Pak. J. Biotechnol. Vol. 14 (1) 37-42 (2017)
www.pjbt.org

ISSN Print: 1812-1837
ISSN Online: 2312-7791

GENETIC ENGINEERING OF POTATO PLANT (*Solanum tuberosum* L.) *cv.* JALA IPAM WITH *MmPMA* GENE ENCODING PLASMA MEMBRANE H$^+$-ATPASE

A Farhanah[1,2], Suharsono[2,3], G A Wattimena[2], Utut Widyastuti[2,3]

[1]Biotechnology Study Program, Graduate School, Bogor Agricultural University, Bogor 16680, Indonesia, [2]Research Center for Bioresources and Biotechnology, Bogor Agricultural University, Bogor 16680, Indonesia. [3]Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Bogor 16680, Indonesia. Email: difarhanah@yahoo.co.id; sony.suharsono@yahoo.com

Article received 4.1.2017; Revised 22.2.2017; Accepted 27.2.2017

ABSTRACT

Plasma Membrane (PM) H$^+$-ATPase has important function as primary transporter driving secondary active transport systems in plant. Some plants overexpressing PM H$^+$-ATPase showed increased light-induced open stomata number and plant growth. This study has an objective to overexpress PM H$^+$-ATPase expected can increase productivity of potato. Genetic transformation was done by cocultivation method using *Agrobacterium tumefaciens* strain LBA4404 carrying pGWB502-*MmPMA*. Efficiency of transformation in this study is 3.46%. Molecular analysis with PCR was carried out by using primer ^{35}S-F and PMA-R1 to ensure integration of *MmPMA* into Jala Ipam potato. PCR analysis showed that all of six putative transgenic Jala Ipam potato lines are containing *MmPMA* gene under the control of ^{35}S CaMV. This study showed that genetic transformation process in Jala Ipam potato with *MmPMA* gene was successful with enhancing light-induced open stomata percentage, open stomata pore width, and root elongation-under low pH condition.

Keywords: Potato, PM H$^+$-ATPase, *Agrobacterium tumefaciens, MmPMA gene*

INTRODUCTION

Genetic improvement of crops through genetic engineering that has to be one way to solve food problem. Genetic engineering is expected to solve agricultural problems that led to the food crisis. The assembly of genetically modified (transgenic) plant is performed to obtain plant that has the quality and quantity further improved than non-transgenic (NT) plant. Planting potato seed good quality is the main factor determining the success of the production of potato plants. Efforts to provide the good quality potato seed must be based on the established seed system (Wattimena 2000).

Plasma membrane H$^+$-ATPase activates secondary transport by producing proton motive force which drives solutes, assimilates, and metabolites crossing PM (Serrano, 1989; Sussman, 1994). Another role of H$^+$-ATPase is to control opening or closing of voltage-gated channels (Morsomme and Boutry, 2000). Wang *et al.* (2014) reported successful transgenic Arabidopsis plants overexpressing H$^+$-ATPase by using a strong promoter of guard cells. They also reported that the transgenic plant open stomata earlier than NT during 30 minutes under light. In addition, cell elongation occurs when plant responded to acidic media (Ijaz *et al.*, 2012; Inoue *et al*. 2016) attributed to effect of H$^+$-ATPase overexpression.

The gene coding PM H$^+$-ATPase isolated from *Melastoma malabathricum* L. was successful cloned (Muzuni *et al.,* 2014) and integrated to *Nicotiana tabacum* SR1 (Ifadatin, 2016). The transgenic *Nicotiana tabacum* SR1 showed increased open stomata precentage, number of reproduction branch, leaf and flower, and earlier flowering than NT. In this study, we aimed to produce transgenic *Solanum tuberosum* L. Over-expressing PM H$^+$-ATPase under the control of strong promoter 35S CaMV to promote stomata opening and root elongation. The transgenic potato plant showed increased open stomata percentage, open stomata pore width, and root elongation under low pH condition. That is expected can enhance productivity of potato cv. Jala Ipam.

MATERIALS AND METHODS

Plant and *Agrobacterium* Preparation: Nodes of Jala Ipam potato plant (collection of Bogor Agricultural University) were cultivated on MS (Murashige and Skoog, 1962) medium for 3-4 weeks at 21^{o}C. Internodes of potato planlet was used as explants for transformation. The explants were cultivated on pre-cultivation medium (MS medium + 2,4D 2mg/L and BA 3mg/L adjusted at pH 5.8) for one day in dark condition at 21^{o}C.

Agrobacterium tumefaciens strain LBA4404 carrying pGWB502-*MmPMA* under control 35S CaMV promoter cultured in liquid LB (*Luria Bertani*) medium with hygromycin 50mg/L, streptomycin 50mg/L, and spectinomycin 50mg/L in dark condition at room temperature for 8-12 hours until

optic density of *Agrobacterium* culture showed 0.4-0.5 in OD_{600}.

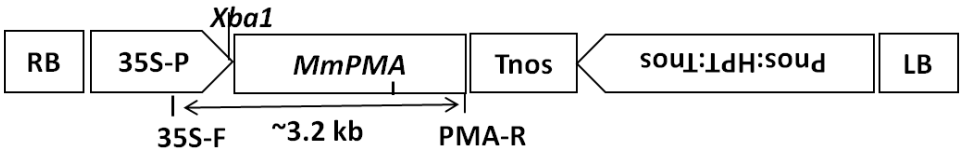

Figure -1: Construction map of pGWB502-*MmPMA* (Ifadatin 2016)

Plant Genetic Transformation: The cultured *Agrobacterium* showing optic density value 0.4-0.5 with OD_{600} was centrifuged at 10000 rpm for 10 minutes. Pellet of cultured *Agrobacterium* was put in liquid cocultivation medium (pre-cultivation medium + acetosyringone 40mg/L, pH 5.8). Explants on preculture medium were put into liquid cocultivation medium containing pellet of Agrobacterium and shake 2-3 times for 10 minutes. Explants were dried on sterile tissue paper for 10 minutes and then cultured on cocultivation medium in dark condition at 21°C for 3 days.

The explants were rinsed by sterile aqua dust for 5 minutes and cefotaxime 100 mg/L for 10 minutes then dried on sterile tissue paper for 10 minutes. Explants were grown on M4 medium (MS medium with IAA 2mg/L, BA 3mg/L, GA3 1mg/L and cefotaxime 100mg/L, pH 5.8) under lighting at 21-22°C for 7-10 days for recovering the explants from *Agrobacterium*. To select transformants carrying *MmPMA* gene, transformants were grown on selective medium (M4 medium with antibiotic hygromycin 40 mg/L, pH 5.8) under lighting at 21-22°C for 30 days until transformants showed bud. The buds were subcultured on MS medium under lighting at 21-22°C for 4 weeks until they grew to be plantlets. Transformation efficiency was obtainned from formula the number of hygromycin resistant callus divided by the number of explants which formed callus. Regeneration efficiency was obtained from formula the number of regenerated callus divided by the number of hygromycin resistant callus.

Total DNA was extracted from leaf of *Solanum tuberosum* L. by modified method of Suharsono (2002). Transgenic plant DNA was identified by PCR with primers ^{35}S-F (5'–AAACCTCCTCGGATTCCATT-3') and PMA-R1 (5'-TCAGGCCCTCCTTGCTGCATCTC-3') (Ifadatin 2016). PCR products were visualized by electrophoresis using 1% agarose soaked in Ethidium Bromide (EtBr) 0.5 µg/mL for 15 minutes. PCR also was done with the same DNA samples using soybean *actin* primers Act-F (5'-ATGGCAGATGCCGAGGATAT-3') and Act-R (5'-CAGTTGTGCGACCACTTGCA-3') (Shah *et al*., 1982) as internal control of potato DNA.

Observation of stomata opening: Observation of stomata opening was done to transgenic and NT plant 3 weeks after aclimatitation with collecting the surface of leaves (adaxial and abaxial) using transparant nail polish at 10.00. After the nail polish was dried, it was taken to preparat glass and observed with microscope Olympus BX51with zoom 600x. Average of open stomata percentage and open stomata pore width were measured on three fields of view, respectively 0.06 mm^2. Aver-age of open stomata percentage was obtained from the open stomata number divided by total of stomata per a field of view Average of open stomata pore width was obtained from measuring all open stomata pore (by application ImageJ 1.48) divided by total of open stomata per a field of view.

Observation of Root Elongation: Transgenic and NT plant, respectively 3 explants, were planted on half-strenght MS medium with pH 4.3 for 10 days. Root lenght of transgenic and NT plants was measured and averaged.

RESULTS AND DISCUSSION

Identification of Transgenic Plant: Identification of transgenic plant was done by selective medium and PCR with primers ^{35}S-F and PMA-R1. We found only 5 of 171 transformated internodes showing buds on selective medium. Some previous studies also showed success of using cocultivation method for tranformating tobacco, potato, and also seeweed (Fatahillah *et al.,* 2016; Paserang *et al.,* 2016; Rajamuddin *et al.,* 2016). The average of transformation and regeneration efficiency in this study are respectively 3.46 and 100%, respectively (table 1). It showed that transformation and gene-ration efficiency were still low if we compared it with previous study (Widiarti, 2016) with effiency transformation 7% and 18% and regeneration efficiency 100% in Jala Ipam. In study Widiarti (2016), potato genetic transformation proccess was almost similar with our study. These results raise presumption that addition of hygromycin 40 mg/L was too high and reduced the ability of explants to form

buds so that regeneration efficiency. Low concentration of antibiotic for may allow escapes to regenerate, and too high concentration may kill the transformed plants expressing moderate levels of resistance (Ijaz *et al.,* 2012). The optimum concentration of selective agents has to be deter-mined a prior by testing a variety of concentrations (Pereira *et al.,* 2016).

Table- 1: Tranformation and regeneration efficiency of Jala Ipam potato

Repitition	Total explants	Number of Hygromycin resistant callus	Number of regenerated callus	Transformation efficiency	Regeneration efficiency
I	80	2	2	3.08%	100%
II	91	3	3	3.85%	100%
Average	85.5	2.5	2.5	3.46%	100%

In this study, there were 5 internodes survived and formed 6 buds on selective medium. The 6 buds were putative transgenic plants then analized by PCR. PCR analysis showed that all of the six buds were transgenic plants (Figure 2A) showing good *actin* bands as internal control (Figure 2B). The results showed that this research method able to produce transgenic plant with regeneration efficiency 100% eventhough the transformation efficiency was still low.

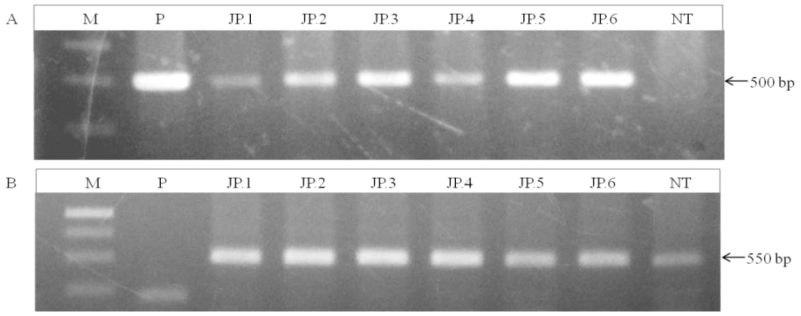

Figure -2: Analysis of *MmPMA* integration to transgenic Jala Ipam potato. A) PCR analysis using 35S-F and PMA-R1 primers. B) PCR analysis using *actin* primers as internal control. M: marka, P: plasmid DNA pGWB502-*MmPMA*, JP1–JP6: transgenic Jala Ipam DNAs, NT = Non-transgenic of Jala Ipam potato.

PM H^+-ATPase Promotes Stomata Opening: Three weeks after aclimatitation, stomata of six transgenic plants were analized. Transgenic plants tended to higher open stomata percentage and stom-ata pore width average than NT (Table 2, Figure 3). These results lead to the expectation that enhance of open stomata percentage and stomata pore width were caused by overexpessing of H^+-ATP- ase. These effects were shown in previous study of Ifadatin (2016), open stomata percentage in trans-genic tobacco was higher ~3.81-16.87 fold than NT. This result was suspected effect of PM H^+ - ATPase overexpression leading to open stomata enhancement. Stomata opening and closing (Kearns and Assmann, 1993; Schulz-Less-dorf *et al.,* 1994) and leaves movement (Cote, 1995) were caused by certain cells which give function in path of PM H^+-ATPase. In guard cell, H^+-ATPase activity leads to PM hyperpolari-tation and subsequent K^+ canal opening, and also precence of anion symporters. The influx of K^+ and Cl^-, and other anions lead to water uptake mediated by canal, turgor enhance-ment, and cell swelling. The cell swelling causes stomata opening. Stomata opening and closing can be achieved by modula-ting at least one protein involved in the proccess (Palmgren, 1998).

Extrusion of proton via plasma membrane is important in most cells for the maintenance of internal pH (Raven and Smith, 1974). This is particularly true for photosynthetic in plant where CO_2 fixation being carbohydrates results in the production of H^+ which must be extruded in exchange for K^+ or other cations (Rayle and Cleland, 1977).

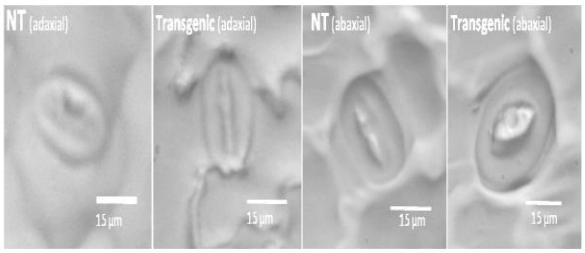

Figure -3: Open stomata taken from adaxial and abaxial of leaf in NT (non-transgenic) and transgenic-*MmPMA* plant.

The enhancement of stomata opening is expected can promotes photosynthesis activity and plant growth. In previous study of Wang *et al.,* (2014) showed that only overexpressing H^+-ATP-ase in guard cell could give signifficant effect to stomata opening. Transgenic Arabidopsis overex-pressing H^+-ATPase in guard cell also showed enhancement of photosynthesis activity and plant growth. Role of H^+-ATPase in this mechanism is to give potensial electrochemistry needed for contro- ling the tension of guard cell opening or closing. It described that the expression of H^+-ATPase was high in guard cell (Becker *et al.,* 1992). On the other hand, Mitchell (1970) has proposed that the proton pump arose as a tool of setting up H^+ gradients which could drive other transport processes coupled to the back diffusion of H^+.

Table-2: Average of open stomata percentage, open stomata pore width, and root lenght in NT and transgenic Jala Ipam potato.

Plant code	Percentage of Open Stomata		Stomata Pore Width (µm)		Root Lenght (cm)
	Adaxial	Abaxial	adaxial	abaxial	pH 4.3
NT	100%	54.72%	2.98 ± 0.64	3.11 ± 0.56	0.40 ± 0.10
JP1	100%	95.83%	4.25 ± 0.69	6.40 ± 2.14	2.39 ± 0.56
JP2	100%	79.17%	4.97 ± 1.99	6.52 ± 0.62	1.58 ± 0.62
JP3	100%	56.67%	4.14 ± 1.56	5.20 ± 1.67	2.78 ± 0.42
JP4	100%	66.67%	4.95 ± 1.23	3.99 ± 0.88	3.53 ± 0.06
JP5	100%	61.18%	3.90 ± 0.24	5.36 ± 1.51	2.03 ± 1.40
JP6	100%	83.01%	3.98 ± 0.08	7.15 ± 0.89	2.27 ± 0.25

PM H^+-ATPase Promotes Root Elongation Under Low pH Condition: PM H^+-ATPase activation promoted root elongation in some plants under low pH condition (Young *et al.,* 1998; Inoue *et al.,* 2016). In this study, we just proved that PM H^+-ATPase really exist in transgenic plants by treating them to low pH condition besides seeing the effect on stomata. To explore the activation of PM H^+-ATP - ase in transgenic potato plant root, we treated NT and transgenic potato plants to half-strenght MS medium pH 4.3. We found that transgenic plants tend to promote more the root elongation in low pH medium (pH 4.3) if we compare them with NT potato plant (Table 2). This result suggests that enhancement of PM H^+-ATPase under the low pH condition leads to root elongation.

Indeed, root elongation in transgenic-*Mm PMA* plant was suppressed markedly under low pH Condition (Figure 4). This result showed that enhanced PM H^+-ATPase activity in low pH condition. Activity of expansin by protein secretion to the wall and by pH change and redox potential of the wall induces stress relaxation and polymer creep needed for wall enlargement and water uptake by cells (Cosgrove, 1997; Rayle, 1992). Result of some in vitro studies, exspecially hypocotyl, showed that external acidification from cell wall can lead to the cell expantion. This effect was transient (Schopfer, 1993; Kutschera, 1994), that was expected that because solution used for modifying apoplastic pH has to cross the cuticular barrier (Niczyj *et al.,* 2016). A previous study has provided evidence that phosphorylation of the penultimate Thr of the H^+-ATPase activates the H^+-ATPase, which stimulates hypocotyl elongation (Takahashi *et al.,* 2012).

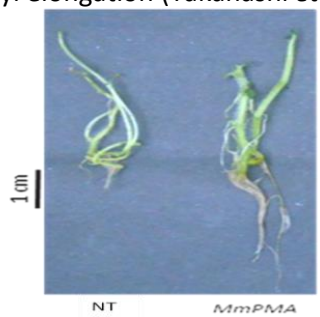

Figure-4: Root appearance of NT (non-transgenic) and transgenic-*MmPMA* plant under low pH condition (pH 4.3).

Inoue *et al.,* (2016) found that ATP hydro-lysis activity and the phosphorylation level of the penultimate threonine of PM H^+-ATPase increased in response to low pH conditions in NT Arabidopsis roots. Root elongation was suppressed slightly by 20% under low pH condition (pH 4.3) compared with normal pH condition. Enhanced PM H^+-ATPase activity in low pH condition is needed to require pH homeostasis for root elongation.

Potato cv. Jala Ipam has been successful to be transformated with *MmPMA* gene to overexpress H^+-ATPase. Overexpressing of PM H^+-ATPase in Jala Ipam potato showed number of stomata tended to open more and wider than NT, and tolerance to low pH medium (pH 4.3) by observing the root lenght. These results reinforce previous

studies stating that overexpression of H^+-ATPase in plant can enhance stomata opening and root elongation in low pH medium. The result of these studies is expected to increase photosynthesis and plant growth.

ACKNOWLEDGEMENT

Authors thank to the mainstay research program for university and industry for providing funds for this research through contract number 079/SP2/ LT/ DRPM/II/2016.

REFERENCES

Becker, D., C. Zeilinger, G. Lohse, H. Depta, R. Hedrich, Identification and biochemical characterization of the plasma-membrane H^+-ATPase in guard cells of *Vicia faba L*. Planta 190:44-50 (1993).

Cosgrove, D.J., Relaxation in a high stress environment: the molecular bases of extensible cell walls and cell enlargement. Plant Cell 9: 1031-1041 (1997).

Cote, G.G., Signal transduction in leaf movement. Plant Physiol. 109: 729-734 (1995).

Fatahillah, Suharsono, U.Widyastuti, Genetic Trans -formation of Potato (*Solanum tuberosum* L.) Cv. Nooksack with *Fbpase/Clran1* Genes Mediated by *Agrobacterium Tumefaciens.* Pak. J. Biotechnol. 13(3): 187 - 192 (2016).

Ifadatin, S., Genetic Engineering of Tobacco and Physic Nut with Gene Encoding H^+-ATPase Plasma Membrane from *Melastoma malabathricum* L. PhD thesis. Bogor (ID): Institut Pertanian Bogor (2016).

Ijaz, S., I.A. Rana, I.A.Khan, M. Saleem, Establishment of an in vitro regeneration system for genetic transformation of selected sugarcane genotypes. Genet. Mol. Res. 11(1):512-530 (2012).

Inoue, S., K. Takahashi, H. Okumura-Noda, T. Kinoshita, Auxin Influx Carrier AUX1 Confers Acid Resistance for Arabidopsis Root Elongation Through the Regulation of Plasma Membrane H^+-ATPase. Plant Cell Physiol. 0(0): 1–8 (2016).

Kearns, E.V., S.M.Assmann, The guard cell environment connection. Plant Physiol. 102:711-715 (1993)

Kutschera, U., The current status of the acid-growth hypothesis. New Phytol. 126: 549–569 (1994).

Mitchell, P., Membranes of cells and organelles: morphology, transport, and metabolism. Syrup. Soc. Gen. Microbiol. 20: 121-166 (1970).

Morsomme,P., M.Boutry, The Plant plasma membrane H (+)-ATPase: structure, function and regulation. Biochim. Biophys. Acta 1465: 1–16 (2000).

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

Muzuni, D. Sopandie, U.W. Suharsono, Suharsono, Isolation and Cloning of the Gene Encoding for PlasmaMembrane H^+-ATPase from *Melastoma malabathricum* L. J. Agron. Indonesia 42(1): 80–8 (2014).

Niczyj, M., A. Champagne, I. Alam, J. Nader, Expression of a constitutively activated plasma membrane H^+-ATPase in Nicotiana tabacum BY-2 cells results in cell expansion. Planta 244 (5): 1109-1124 (2016).

Palmgren, M.G., Proton gradients and plant growth: role of the plasma membrane H^+-ATPase. Adv. Bot. Res. 28: 1-70 (1998).

Paserang, A.P., A. Tjahjoleksono, U. Widyastuti, Suharsono, Genetic Transformation of Tobacco Plant Using Inhibitor of Meristem Activity (*IMA*) Genes. Pak. J.Biotechnol. 13(2): 125-131 (2016).

Pereira, A.V.C., L.G.E.Vieira, A.F. Ribas, Optimal Concentration of Selective Agents for Inhibiting In Vitro Growth of Urochloa brizantha Embryogenic Calli. Afr.J.Biotechnol. 15(23):1159-1167 (2016).

Rajamuddin, M.A.L., Alimuddin, U. Widyastuti, E. Harris, Transformation of *Kappa (κ)-Carrageenase* Gene From *Pseudoalteromonas* in Seeweed *Kappaphycus alvarezii* [DOTY]. Pak.J. Biotechnol. 13(3): 157-163 (2016).

Raven, J.A., F.A. Smith, Significance of hydrogen ion transport in plant cells. Can. J. Bot. 52: 1035–1048 (1974).

Rayle, D.L., R.E. Cleland, The Acid Growth Theory of Auxin-induced Cell Elongation Is Alive and Well1. Plant Physiol. 99: 1271-1274 (1992).

Rayle, D.L., R. Cleland, Control of plant cell enlargement by hydrogen ions. Curr. Top. Dev. Biol. 11: 187-214 (1977).

Schopfer, P., Determination of auxin-dependent pH changes in coleoptile cell walls by a null-point method. Plant Physiol. 103: 351–357 (1993).

Schulz-Lessdorf,B., P.Dietrich, I.Marten, G. Lohse, H.Busch, R.Hedrich, Coordination of plasma mem brane and vacuolar membrane ion channels during stomata movement. Symposia of the Society for Experimental Biology 48: 99-112 (1994).

Serrano, R., Structure and Function of Plasma Membrane ATPase. Ann. Rev. Plant Physiol. Plant Mol. Biol. 40: 61-94 (1989).

Shah D.M., R.C. Hightower, R.B. Meagher, Complete nucleotide sequence of a soybean actin gene. Proceeding of the National Academic of Science of the USA. 79 (4): 1022- 1026 (1982).

Suharsono, Construction of Genomic Library of Soybean Cultivar Slamet. Hayati 9(3):67-70 (2002).

Sussman, M.R., Molecular analysis of proteins in the plant plasma membrane. Ann. Rev. Plant Physiol. Plant Mol. Biol. 45: 211-234 (1994).

Takahashi, K., H. Okumura-Noda, T. Kinoshita, Auxin Activates the Plasma Membrane H^+-ATPase by Phosphorylation during Hypocotyl Elongation in Arabidopsis. Plant Physiol. 159: 632-641 (2012).

Wang, Y., K.Noguchi, N.Ono, S.Inoue, I. Terashima, T.Kinoshita, Overexpression of plas-ma membrane H^+-ATPase in guard cells promotes light-induced stomata opening and enhances plant growth. PNAS. 111(1): 533-538 (2014).

Wattimena, G.A., Propagule Development of Qualified and Superior Potato Cultivar for Improving Potato Production in Indonesia. Full-time Professorship Scientific paper in Horticultural Science, Faculty of Agriculture, Bogor Agricultural University (2000).

Widiarti, W. Genetic transformation of potato (*Solanum tuberosum L.*) cv Jala Ipam by using *Hd3a* gene. Thesis. Bogor (ID): Institut Pertanian Bogor (2016).

Young, J.C., N.D. DeWitt, M.R. Sussman, A transgene encoding a plasma membrane H^+-ATP ase that confers acid resistance in *Arabidopsis thaliana* seedlings. Genetics 49: 501–507 (1998).