into 25 ml 80% ethanol.

2. Pipette 100 µl of 80% ethanol extracted sample and 50 µl of rhammose (100 mg of rhammose in 25 ml of 80% ethanol) as internal standard, put into a vial, evaporate until dry, then dehydrate by vacuum for 1 – 2 hrs.

3. Pipette 50 µl of standard solution, puts into a vial, evaporate and dehydrate.

4. Add 50 µl of TMSI-H (N-trimethylsilylimidazole) into the vial of sample, and standard. Cap with the parafilm, leave it overnight at room temperature.

5. Inject 10 µl of sample to Gas Chrsomatograph (GC).

6. The analytical conditions of GC

Column for Fructose	OV-17
Column initiation temperature	120°C
Column final temperature	280°C
Temperature rate	10°C
Injection temperature	300°C

Note : Use column SE-30 for Glucose and Mannose analysis

Initiation temperature	110°C
Temperature rate	5°C

<u>Appendix G</u> Total nitrogen analysis by modified Kjeldahl method (Ohyama, 1985)

Reagent

- Reagent A (EDTA reagent);

  Dissolve 6 g of EDTA (ethylenediaminetetra acetic acid disodium salt) into 80 ml of deionized water, adjust pH about 7, mix well and dilute to a final volume of 100 ml.

- Reagent B (1 M of KH_2PO_4);

  Dissolve 136.09 g KH_2PO_4 and 2.75 g benzoic acid into 1 L of deionized water.

- Reagent C (Phenol-nitroprusside reagent);

Dissolve 100 mg sodium nitroprusside into 10.25 ml phenol, dilute to a final volume of 1 L with deionized water (Use the sodium nitroprusside as a catalyst).

- Reagent D (Buffer hypochlorite reagent);

  Put 10 g NaOH (adjusts pH 10 by 10 N of NaOH), 7.06 g Na_2HPO_4 and 31.8 g $Na_3PO_4.12H_2O$ into a 500 ml beaker, dissolve in deionized water and transfer to 1 L of volumetric flask, add 10 ml of sodium hyperchlorite, dilue to 1 L of flask with deionized water.

- Standard ammonium solution;
- Dissolve 471.7 mg $(NH_4)_2SO_4$ in 1 L of 0.5 N H_2SO_4 for 100 mg/l of a stock solution. Make standard concentration 0 -0.7 mg/l.

Analytical method

- Pipette sample solution of the H_2SO_4 digested solution 0.1 – 2 ml into a 25 ml of volumertric flask, add 0.5 ml of reagent A and 0.5 ml of reagent B.
- Add a small amount of 2 N NaOH, for pH adjust, until color changed, add 2.5 ml of reagent C, follow by 2.5 ml of reagent D, and then fill up flask to volume with deionized water and mix well.
- Maintain the flask at 30^oC for 3 hrs and determine the absorbance of the colored complex at a wavelength of 625 nm. Do the same method for blank solution and standard.
- Determine the NH_4^+-N concentration of the sample by reference to a calibration curve plotted form the results obtained with a standard curve.

Appendix H Total amino acid analysis (Takahashi *et al*., 1993)

Amide-N concentration was determined by the ninhydrin method. 50 µl of sample was taken into a test tube, and 1.5 ml of citrate buffer (comprised of 56 g of citrate and 21.3 g of NaOH and dissolved in 1 L of water) was added. Then 1.2 ml of ninhydrin solution (0.958 g of ninhydrin and 33.4 mg of ascorbate was dissolved in 3.2 ml of water). And methoxyethanol (methylcellosolve) was added to 100 ml. The tubes were heated in boiling water for 20 min with aluminium foil lid. After 3 ml of 60% ethanol was added and mixed, then cooled to room temperature. After 10 min incubation, OD_{570} was measured by optical spectrometry. Standard solution was made by dissolving 165 mg of asparagines (or 188 mg of asparagines monohydrate) plus 183 mg of glutamine in 250 ml of water, which contains 280 *µg*-N/ml. Diluted standard solution and simultaneously measured with samples.

Appendix I Gene expression in the rhizome formation by differential display

RNA extraction

The new rhizomes were washed immediately with tap water, frozen in liquid nitrogen and ground to a fine powder. The ground samples were freeze-dried and stored at -80° C. The RNA extraction was modified by Sueyoshi (1999).

Extraction buffer

- 0.1 M Tris-HCl (pH 9.0)
- 0.1 M NaCl
- 1% SDS

Protocol

1. Cool a mortar and pestle by pouring a little liquid nitrogen over it.
2. Weight 2.0 g of freeze-dried sample.
3. Grind plant tissue in the mortar and pestle until tissue becomes a fine powder.
4. Transfer the frozen powder into a 15 ml polypropylene centrifuge tube containing 20 ml extraction buffer and 0.4 ml β-mercaptoethanol. Dispense and thaw the content by vortexing.
5. Add 0.6 – 1.0 ml of phenol, 0.6 – 1.0 ml of chloroform:isoamyl alcohol mix (24:1) and 0.15 – 0.2 ml 2M Na-acetate.
6. Mix well and spin at 15,000 rpm for 10 min at 4^{o}C.
7. Transfer the supernatant into a new tube and extract with an equal volume of isopropylalcohol and incubate for 15 min at -80^{o}C.
8. Spin at 15,000 rpm for 10 min at 4^{o}C.
9. Discard supernatant and add 1 ml Na-acetate (pH 5.2).
10. Mix well by vortex.
11. Spin at 15,000 rpm for 10 min at 4^{o}C.
12. Discard supernatant and wash with 70% ethanol.
13. Dry at room temperature and add 300 µl DEPC-treated H_2O, 30 µl 3M Na-acetate after incubate at -80^{o}C for 10 min (sometime for overnight).
14. Spin at 15,000 rpm for 10 min at 4^{o}C.
15. Wash with 70% ethanol about thrsee times.
16. Dry at room temperature for 10 min and add 50 µl DEPC-treated H_2O.
17. After that the RNA cleanup was done using RNeasy® Kit (QIAGEN).

18. The pellets are dissolved in 50 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) and stored at -80^{o}C.

Analysis of RNA quality

A 1.2 % denaturing agarose gel (w/v) was prepared by adding 5 ml of 10X MOPS/EDTA buffer (0.2 M MOPS (3-(N-Morpholino) propanesulphonic acid), 50 mM EDTA, pH 7), and 43 ml DEPC-treated H_2O to an RNase-free flask. The agarose was dissolved and once the gel solution had cooled down to 50^{o}C, 2.55 ml of 37% formaldehyde was introduced, in a fume hood, and the solution was made up to a final volume of 50 ml. The gel was run in a 1X MOPS buffer. Samples (5 µg) were denatured at a ratio of 1:5 (v/v) in electrophoresis sample buffer (0.75 ml deionised formamide, pH7, 0.15 ml 10X MOPS, 0.24 ml formaldehyde, 0.1 ml DEPC-treated H_2O, 0.1 ml glycerol and 0.08 ml 10% (w/v) bromophenol blue) at 65^{o}C for 15 min. Prior to loading the solidified gel, 1 µl of a 1 mg ml-1 EtBr solution was introduced to the samples. The gel was electrophoresed at 60 voltage in a Midi-Gel electrophoretic system (Sigma Chemical Co., Japan) and visualized on a UV transilluminator (312 nm wavelength). The gels were photographed by using an orange filter (Kodak Wratten) and Panchrsomatic type Fujifilm (Fuji Photofilm Co.).

Reverse Transcription of mRNA

The reverse transcription (RT) reactions, for each RNA sample, were set up on ice in separate 500 µl, thin walled PCR tubes. For the RT reaction, the one-base anchor primer ($GT_{15}C$, 50 pmol) was by mRNA Fingerprinting Kit version 1.0 SG (Nippon gene Co.LTD., Japan). The sample were mix 1 µl (2.5 µg μl^{-1}) of purified total RNA,

8 µl DEPC-treated H_2O and 1 µl anchor primer. The reaction mix was heated at 70°C for 5 – 10 min and keep to ice. To initiate the RT reaction, 10 µl of the sample were used to which M , 1 µl Reverse Transcriptase (M-MLV and RNase), 1µl dNTP (10 mM), 2 µl DTT (100 mM), 2 µl $MgCl_2$ (25 mM), 2 µl 10X RT buffer and 2 µl DEPC-treated H_2O. The cycling conditions consisted of pre-denaturing at 25°C for 10 min, 42°C for 50 min and 70°C for 15 min. The final extension was performed at 4°C for infinitive.

Polymerase Chain Reaction

To systematically amplify most of the mRNA, a limited number of short arbitrary primers in combination with the same, one(single) base achor primer, which produced the duplicate cDNA samples, were used. For DD the arbitrary primers were designed to have an optimal length of 10 bases. The series of 25 primers of arbitrary but defined sequence were obtained at base concentrations of 50 pmoles:

No.	Sequence
AP-A-01	TACAACGAGG
AP-A-02	TGGATTGGTC
AP-A-03	CTTTCTACCC
AP-A-04	TTTTGGCTCC
AP-A-05	GGAACCAATC
AP-A-06	AAACTCCGTC
AP-A-07	TCGATACAGG
AP-A-08	TGGTAAAGGG
AP-A-09	TCGGTCATAG
AP-A-10	GGTACTAAGG
AP-A-11	TACCTAAGCG
AP-A-12	CTGCTTGATG
AP-A-13	GTTTTCGCAG
AP-A-14	GTACAAGTCC
AP-A-15	GTACCAGTAC
AP-A-16	GATCACGTAC
AP-A-17	GATCTGACAC
AP-A-18	GATCTCAGAC
AP-A-19	GATCATAGCC
AP-A-20	GATCAATCGC
AP-A-21	GATCTAACCG
AP-A-22	GATCGCATTG
AP-A-23	GATCTGACTG
AP-A-24	GATCATGGTC
AP-A-25	GATCATAGCG

The RT reaction 2 µl was added to 1 µl dNTP (25 µM), 2 µl 10X Gene *Taq* FP Buffer (5 U/µl), 1 µl arbitrary primers (10 µM), 0.2 µl DNA Polymerase Gene *Tap* in a final volume of 20 µl. The modified cycling conditions consisted of pre-denaturing at 1 cycle of 95°C for 3 min, 40°C for 5 min and 72°C for 5 min, followed by 24 cycles of 95°C for 15 s, 40°C for 2 min and 72°C for 1 min. The final extension was performed at 72°C for 5 min.

Agarose gel electrophoresis

The successful reamplification of the excised DNA was verified in a 1.5% (w/v) agarose gel in 1X TBE-buffer. Ethidium bromide was added to the buffer and gel to a final concentration of 0.5 µg ml-1. The samples (6 µl) were added to 1 µl 6X loading buffer (Promega). The sample were then loaded on an agarose gel and electrophoresed for approximately 1 hrs at 30 Vole. The gels were visualized on a UV transilluminator and the size of the bands were determined by comparison to a 100 bp and 1 Kb ladder (Promega). The gels were photographed using an orange filter (Kodak Wratten, Japan) and Panchrsomatic type Fujifilm (Fuji Photofilm Co., Japan).

DNA sequencing and sequence analysis

The nucleotide sequences of both strands were determined by the chain termination method and inserted randomly into the plasmid of *Escherichia coli*. The transformants were retrieved from *E. coli* cells using a QIAprep Spin Miniprep Kit (Qiagen) and screened using a restriction enzyme BamHi (Promega). The samples were digested overnight in a 37°C water bath and analyzed on 1% agarose gel the following day. In order to facilitate sequencing, digested fragments of samples were subcloned in to the

pGEM®-T Easy vector. Based on the results transformants were selected for lager preps using the GoTaq® Green Master Mix to increase plasmid concentration. PCR was performed with the SequiTherm EXCEL™*II* DNA Sequencing Kit-LC (Epicentre Technologies, Japan). Specific labeled forward and reverse primers (LI-COR) for the transposon allowed bidirectional sequencing of the plasmid DNA. The resulting sequencing reactions were separated on a 4% polyacrylamide (66 cm) gel, used with the LI-COR 4000L automated sequencer (Fig. 5.4). The nucleotide sequence reported were submitted to the GenBank BLASTN program of NCBI and to identify similarity with gene products already present in the database.

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