Research Article

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Physico-chemical properties of *Arabidopsis* Ca^{2+}/H^{+} antiporter transgenic rice grain.

G. H. Yi[1], H. S. Lee[2], J. K. Sohn[2], K. M. Kim[2*]

[1]Department of Functional Crop, National Institute of Crop Science, Milyang, 627-803, **Republic of Korea**
[2]School of Applied Biosciences, Kyungpook National University, Daegu, 702-701, **Republic of Korea.**
***Corresponding author**

In this study, calcium contents, physico-chemical compositions, and grain related cytological & agronomic traits of *CAX*1 (CAtione Xange) transgenic rice plants were examined. The average content of calcium in brown seeds of transgenic plants was 183.3ppm, which was elevated to 71% more than that of the parental cultivar 'Ilpum'. CLMS analysis revealed that the starch granule compound was more compact and dense in the endosperm of the *CAX*1 transgenic grain. Green fluorescence was observed having strong signals in the cell and the vacuolar membranes, respectively. The grain size of *CAX*1 transgenic rice was similar to the parental cultivar; however, one thousand grain weight was reduced to 21.8g from that of 25.9g in the parental cultivar 'Ilpum'. Physico-chemical properties of the endosperm such as amylose, protein and lipid content were similar to the parental cultivar 'Ilpum'. The results indicate that *Arabidopsis CAX*1 is successfully expressed in rice and found to increase calcium contents in the endosperm. Among the transgenic plants, elite plants can be selected with elevated calcium content of about 2.4 times more than parental cultivar, 'Ilpum'. These lines can be used as a good genetic resource for releasing calcium fortified rice in the future.

Key words: *CAX*1, Rice, Green fluorescence, Calcium, Transgenic rice, Ilpum

Calcium is both a signaling molecule and a component in providing the plant cell with its structural strength. Regulated Ca^{2+} fluctuations are involved in plant growth and responses to environmental stimuli (Zhao et al. 2009). Ca^{2+} transporters play an important role in regulating these biological processes and the ability to manipulate these transporters is an important component in strategies designed to manipulate plant productivity (Hirtschi et al. 1996; Shigaki et al. 2000; Cheng et al. 2005). Calcium ions containing intracellular vacuoles present in many cells of plant organs and regulates the Ca^{2+} concentrations in cell compartments, the plant possess calcium-binding proteins, Ca^{2+} channels, Ca^{2+} ATPase and Ca^{2+}/H^{+} exchangers(CAXs) (Shigaki et al. 2001).

In *Arabidopsis*, there are six CAX genes belonging to two clades, CAX1, 3, and 4 within clade 1-A and CAX2, 5, and 6 within clade 1-B (Shigaki and Hirschi, 2006). *CAX*1 and *CAX*3 of *A. thaliana* were identified as proteins that suppressed the Ca^{2+}-sensitive phenotype of yeast. *CAX*3 is 77% identical (93% similar) to *CAX*1, and when expressed in yeast, localizes to the vacuole but and does not suppress yeast mutants defective in vacuolar Ca^{2+} transport (Shigaki et al. 2002; Manohar et al. 2011).

In plants, *CAX*1 contains an additional 36 amino acid at the N-terminus which is not present in s*CAX*1 (short CAtione Xchanger) (Mei et al., 2007). *CAX*1 contains an N-terminal auto-inhibitory domain, and expression of N-terminal truncations of *CAX*1 (s*CAX*1) in tobacco increases the Ca levels of the plants (Hirsch, 1999). Ectopic expression of the N-terminal truncated *CAX*1, s*CAX*1, increases Ca levels in several plants. In order

to develop transgenic potato tuber with enhanced calcium content, the s*CAX*1 gene was introduced into potato, (Park et al*.,* 2005b). Utilizing yeast assays have characterized the transporters properties of variants of *CAX*4 and the effect of s*CAX*1 and *CAX*4 expression in tomato (Park et al. 2005a; Chung et al*.* 2010).

Modulation of *CAX*1 could make an important contribution toward increasing the value of various agriculturally important crops. Transgenic tobacco shows that constitutive expression of *CAX*1, a putative vacuolar Ca^{2+}/H^{+} transporter, can alter the growth, increase the sensitivity of the plant to various ions, and alter the plant response to chilling (Hirschi et al*.* 1996). In the study by Cheng et al. (2004), they found out that all of the cloned plant's *CAX* proteins were determined to be localized to vacuolar membranes by demonstrating the *CAX* protein fused to green fluorescent protein (GFP).

In addition, calcium is an important mineral that comprises human bones. Lack of calcium in the body is known to inhibit growth in youth and children, osteoporosis induction and can result in weakened immune system (Bachrach, 2001). Consumption of adequate dietary Ca can be accomplished through intake of a variety of Ca-rich vegetables and crops.

Transgenic potato bulbs using s*CAX*1 genes isolated from *Arabidopsis*, increased the calcium to three times (Park et al*.,* 2005a; Park et al. 2005b), contained 2-fold-higher calcium content in the edible portions of the carrot (Connolly 2008, Morris et al. 2008) and 25%-32% more calcium in the lettuce than controls (Park et al*.,* 2009). In rice plants, *CAX1* was introduced into japonica cultivars of rice by *Agrobacterium*-mediated transformation, and T_1 of *CAX*1 transgenic plants were produced (Kim et al. 2005).

This study suggests that *CAX*1 transgenic plants could make the target system related to the calcium content of brown rice, physico-chemical traits and analysis of starch granule compounds and arrangement in endosperm. We have also characterized the sub-cellular localization of *CAX*1 fused to green fluorescent protein (GFP) for increasing the Ca^{2+} levels of rice through expression of *CAX*1.

MATERIALS AND METHODS

Transgenic plant source and cultivation

This study was conducted during the rice-growing season on experimental fields at Kyungpook National University from 2006 to 2008. Field management was conducted according to the standard cultivation practices recommended by the Rural Development Administration (RDA) with fertilizer at the rate of 110kg N ha^{-1}, 45 kg P_2O_5 ha^{-1}, and 57 kg K_2O ha^{-1}. A total of thirty four lines T_3lines of *CAX*1 transgenic plants were obtained on the PCR analysis. Seven lines (T_4 to T_7) from the thirty four lines of *CAX*1 transgenic plants were selected on the basis of their performance in calcium contents and the PCR analysis.Using a spacing pattern of 15×30cm between plants and rows, approximately 30-day-old seedlings from each *CAX*1 transgenic plants consisting of six rows with fifty plants per row were grown, with the parental cultivar 'Ilpum' placed between *CAX*1 transgenic plants as the Control.

Analysis of calcium content in CAX1 transgenic brown rice

The harvested rice grains were collected from each of *CAX*1 transgenic plants and parental cultivar, 'Ilpum', dried naturally, and then was stored at room temperature. For analysis of calcium and inorganic elements, freeze-dried brown rice of transgenic plants and parental cultivar, 'Ilpum', were grinded in homogenizer (IKA: Package DI 18 ™) 2000rpm, 15 g of the powder was placed with sterilized water, left to stay for thirty minutes and then mixed every five minutes three times in the vortex. After sinking, 10ml of supernatant was placed at room temperature for one day. Afterwards, it was vigorously mixed in the vortex for one hour then 1.5ml of the supernatant was transferred to the e-tube (eppendorf tube). Finally, the solution was centrifuged at 1200rpm for two minutes and diluted to a ratio of 1:3 supernatant and sterilized-water solution. Inorganic ions including calcium were analyzed with CULTURYZER-mini Technomedica Co. (Japan).

Structure of starch granules in *CAX*1 transgenic brown rice

Starch structure of brown rice in *CAX*1 transgenic plants and parental cultivar, 'Ilpum', were commissioned to the National Institute of Crop Science. Samples were then pretreated for electron microscopic observation. After performing the sideways cut of the brown rice, it was then cooled in

phosphoric acid at 4℃. For a fixed sample of 2-3% glutaraldehyde, after 2 hours of immersion in a phosphoric acid of 1% OsO_4, the phosphate solution was placed for 2 hours in fixed-samples. Fixed-samples were dehydrated in acetone and then vacuumed in gold-palladium for 60 seconds after which a coating of starch structure was observed. The starch structure of brown rice in *CAX*1 transgenic plants and parental cultivar, 'Ilpum', were observed by using a scanning electron microscope (SEM, Scanning Electron Microscope 4300, Hitachi, Tokyo, Japan).

Fluorescence analysis for tissues of *CAX*1 transgenic plants

Fluorescence of the GFP expression in *CAX*1 transgenic plants and parental cultivar, 'Ilpum' were investigated by visualizing the fourth leaves and roots tissues through the self-fluorescence techniques of the Confocal Laser Scanning Microscope (Nicon, A1RSI, Japan). For each type of tissue, the setting of the microscopic system was adjusted so that the fluorescence signals from samples should not be saturated, and the signal from control should be at the background level. Area measurements of the vascular elements were performed using an Olympus analySIS TS Lite software. The eGFP signal was induced with the red circle between the eGFP reference data and the background, wherein emission was collected from 450nm~500nm. Differential Interference Contrast (DIC) images were recorded simultaneously and compared with the fluorescence images to locate the cells or tissues that generate fluorescence.

The grain shape and physicochemical constituents of *CAX*1 transgenic brown rice

An instrument designed to measure moisture, GMK-303, was applied in surveying the water content of the brown rice. The moisture level of 12-13% was controlled. Damaged grains, red grains, green grains and broken grains were removed from the brown rice before testing. One thousand seeds of the grain weight were measured three times. Grain length, width, ratio of length to width, and grain thickness of 300 grains for each line were determined by using a vernier caliper (Series 500). The physicochemical properties such as amylose, protein, fat, moisture and starch content were analyzed with microscope using a NIRS (near-infrared spectroscopy, Foss 6500).

RESULTS

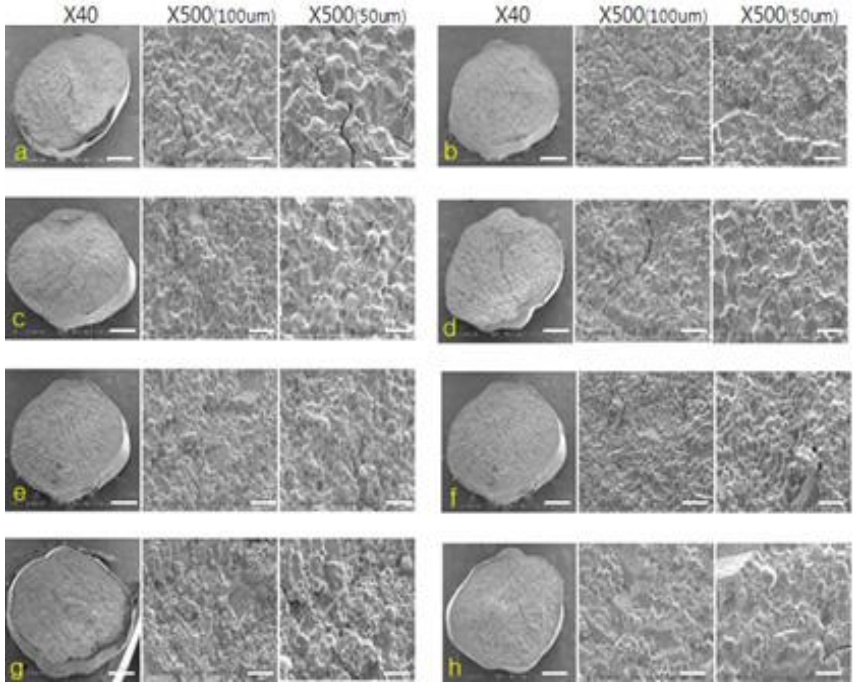
Among the thirty four lines in T_3 generation, five lines of T_4 *CAX*1 transgenic plants were selected for calcium content and agronomic traits. To evaluate the genetic stability of *CAX*1 transgenic rice, we planted five lines and its self-fertilized offspring for 6 successive years from 2003 to 2008.

*CAX*1 transgenic rice was characterized by an average 183.3ppm increased to 52.6% than 96.4ppm parental cultivar, 'Ilpum'. *CAX*1 transgenic plants yielded significant differences on the average calcium content from 253ppm to 139.8ppm. All *CAX*1 transgenic plants were higher than that of the parental cultivar, 'Ilpum'. The range of magnesium content in the *CAX*1 transgenic plants showed a distribution of 29.4 ~ 58.1 ppm which were all slightly lower, compared with the 64.8ppm in parental cultivar, 'Ilpum'. The range of potassium content in the *CAX*1 transgenic plants showed a distribution range value of 153.8 ~ 175.3 ppm which tend to be slightly higher, compared with 136ppm of parental cultivar, 'Ilpum'(Table 1).

Starch granules of *CAX*1 transgenic plants and parental cultivar, 'Ilpum', were observed to vary in particle size and uniformity (Fig. 1). In 'Ilpum', the fractured starch granule of the endosperm displayed comparatively standardized shape and size (Fig. 1a). The T-14 and T-15 endosperm showed less dense arrangement compare to the parental cultivar, Ilpum endosperm of round starch granule (Fig. 1b,1c) while theT-17 endosperm showed small and un-separated starch granules containing some pore(Fig.1d). In T-18 endosperm, there were more small starch granules than large starch granules displayed (Fig.1e). T-19 showed the smallest, sparse and un-separated starch granules containing protein body compared to 'Ilpum' endosperm (Fig.1f). The T-20 showed small, sparse and separated starch granules compared to 'Ilpum' endosperm (Fig.1g) and the T-24 endosperm showed relatively large, sparse and un-separated starch granules compared to Ilpum endosperm (Fig.1h).

The *CAX*1 transgenic tissues imaging aspects in calcium ions were investigated to determine the GFP (green fluorescent

Figure 1. SEM (Scanning Electron Microscope) analysis of endosperm of *CAX*1 transgenic rice lines and 'Ilpum' in brown rice showing starch granules of compounds and arrangement. a. Ilpum, b. T_6-14(T_7 seed), c. T_6-15(T_7 seed), d. T_6-17(T_7 seed), e. T_6-18(T_7 seed), f. T_6-19(T_7 seed), g. T_6-20(T_7 seed), h. T_6-24(T_7 seed). White vertical arrow, comparable point with 'Ilpum' that is control.



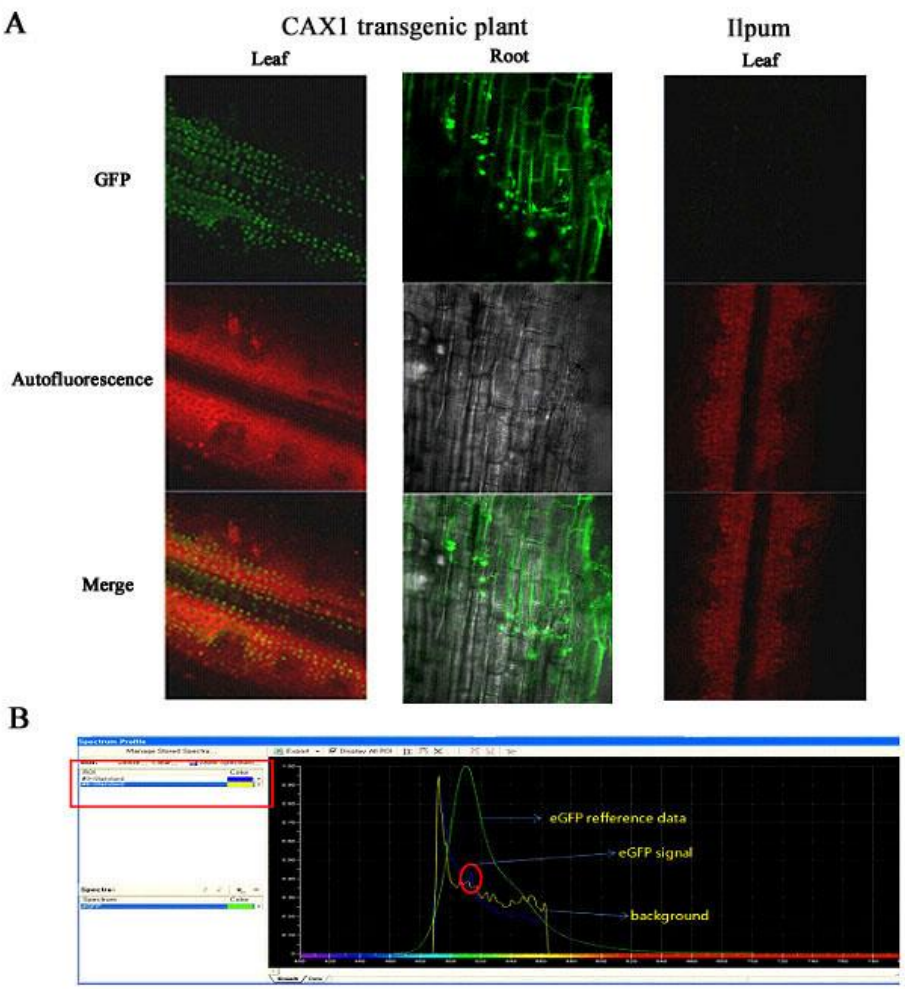
protein). Expression sites were investigated using a confocal microscope. The main expression of *CAX*1 in leaf and root tissue showed signal of the cell membrane and vacuoles compared to parental cultivar, 'Ilpum' (Fig. 2). The leaf tissue strongly responded to the vacuole, the root expressed in the cell membrane and the intercellular spaces indicated that GFP imaging was achieved (Fig. 2a). The eGFP signal was induced with the red circle between the eGFP reference data and background with the range from 450nm~500nm (Fig. 2b). These cells might function as Ca^{2+} buffer space by absorbing or releasing Ca^{2+} in response to stimuli.

The five selected lines in terms of the grain size revealed that most of the length/width ratio was similar to parental cultivar, 'Ilpum'. However, the length/width ratio of T_6-19 and T_6-20 lines showed less than 1.94 and has decreased more as compared to the parental cultivar, 'Ilpum', in one thousand grain weight to an average of 21.8g, 25.9g, respectively (Table 2). In *CAX*1 transgenic plants, the physico-chemical composition such as amylose, protein, and lipid were analyzed to be similar with the parental cultivar, 'Ilpum'. Amylose content showed the range of 20.0 to 21.1%, and the tendency was similar to 20.4%, compared with 66.9% starch parental cultivar, 'Ilpum', with range of 67.3 to 69.8%, protein content of 7.1 to 8.7% and 8.3% similarity to the parental cultivar, 'Ilpum'. In this experiment, except T_6-17 line, one thousand grain weight on all *CAX*1 transgenic plants decreased compared to parental cultivar, 'Ilpum' (Table 3).

DISCUSSION

The *Arabidopsis CAX*1 one of Ca^{2+}/H^+ transporter is known to play an important role, both as regulators of Ca^{2+} and in providing structural strength to cell components. Transgenic plants expressing *CAX*1 demonstrated increased Ca^{2+}accumulation and altered activity of the tonoplast-enriched Ca^{2+}/H^+ transporter in several crops. For example, in tobacco roots the increase is two times and 0.3 times more in leaf tissue (Hirschi, 1999), while in tomato fruit from 150% (Park et al. 2005a; Chung et al*.,* 2010). In carrot, it has been reported to increase of 2-fold-higher calcium content in the edible portions (Connolly, 2008, Morris et al. 2008).

Figure 2. Expression of GFP (Green Fluorescence Protein) in *CAX*1 transgenic plants. Green fluorescence of the GFP was viewed using confocal laser microscopy'A1Rsi'. eGFP(enhanced Green Fluorescent Protein) signal (green), DIC(Differential Interference Contrast)picture and merged view of eGFP signal and DIC(a). Area measurements of the vascular elements were performed using an Olympus analySIS TS Lite software. eGFP signal was induced with the red circle between the eGFP reference data and background. The relative Ca^{2+}localization in the red circle could be observed with the range from 450nm~500nm(b)



In this work, we have analyzed the calcium content and PCR of *CAX*1 transgenic plants from T_4 to T_7generation,selected five lines of T-17, T-18, T-19, T-20, T-25 and characterized the parental cultivar, 'Ilpum' having an average 183.3ppm increased to 71% at 106.9ppm. The significant differences on the average of calcium content from 253ppm to 139.8ppmin *CAX*1 transgenic plants imply that the *CAX*1 transgenic rice by *Agrobacterium*-mediated gene transfer can be genetically transferred to progenies. The average calcium content of each *CAX*1 transgenic plants were observed for yearly variations depending on the recognition that some of these causes were introduced to gene copy number, homogeneity, or breeding for the agricultural transgenic lines.

Currently, most people obtain their dietary Ca^{2+} from milk-related products. Calcium-rich rice can contribute significantly to Ca^{2+} intake as staple food for over half of the world's population. Likewise, increasing the endogenous levels of Ca^{2+} in commonly consumed rice should help yield improved dietary Ca^{2+}-intakes within many populations. Studies on the bioavailability in Ca^{2+}-related products are needed to determine as to what extent these mineral changes in seeds can translate into improved Ca^{2+} bioavailability and nutritional quality (Park et al. 2005a; 2005b).

Table 1. Ca^{2+} contents of *CAX*1 transgenic lines and 'Ilpum' in brown rice.

Transgenic lines	Grain generation						
	T_7			T_6	T_5	T_4	Average
	Mg^{2+}	K	Ca^{2+}	Ca^{2+}	Ca^{2+}	Ca^{2+}	Ca^{2+}
T-17	37.2d*	161.3b	219.6b (63.2)**	154.0c (52.5)	98.7b (-18.4)	87.0d (22.2)	139.8d (30.8)
T-18	50.7c	155.2c	226.1a (68.0)	388.0a (284.2)	124.1a (2.7)	273.6a (284.3)	253.0a (136.6)
T-19	32.6e	155.8c	205.9c (53.0)	154.5c (53.0)		91.0c (27.8)	140.9d (31.8)
T-20	58.1b	175.3a	152.6e (13.4)	154.0c (52.5)	-	-	153.3c (43.4)
T-24	29.4f	153.8c	202.1d (50.1)	350.0b (246.5)		260.0b (265.2)	229.6b (114.7)
Ilpum	64.8a	136.0d	134.6f (0.0)	101.0d (0.0)	120.9c (0.0)	71.2e (0.0)	96.4e (0.0)

*part per million, In a column, means followed by a common letter not significantly different at the 1 or 5 % level by DMRT.
**rate of calcium increase.

Table 2. Grain size of *CAX*1 transgenic rice and 'Ilpum' in brown rice.

Transgenic lines & cultivars	Length (mm)	Width (mm)	Thickness (mm)	Length /Width	Weight of 1000seeds(g)
T_6-14	5.28±0.15[a]	2.85±0.12	1.90±0.08	1.86	18.9
T_6-15	5.27±0.15	2.89±0.17	1.95±0.07	1.82	21.5
T_6-17	5.83±0.25	3.02±0.09	2.18±0.08	1.93	26.2
T_6-18	5.44±0.12	2.95±0.12	1.94±0.06	1.85	22.2
T_6-19	4.68±1.23	2.91±0.09	2.07±0.06	1.61	21.0
T_6-20	5.07±0.17	3.15±0.09	2.18±0.07	1.61	24.1
T_6-24	5.09±0.20	2.73±0.13	1.92±0.06	1.86	19.1
Average				1.79	21.8
Ilpum	5.91±0.16	3.05±0.11	2.08±0.08	1.94	25.9

[a]Mean ± SD.

The dense structure of starch showed to relate to rice heating time higher hardness and stickiness, and reduced rate of pore expansion rate, as well as high eating quality. Generally, starch grains are arranged uniformly and estimated to have a texture and physical properties of rice that have high eating quality and good value (Kim et al., 2005). Among *CAX*1 transgenic plants, starch grains of the brown rice showed that the starch granules and compound of the *CAX*1 transgenic plants were less densely arranged compared with that of the parental cultivar, 'Ilpum'. Systematic study should be carried out to further investigate the relationship of calcium in starch grains structure, size and eating quality of rice. The main expression of *CAX*1 in leaf and root tissues showed signal of the cell membrane and vacuoles compared to the parental cultivar, 'Ilpum' through GFP

Table 3. Physico-chemical constituents of *CAX*1 transgenic rice and 'Ilpum' in brown rice

Transgenic lines & cultivars	Amylose (%)	Starch (%)	Protein (%)	Lipid (%)
T_6-17	21.06±0.56 [a]	67.31±0.27	8.23±0.07	1.58±0.05
T_6-18	20.46±0.37	68.07±1.16	7.93±0.14	1.58±0.10
T_6-19	20.16±0.54	67.60±0.64	8.73±0.13	1.62±0.12
T_6-20	20.88±0.41	69.76±0.92	7.06±0.06	1.71±0.01
T_6-24	20.04±0.51	68.11±0.59	8.32±0.14	1.74±0.06
Ilpum	20.38±0.44	66.86±0.35	8.33±0.07	1.58±0.05

[a]Mean ± SD.

imaging. These cells might function as Ca^{2+} buffer space by absorbing or releasing Ca^{2+} in response to stimuli. The calcium contents of leaves were accumulated in the coleoptile tissues compared to the older leaf. In rice, *CAX*1 are transported from the root tissue and absorbed to the vacuole in plant cells and stored and maintained at certain level in the leaves. Consistent with this observation, OsCAX1 might maintain the vacuolar and trichomes function of a Ca^{2+} stored in these cells (Kamiya et al*.* 2006). Cell-specific vacuolar calcium storage mediated by *CAX*1 regulates apoplastic calcium concentration in *Arabidopsis* (Conn et al. 2011). CXIP4 can activate *CAX*1 in a yeast growth assay; characterize the sub cellular localization of the protein by making a fusion of the plant gene product with the green fluorescence protein (Cheng et al*., 2004*). We have also characterized the specificity of this activation and the region of *CAX*1 which is involved in the association between *CAX*1 and GFP protein.

The 5 lines selected in T_6 investigated the grain size, where in most of the length/width ratio was similar to the parental cultivar, 'Ilpum', and the length/width ratio of T_6-19 and T_6-20 lines showed less than 1.94 to the parental cultivar, 'Ilpum'. In terms of grain weight, an average decrease in one thousand grain weight of 21.8g, 25.9g, respectively, was obtained compared to parental cultivar, 'Ilpum'. More in-depth studies are needed understand further the cause of the decrease either due to somatic mutation in tissue culture or physiological function of calcium. These behaviors may be due to the increase in the variations in 'Calrose 76', as the main traits in the agricultural transgenic anther culture, which may also control the increased weight of one thousand grain weight compared to the parental cultivar (Schafer et al*.*, 1996).

*CAX*1 in transgenic potato to the size of the bulb were similar to that of parental variety (Park et al. 2005b). The 'Hwayoung' variety derived from the anther culture subsequent analysis of the physicochemical properties of brown rice did not show any significant difference. Between the *CAX*1 transgenic plants and parental cultivar, 'Ilpum', reported similar physico-chemical constituents in brown rice. Additionally, *CAX*1 transport in rice would measure to be resistant to blast, cold, chlorophyll value, and water solution (Kim, 2009). Result of this study revealed that *CAX*1 isolated from *Arabidopsis* yielded an average calcium contents in brown rice which was higher by 70% than the parental cultivar, 'Ilpum', while T-18 of *CAX*1 transgenic plants were confirmed to express 2.4 times higher. Following the results and combining these to develop intermediate rice breeding program can further increase the calcium content in rice.

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REFERENCES

Bachrach LK. 2001. Acquisition of optimal bone mass in childhood and adolescence. Trends Endocrin Met 12: 22-28

Cheng NH, Pittman JK, Shigaki T, Lachmansingh J, Leclere S, Lahner B, Salt DE, Hirschi KD. 2005. Functional association of *Arabidopsis CAX*1 and *CAX*3 is required for normal growth and ion homeostasis. Plant Physiology138: 2048-2060

Cheng NH, Liu JZ, Nelson RS, Hirschi KD. 2004. Characterization of *CXIP*4, a novel *Arabidopsis* protein that activates the H^+/Ca^{2+} antiporter, *CAX*1. FEBS Letters 559: 99-106

Chung MY, Hans JS, Giovannoni J, Liu Y, Kim CK, Lim KB, Chung JD. 2010. Modest calcium increase in tomatoes expressing a variant of Arabidopsis cation/H^+ antiporter. Plant Biotech Rep 4:15-21

Conn SJ, Gilliham M, Athman A, Schreiber AW, Baumann U, Moller I, Cheng NH, Stancombe MA, Hirschi KD, Webb AAR, Burton R, Kaiser BN, Tyerman SD, Leigh RA. 2011. Cell-specific vacuolar calcium storage mediated by *CAX*1 regulates apoplastic calcium concentration, gas exchange, and plant productivity in *Arabidopsis.* The Plant Cell 23: 240-257

Connolly EL. 2008. Raising the bar for biofortification: enhanced levels of bioavailable calcium in carrots. Trends Biotechnol 26: 401-413

Hirschi KD.1999. Expression of *Arabidopsis CAX1* in tobacco: altered calcium homeostasis and increased stress sensitivity. The Plant Cell 11: 2113-2122

Kamiya T, Akahori T, Ashikari M, Maeshima M. 2006. Expression of the vacuolar Ca^{2+}/H^+ exchanger, OsCAX1a, in rice: cell and age specificity of expression, and enhancement by Ca^{2+}. Plant and Cell Physiology 47:96-106

Kim KM. 2009. Major character analysis of *CAX*1 (cation exchanger 1) transgenic rice plants *in Vivo*. Korean J. Crop Sci 54: 375-383

Kim KM, Park YH, Kim CK, Hirschi KD, Sohn JK. 2005. Development of transgenic rice plants over expressing the *Arabidopsis* H^+/Ca^{2+} antiporter *CAX*1 gene. Plant Cell Rep 23: 678-682

Manohar M, Shigaki T, Mei H, Park S, Marshall J, Aguilar J, Hirschi KD. 2011. Characterization of *Arabidopsis* (Ca2+)/H(+) exchanger CAX3. Biochemistry 50: 6189-6195

Mäser PS, Thomine JI, Schroeder JM, Ward JM, Hirschi KD, Sze H, Talke IN, Amtmann A, Maathuis FJM, Sanders D, Harper JF, Tchieu J, Gribskov MM, Persans W, Salt DE, Kim SA, Guerinot ML 2001. Phylogenetic relationships within cation transporter families of *Arabidopsis.* Plant Physiol 126:1646-1667

Mei H, Zhao J, Pittman JK, Lachmansingh J, Park S, Hirschi KD. 2007. *In planta* regulation of the *Arabidopsis* Ca^{2+}/H^+ antiporter CAX1. Journal of Experimental botany 58:3419-3427

Morris J, Hawthorn KM, Hotze T, Abrams SA, Hirschi KD. 2008. Nutritional impact of elevated calcium transport activity in carrots. Proc Nati Acad Sci USA 105:1431-1435

Park S, Elless MP, Park J, Jenkins A, Lim W, Chambers E 4th, Hirschi KD. 2009. Sensory analysis of calcium-biofortified lettuce. Plant Biotechnol J 7:106-117

Park SH, Kim CK, Pike LM, Smith RH, Hirschi KD. 2004. Increased calcium in carrots by expression of *Arabidopsis* H^+/Ca^{2+} transporter. Mol Breed 14:275-282

Park SH, Cheng NH, Pittman JK, Yoo KS, Park JG, Smith RH, Hirschi KD. 2005a. Increased calcium levels and prolonged shelf life in tomatoes expressing *Arabidopsis* H^+/Ca^{2+} transporters. Plant Physiology 139:1194-1206

Park SH, Kang TS, Kim CK, Han JS, Kim SG, Smith RH, Pike LM, Hirschi KD. 2005b. Genetic manipulation for enhancing calcium content in potato tuber. J. Agri Food Chem 53: 5598-5603

Schafer SL, Barrett WC, Kallarakal AT, Mitra B, Kozarich JW, Gerlt JA, Clifton JG, Petsko GA, Kenyon GL. 1996. Mechanism of the reaction catalyzed by mandelate racemase; structure and mechanistic properties of the D270N mutant. Biochemistry 35: 5662-5669

Shigaki T, Cheng NH, Pittman JK, Hirschi KD.2001. Structural determinant of Ca^{2+}

transport in the *Arabidopsis* H^+/Ca^{2+} antiporter*CAX*1. J. Biol. Chem. 276: 43152-43159

Shigaki T, Hirschi KD. 2000. Characterization of CAX-like genes in plants; implications for functional diversity. Gene 257:291-298

Shigaki T, Hirschi KD. 2006. Diverse functions and molecular properties emerging for CAX cation/H+ exchangers in plants. Plant Biol (Stuttg) 8:419–429

Shigaki T, Sreevidya C, Hirschi KD. 2002. Analysis of the $Ca2^+$ domain in the Arabidopsis H^+/Ca^{2+} antiporters CAX1 and CAX3.Plant Mol Biol50:475-483

Zhao J, Connorton JM, Guo YQ, Li X, Shigaki T, Hirschi KD, Pittman JK. 2009. Functional studies of spilt *Arabidopsis* Ca^{2+}/H^+ exchangers. J. Biol. Chem 284: 34075-34083

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