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In Silico Analysis of Expression Data for Identification of Genes Involved in Spatial Accumulation of Calcium in Developing Seeds of Rice

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Abstract

The calcium (Ca^{2+}) transporters, like Ca^{2+} channels, Ca^{2+} ATPases, and Ca^{2+} exchangers, are instrumental for signaling and transport. However, the mechanism by which they orchestrate the accumulation of Ca^{2+} in grain filling has not yet been investigated. Hence the present study was designed to identify the potential calcium transporter genes that may be responsible for the spatial accumulation of calcium during grain filling. *In silico* expression analyses were performed to identify Ca^{2+} transporters that predominantly express during the different developmental stages of *Oryza sativa*. A total of 13 unique calcium transporters (7 from massively parallel signature sequencing [MPSS] data analysis, and 9 from microarray analysis) were identified. Analysis of variance (ANOVA) revealed differential expression of the transporters across tissues, and principal component analysis (PCA) exhibited their seed-specific distinctive expression profile. Interestingly, Ca^{2+} exchanger genes are highly expressed in the initial stages, whereas some Ca^{2+} ATPase genes are highly expressed throughout seed development. Furthermore, analysis of the cis-elements located in the promoter region of the subset of 13 genes suggested that Dof proteins play essential roles in regulating the expression of Ca^{2+} transporter genes during rice seed development. Based on these results, we developed a hypothetical model explaining the transport and tissue specific distribution of calcium in developing cereal seeds. The model may be extrapolated to understand the mechanism behind the exceptionally high level of calcium accumulation seen in grains like finger millet.

Introduction

Calcium (Ca^{2+}) is a divalent cation that is among the most versatile in eukaryotic organisms, and one that has sparked considerable interest in recent years. It has roles in nearly all plant development processes, and participates in many regulatory and signal transduction processes, such as responses to various stresses, hormones, and pathogens (Jörg Kudla et al., 2010). These calcium-mediated cellular responses are brought about through transient elevations in cytosolic Ca^{2+} concentrations, which are sensed by Ca^{2+} binding proteins that decode and relay the information encoded by Ca^{2+} signatures into specific protein-protein interactions, defined as phosphorylation cascades or transcriptional responses (Batistic and Kudla, 2004; Finkler et al., 2007; Luan et al., 2002; Sanders et al., 2002). Since, Ca^{2+} can easily form complexes with proteins, membranes, and organic acids, and can readily form insoluble complexes with phosphate (as seen in ATP), cells require efficient transport systems to carefully regulate Ca^{2+} concentrations in different cellular compartments. Cytosolic calcium concentrations are tightly regulated by three classes of membrane transporters, namely Ca^{2+} channels (Berridge et al., 2003), Ca^{2+} ATPases (Maclennan et al., 1997; Sze et al., 2000), and cation/Ca^{2+} exchangers, consisting of Na^{+}/Ca^{2+} exchangers (NCX; Blaustein and Lederer, 1999; Philipson and Nicoll, 2000) and H^{+}/Ca^{2+} exchangers (Hirschi, 2001). Following a stimulus, calcium enters cells through calcium channels located in the plasma membrane (PM) or endomembranes (vacuoles) to maintain homeostasis. Calcium is sequestered or pumped back either out of the cell, or to vacuoles, the endoplasmic reticulum (ER), and chloroplasts through the action of active calcium transporters at the plasma membrane or in the secretory pathway (Nagata et al., 2004; Wuytack et al. 2002). Members of the high-affinity Ca^{2+} ATPases belong to two phylogenetic types: (1) type IIA Ca^{2+}-ATPases similar to animal Ca^{2+}-ATPases of the sarcoplasmic or ER; and (2) type IIB Ca^{2+}-ATPases similar to animal calmodulin (CaM)-stimulated Ca^{2+}

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ATPases found in the PM (Askerlund and Sommarin, 1996; Axelsen and Palmgren, 1998; Evans and Williams, 1998). Calcium channels display selective permeability to calcium ions, although there are also ligand-gated calcium channels. Type IIA and type IIB Ca^{2+}-ATPases are found both in endo-membranes and in the PM, and can coexist in the same membrane system (Evans, 1994; Geisler et al., 2000; Sanders et al., 1999). Ca^{2+}/H^{+} exchange is driven by the H^{+} gradient at the membrane, which is generated by specific proton pumps, such as H^{+}-ATPases. Several cDNAs from different plants encoding these transporter proteins have been identified and cloned. However, understanding of the biological roles of these transporters in cell growth and in response to environmental stresses is only beginning to emerge.

Calcium not only plays important roles in mediating cellular signaling, it also accumulates as a principal cation in the grains of many monocots and dicots during grain filling. This accumulated Ca^{2+} is later used in the activation of many hydrolytic enzymes and signal transduction pathways during germination. Elemental accumulation is a complex process that impacts almost every aspect of plant growth, development, and survival (Baxter, 2009). At the whole-plant level, it has been well documented that there is a complex interplay among various ions. Ca^{2+} and other cations such as PO_4^{3-}, Mn^{2+}, Zn^{2+}, and Mg^{2+} can accumulate to millimolar levels in the vacuole, whereas the concentrations of these cations are maintained in the micromolar range in the cytosol (Cheng et al., 2005).

Although much of the research efforts have been directed toward understanding the role of calcium in controlling diverse biological processes, the uptake, assimilation, and accumulation of calcium in developing cereal grains is not clearly understood. The reports published so far suggest that the accumulation of calcium in the seed is governed by both environmental and genetic factors. We recently found that in developing finger millet grains, calcium is mainly deposited in the aleurone layer of the seed, leading to the assumption that there may be specific calcium transporters operative in the seed to carry out such differential spatial distribution (Nath et al., 2010). However, molecular studies so far conducted have primarily focused on calcium exchangers (CAXs) as drivers of Ca^{2+} accumulation in plant tissues, and engineering CAXs is thought to have great potential in biofortification (Hirschi, 2001). For example, overexpressing CAX1 or CAX3 and CAX1/CAX3 genes in *Arabidopsis* increased concentrations of Ca in both the seed coat and the embryo (Punshon et al., 2012). In another study, the double mutants of these genes, rather than displaying a severe reduction in growth, showed increased concentrations of PO_4^{3-}, Mn^{2+}, and Zn^{2+}, and reduced concentrations of Ca^{2+} and Mg^{2+} in shoot tissue (Cheng et al., 2005). Similarly, overexpressing *Arabidopsis* CAX1 in rice has been shown to increase seed calcium content by 2.4% without affecting the physicochemical properties of the endosperm such as amylose, protein, and lipid content (Yi et al., 2012). This indicates that engineering plants with calcium exchangers could be an effective strategy to increase calcium content in cereal grains. However, CAXs represent only one class of Ca transporters, whose members have been reported to contribute to the accumulation of calcium during grain filling. Therefore, it is pertinent to study the expression patterns of all the Ca transporter genes during grain filling that would allow us to better understand the accumulation and distribution of calcium inside the seed. Since, calcium is an important macronutrient in the human diet, and the accumulation of calcium in some cereal grains like finger millet is high, it is necessary to understand the molecular basis of the high calcium levels seen in cereal grains. This knowledge will enable us to formulate effective strategies to increase the levels of calcium in grains. Therefore, in the present investigation rice whole genome gene expression data have been studied to identify possible candidate genes that may be responsible for the accumulation of calcium in the developing grain.

Materials and Methods

In silico *expression analysis of calcium transporters*

Using MPSS signature sequences. In order to overcome sample-to-sample variability and experimental platform-related artefacts, we utilized two different measures of expression: MPSS and microarray. While microarray is hybridization-based, MPSS is based on nucleotide sequencing technology. For MPSS, the rice expression atlas (http://mpss.udel.edu/rice; Nakano et al., 2006) is hosted at the University of Delaware website. In this atlas 17-nucleotide-long signature sequences were used for viewing the expression level of calcium transporters in tissue-specific libraries. The tissues and developmental stage expression libraries selected were: NYR, 14 days; Young Roots, NRA, 60 days; Mature Roots, Replicate A, NRB, 60 days; Replicate B, NGD, 10 days; Germinating seedlings grown in the dark, NST, 60 days; Stem, NYL, 14 days; Young leaves, NLA, 60 days; Mature Leaves, Replicate A, NLB, 60 days; Mature Leaves, Replicate B, NLC, 60 days; Mature Leaves, Replicate C, NLD, 60 days; Mature Leaves, Replicate D, NPO, Mature Pollen, NIP, 90 days; Immature panicle, NGS, 3 days; Germinating seed, NCA, 35 days; Callus, I9RO, Roots, I9RR, Roots Replicate I9LA, Leaves, I9LB, Leaves Replicate I9LC, Leaves, I9LD, Leaves Replicate I9ME; Meristematic Tissue, PSC, rice developing seeds, 6-day-old cypress high milling (99–1710); PSI, rice developing seeds, 6 days old; Ilpumbyeo, High Taste, PSL; rice developing seeds, 6 days old; LaGrue-Low Milling, PSN rice developing seed, 6 days old; Nipponbare-grain quality control, PSY; rice developing seeds, 6 days old; YR15965Acp33, Low Taste. The MPSS signature sequence counts across the libraries ranging from 0 to >400, and were fetched using the online query system by providing the 31 transporter gene names. Log-transformation, which is a prerequisite step before normalization, only works for values >0. Hence all signature counts were scaled by +1. Quantile normalization was applied subsequent to log transformation, and graphs were plotted for the expression patterns of the transporters. For determining the distinctive expression patterns of calcium transporters, we tested whether the seed tissue could be categorized apart from the rest based on MPSS signature sequence counts of the constituent transporters. This was achieved by using the PCA method. Normalized signature counts for the transporters were imported into the R statistical framework (version 2.13.2; R Development Core Team, 2010), and the principal component loadings were calculated using the *prcomp* command, which uses the singular value decomposition method alternative for numerical accuracy. The principal component loadings were exported out of R for

graphical plotting. SPSS was then used to create a 3D scatterplot using the first three principal components.

Using Affymetrix Gene Chip data. Calcium transporters in rice have been documented in the literature (Goel et al, 2011). To determine their expression patterns across tissues using a microarray platform we used the online repository of gene expression studies, GEO. The GSE6893 dataset available in the Gene Expression Omnibus (GEO) database of the National Center for Biotechnology Information (NCBI) was chosen. This dataset contains rice gene profiles across different vegetative stages, along with panicle and seed developmental stages queried on the Affymetrix Rice Genome array, consisting of a >57,000 probe platform. The strategy was to compare the expression pattern of calcium transporters in the seed tissue versus the other tissues to look for seed-specific calcium transporters as markers for involvement in the development of seed. For this we downloaded the Series Matrix data and imported it into the R (version 2.13.2) statistical framework. The dataset comprising 45 different samples was normalized by log transformation, followed by a quantile normalization procedure (Quackenbush, 2002). We used the Affymetrix array annotation file from NetAffx (http://www.affymetrix.com/analysis/netaffx/probematch) to fetch the probes targeting the 31 calcium transporter genes. Using a Perl script, we parsed the probe annotation column and found 45 Affymetrix probes that corresponded to the known calcium transporters. We found that some of the probes targeted multiple genes, while some genes were targets of more than one probe. We made the probe list unique for downstream analysis, and that resulted in the generation of 41 unique probes targeting 28 out of the 31 calcium transporters. Normalized expression values for these 41 probes were then used for further statistical analyses. As a first step toward studying seed enrichment by calcium transporter genes, we used Z-score transformation to arrive at independent measures of expression values in the seed tissue replicates. For filtering the data, calcium transporter genes were selected that had z-scores ≥ 1.64 and ≥ 1.28 (corresponding to 90% and 80% confidence levels, respectively). To address the question of statistical confidence, we used the comparative procedure of ANOVA. We categorized the 45 samples into 5 broad tissue categories (3 root, 6 leaf, 3 SAM, 18 inflorescence, and 15 seed samples). Equality of variances, a condition for the ANOVA test, was found to be true (variance test p value not significant). Using a loop in R, ANOVA was applied to each of the 41 probes across the 5 tissue categories. Strip plots were made for visual interpretation of the ANOVA results.

Promoter element analysis using the PLACE database

Using individual CDS sequences as query, a 1-kb-upstream DNA sequence for each of the 31 calcium transporter genes was extracted by performing BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi). *Cis*-acting regulatory elements within the upstream region were analyzed using PLACE Signal Scan Search software (http://www.dna.affrc.go.jp/PLACE/signalscan.html; Higo et al., 1999). Counts of all forward cis-elements in each of the promoters for the 31 Ca^{2+} transporters and the 13 seed preferential Ca^{2+} transporter genes were summarized by statistical analysis, resulting in cis-elements with higher occurrence frequencies, and these were matched with annotated cis-acting regulatory DNA elements from the PLACE database.

Results

In silico *expression analysis of calcium transporters*

Based on the MPSS database. The rice genome encodes at least 31 calcium transporter genes, comprised of 1 Ca^{2+} channel, 14 Ca^{2+} ATPases, and 16 Ca^{2+} exchangers (Table 1). To study the expression pattern of the calcium transporters using the MPSS database, the mRNA locus numbers for all 31 genes were used for performing bulk queries. No signatures could be detected for three genes (Os11g43860, Os12g42910, and Os03g08230), therefore their expression could not be studied using MPSS. This may be because of the limitations arising in generating the MPSS data for, for example the absence of the signature sequence or the Sau3A site (Nakano et al., 2006). The results of the rice MPSS search are shown in Supplementary Material 1 (see online supplementary material at http://www.liebertonline.com). Though it has an ample number of tissue libraries, the expression atlas lacks replicates for any of them. Hence to be confident of the signature sequence count observed, we applied a threshold criterion of presence in more than one tissue library. To make the MPSS signature sequence counts comparable between libraries, we log transformed the data and performed quantile normalization. We observed that there are only seven calcium transporter genes (i.e., three IIB-type Ca^{2+} ATPases [Os02g08018, Os04g51610, and Os12g39660], two IIA-type Ca^{2+} ATPases [Os03g17310 and Os03g52090], and two Ca^{2+} exchangers [Os01g37690 and Os011g05070]), that were expressed during the seed development stages. Nevertheless, we also observed that those calcium transporter genes that were found to be expressed in the seed were also expressed in the other parts of the plant, including the roots, stem, and leaves. Similarly, the transcript abundance of four genes (Os04g51610, Os12g39660, Os03g17310, and Os01g37690) were found to be high in the immature panicle. The transcript abundance of 7 calcium transporters in seed-specific libraries of the MPSS database is shown in Figure 1.

Based on Rice Affymetrix Gene Chip data. Affymetrix Gene Chip microarray data (GSE6893) on the expression profiling of rice, available at GEO of the NCBI, was used to study the calcium transporter genes in four vegetative stages, as well as the panicle (stage P1–P6), and seed (stage S1–S5) developmental stages. The dataset was comprised of 45 samples, including 3 replicates each from different developmental stage or tissue type. A meta-analysis was performed by quantile normalization of signal values followed by Z-score conversion. Using the array annotation available from the NetAffx web resource (http://www.affymetrix.com/analysis/index.affx), probes targeting the known calcium transporters were determined. Of the 31, only 28 calcium transporters had probes available on the microarray platform. Thus the expression of three calcium transporter genes (Os02g08018, Os03g01330, and Os12g42910) could not be studied using this strategy. The Z-scores of all 28 transporter genes in triplicate for each developmental stage are provided in Supplementary Material 2 (see online supplementary material at http://www.liebertonline.com). At the 90% confidence level for the Z-scores (i.e., $Z \geq 1.68$), only 5 calcium

Table 1. Calcium Transporter Genes of Rice

S. no.	Gene	Function	Cellular localization
1	Os01g48680	Two pore calcium channel	Plasma membrane
2	Os03g17310	Calcium ATPase2, IIA type	Endoplasmic reticulum (ER)
3	Os03g52090	Calcium ATPase3, IIA type	Endoplasmic reticulum
4	Os05g02940	Calcium l ATPase2, IIA type	Endoplasmic reticulum
5	Os01g71240	Calcium ATPase 11,IIB type	Plasma membrane
6	Os02g08018	Calcium ATPase10, IIB type	Plasma membrane
7	Os03g42020	Calcium l ATPase2, IIB type	Chloroplast
8	Os03g10640	Calcium l ATPase2, IIB type	Plasma membrane
9	Os04g51610	Calcium l ATPase9, IIB type	Plasma membrane
10	Os05g41580	Calcium l ATPase4, IIB type	Plasma membrane
11	Os08g40530	Calcium l ATPase9, IIB type	Plasma membrane
12	Os10g28240	Calcium l ATPase13, IIB type	Plasma membrane
13	Os11g04460	Calcium l ATPase4, IIB type	Plasma membrane
14	Os12g04220	Calcium l ATPase4, IIB type	Plasma membrane
15	Os12g39660	Calcium l ATPase2, IIB type	Plasma membrane
16	Os01g11414	Calcium exchanger	Plasma membrane
17	Os01g37690	Cation/proton exchanger 1a	Plasma membrane
18	Os02g04630	Sodium/calcium exchanger l 4	Plasma membrane
19	Os02g21009	Cation/proton exchanger 1c	Vacuole
20	Os02g43110	Sodium/calcium exchanger 1	Plasma membrane
21	Os03g01330	Sodium/potassium/calcium exchanger 1	Nucleus
22	Os03g08230	Calcium exchanger	Plasma membrane
23	Os03g27960	Cation/proton exchanger 2	Plasma membrane
24	Os03g45370	Calcium l exchanger	Chloroplast
25	Os04g55940	Cation/proton exchanger 3	Plasma membrane
26	Os05g51610	Cation/proton exchanger 1b	Plasma membrane
27	Os10g30070	Sodium/potassium/calcium exchanger 6	Plasma membrane
28	Os11g01580	Sodium/potassium/calcium exchanger 6	Chloroplast/ER
29	Os11g05070	Sodium/potassium/calcium exchanger 6	Plasma membrane
30	Os11g43860	Calcium l exchanger	Vacuole
31	Os12g42910	Calcium l exchanger	Plasma membrane

transporter genes, comprised of 2 calcium exchangers, 2 ER-type Ca^{2+} ATPases, and 1 PM-type ATPase, were found to express differentially and at higher levels during developing stages of seed. Further, 4 additional calcium transporter genes (2 exchangers and 2 PM-type ATPases) were identified at the 80% confidence level (i.e., $Z \geq 1.28$). Five out of these 9 transporters (1 exchanger, 2 ER-type, and 2 PM-type ATPases) were found to be expressed up to the S5 stage. The remaining 3 exchanger and 1 PM-type ATPase were found to be expressed highly in the initial stages (S1 and S2) of seed development, but declined in the subsequent stages. Expression patterns of the 9 calcium transporter genes expressed during the S1–S5 seed developmental stages are shown in Figure 2, and the expression values (Z-scores) of these same genes are shown in Table 2.

Statistical analysis of MPSS data

MPSS data were analyzed to test the presence of seed-specific characteristics in the expression pattern of the calcium transporters. The hypothesis proposed was that if these genes had a distinct seed-specific expression pattern, this could act as a classifier to categorize the seed tissue and distinguish it from the rest of the tissues. Our selected MPSS data has 28 calcium transporter genes spread across 26 different libraries belonging to 9 tissue types, namely callus, leaf, meristematic, panicle, pollen, root, seedling, seed, and stem. We henceforth applied PCA to categorize the tissues (as factors) on the basis of constituent gene expression (tag counts; as cases). MPSS signature counts ranged from <5 to >400 across the libraries. We applied log transformation to scale the data and make tag signature counts comparable for every gene. Five of the transporters (Os11g04460, Os12g04220, Os02g43110, Os03g01330, and Os11g01580) were excluded, as they had 0 tag count across all the libraries, and hence were of no use in testing a classifier. We used the *prcomp* command in the R statistical package to calculate the principal components. The first three components, PC1, PC2, and PC3, could cumulatively explain $>50\%$ of the variation in the data. From the scree plot it is also evident that the first three components are the major contributors to the variation in the data, and the later components have only marginal contributions. Subsequently we extracted the factor scores/rotated data for each of the factors (i.e., tissue library) across the 22 principal components (for 22 genes). We used the graphical capability of SPSS to plot a 3D scatterplot of the first three principal components. The data points have been assigned distinct colors for distinct tissue types. The 3D scatterplot of PC1, PC2, and PC3 in the x, y, and z axis, respectively, clearly shows the classification of the tissues on the basis of MPSS tag signature counts (Fig. 3).

Statistical analysis of microarray data

For the five tissue categories we had 45 samples in total (3 root, 6 leaf, 3 SAM, 18 inflorescence, and 15 seed samples).

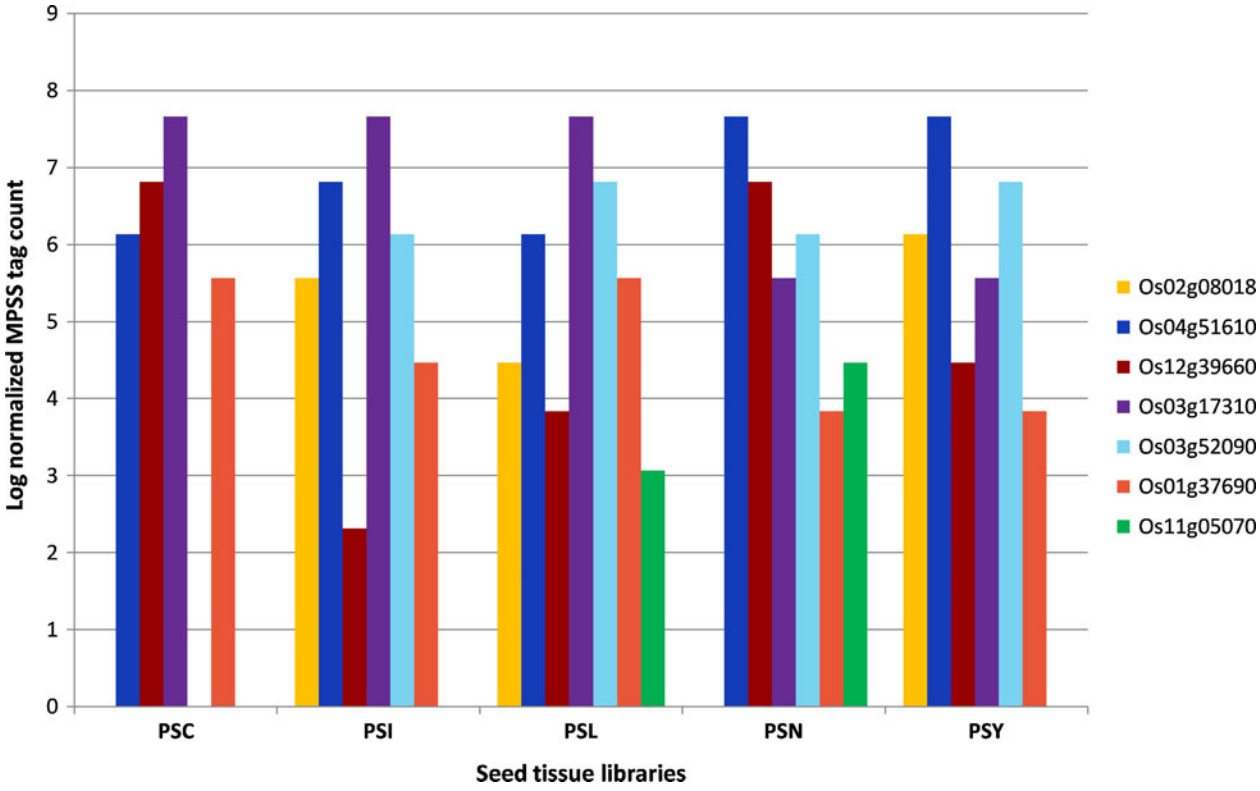

FIG 1. Transcript abundance of 7 calcium transporters in seed-specific libraries of the massively parallel signature sequencing (MPSS) database. Different libraries in the MPSS database were analyzed for the expression level of calcium transporters (PSC: rice developing seeds, 6-day-old cypress high milling (99–1710); PSI: rice developing seeds, 6 days old, Ilpumbyeo, High Taste; PSL: rice developing seeds, 6 days old, LaGrue-Low Milling; PSN: rice developing seed, 6 days old, Nipponbare-Grain quality control; PSY: rice developing seeds, 6 days old, YR15965Acp33-Low Taste).

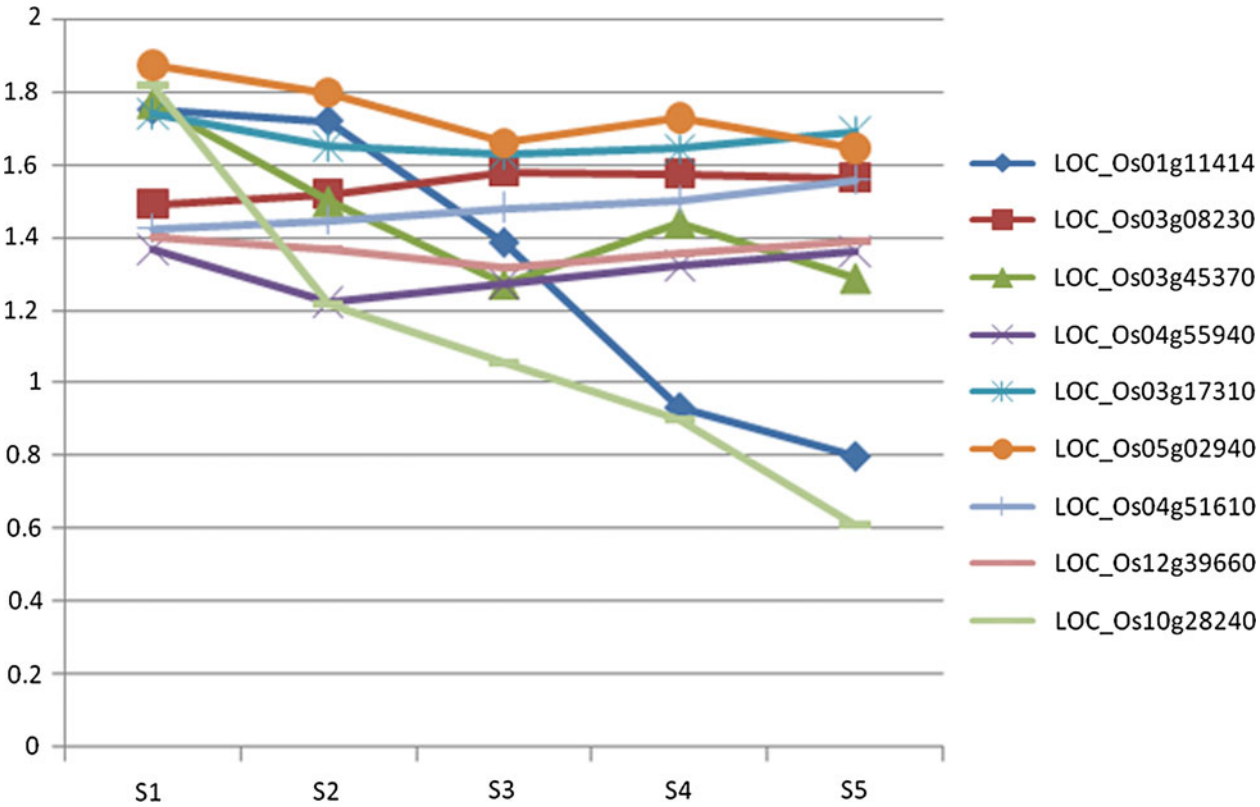

FIG 2. Expression patterns of 9 calcium transporter genes expressed during the S1–S5 seed development stages.

Table 2. Expression Values (Z Scores) of 9 Calcium Transporter Genes in Seed Development Stages S1–S5

Function	*Gene*	*S1*	*S2*	*S3*	*S4*	*S5*
Sodium/calcium exchanger	Os01g11414	1.7491357	1.7184793	1.384826	0.9333046	0.7993739
Sodium/calcium exchanger	Os03g08230	1.4914622	1.5186212	1.5805527	1.5760795	1.5638108
Sodium/calcium exchanger	Os03g45370	1.7687161	1.4999713	1.2723863	1.4416716	1.2886707
Sodium/calcium exchanger	Os04g55940	1.3661351	1.2230504	1.2745208	1.3207957	1.3622256
Calcium-transporting ATPase,ER-type	Os03g17310	1.7382954	1.6498512	1.6255868	1.6410098	1.688297
Calcium-transporting ATPase 2, ER	Os05g02940	1.874983	1.7984433	1.6608688	1.7291708	1.6452644
Calcium-transporting ATPase, PM- type	Os04g51610	1.4245683	1.4472431	1.4820671	1.5042294	1.5581981
Calcium-transporting ATPase	Os12g39660	1.4005476	1.3666201	1.3182601	1.3550484	1.3899052
Calcium-transporting ATPase, PM- type	Os10g28240	1.8154164	1.2162776	1.0537968	0.8983787	0.6078782

Values ≥1.64 have a 90% confidence level, while values <1.64 but ≥1.28 have a 80% confidence level.

Equality of variances, a condition for the ANOVA test, was found to be true (variance test p value not significant). Using a loop in R, ANOVA was applied to each of the 28 probes across the 45 samples. At a threshold of ≤0.05, we found 26 of the 28 transporter genes to be statistically significant for differential expression across the five tissue categories. p Values of all calcium transporters are provided in Table 3. Strip plots (Supplementary Material 3; see online supplementary material at http://www.liebertonline.com) show that many of the calcium transporters are highly expressed either solely in the seed tissue or in combination with other tissues. Note that the different expression patterns of the calcium transporters across the tissues signify different combinatorial control/enhancer elements present upstream of them.

Synthesis of Affymetrix array and MPSS in silico *expression analyses*

Our aim was to comprehensively profile expression patterns of calcium transporters with special emphasis on the seed tissue. Hence datasets were chosen from two different methodologies. After the separate analyses, we decided to synthesize the findings. For this we summarized together the calcium transporter genes found in MPSS and microarray.

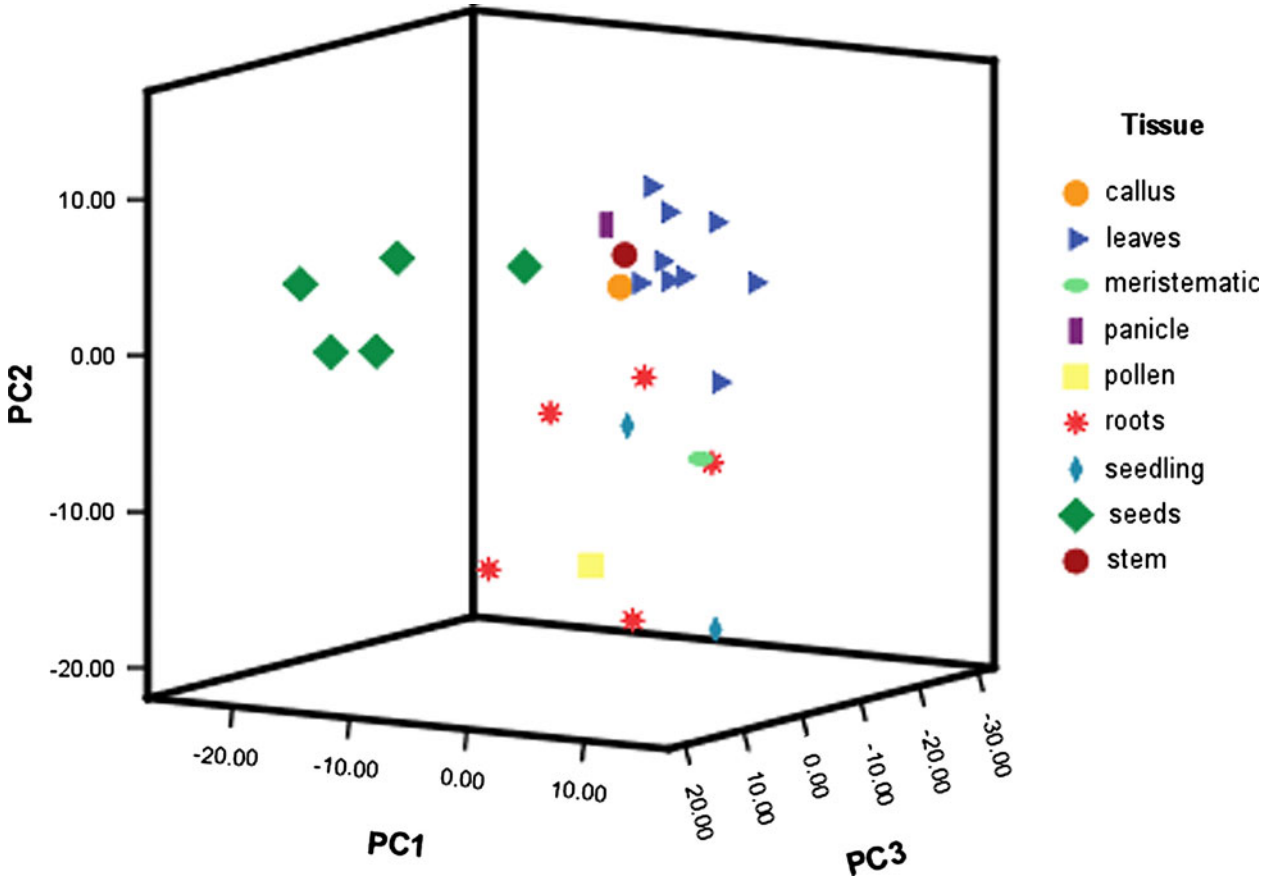

FIG 3. Principal component analysis (PCA) for calcium transporters across different libraries. First 3 principal components segregate the seed tissue from the rest.

Table 3. Analysis of Variance (ANOVA) Results: Affymetrix Probes for Calcium Transporter Genes with Their Test-Statistic and *p* Value

S. no.	*Probe ID*	*Gene name*	*Test statistic*	p *Value*
1	Os.3917.2.S1_x_at	LOC_Os01g11414	14.38657736	2.31E-07
2	Os.19755.2.S1_a_at	LOC_Os01g37690	4.328029932	0.0053
3	Os.27644.1.S1_at	LOC_Os01g48680	44.97214875	2.73E-14
4	Os.34808.1.S1_at	LOC_Os01g71240	1.4354174	0.24006
5	OsAffx.11891.1.S1_at	LOC_Os02g04630	1.99075627	0.11445
6	OsAffx.12141.1.S1_at	LOC_Os02g21009	15.60541628	8.99E-08
7	Os.38169.1.S1_a_at	LOC_Os02g43110	12.10440277	1.54E-06
8	Os.40052.1.A1_s_at	LOC_Os03g08230	4.794898065	0.00296
9	Os.50449.1.S1_at	LOC_Os03g10640	7.318588624	0.00016
10	Os.17526.1.S2_at	LOC_Os03g17310	32.7192058	3.98E-12
11	Os.21823.1.S1_at	LOC_Os03g27960	27.96334259	4.07E-11
12	Os.24860.1.S1_at	LOC_Os03g42020	55.36655114	8.85E-16
13	Os.20354.1.S1_at	LOC_Os03g45370	18.10386619	1.47E-08
14	Os.33998.1.S1_at	LOC_Os03g52090	15.59378951	9.07E-08
15	Os.28422.1.S1_at	LOC_Os04g51610	27.59875456	4.92E-11
16	Os.14873.1.S1_at	LOC_Os04g55940	27.84239741	4.33E-11
17	Os.3895.1.S1_at	LOC_Os05g02940	7.441347659	0.00014
18	Os.27028.1.A1_at	LOC_Os05g41580	10.7127423	5.37E-06
19	Os.27828.1.S1_a_at	LOC_Os05g51610	12.88235793	7.92E-07
20	Os.10351.1.S1_a_at	LOC_Os08g40530	10.88218097	4.59E-06
21	Os.46832.1.S1_at	LOC_Os10g28240	11.22163417	3.37E-06
22	Os.46449.1.S1_at	LOC_Os10g30070	2.613130579	0.04952
23	OsAffx.11747.1.S1_at	LOC_Os11g01580	4.910060547	0.00257
24	OsAffx.18661.1.S1_at	LOC_Os11g04460	22.8238207	7.07E-10
25	Os.55533.1.S1_at	LOC_Os11g05070	7.507926108	0.00013
26	Os.38169.1.S1_a_at	LOC_Os11g43860	14.41441466	2.26E-07
27	OsAffx.18661.2.S1_x_at	LOC_Os12g04220	22.8238207	7.07E-10
28	Os.9632.1.S2_at	LOC_Os12g39660	12.53508297	1.06E-06

From the MPSS dataset, 7 genes (2 type IIA Ca^{2+} ATPases, 3 type IIB Ca^{2+} ATPases, and 2 Ca^{2+} exchangers) had signature sequence counts in at least three seed tissue libraries. On the other hand, in the Affymetrix dataset 9 genes (2 type IIA Ca^{2+} ATPases, 3 type IIB Ca^{2+} ATPases, and 4 Ca^{2+} exchangers) were found with significant expression in the developmental stages of seed. Combining them together we found 13 calcium transporter genes (3 type IIA Ca^{2+} ATPases, 4 type IIB Ca^{2+} ATPases, and 6 Ca^{2+} exchangers; Table 4) to be significantly expressed in seed tissues. Further, we looked into the upstream promoter elements for this subset of genes to find possible seed-specific and stress-related transcription regulatory elements.

Cis-regulatory element analysis of the upstream region of seed-expressed calcium transporter genes

To further explore the transcription regulation of all 31 calcium transporter genes (1 Ca^{2+} channel, 14 ATPases, and 16 Ca^{2+} exchangers), as well as 13 seed-preferential calcium

Table 4. A Total of 13 Calcium Transporter Genes Found by Summarizing Our *In Silico* Expression Analyses of Microarray and MPSS Data

S. no.	*Gene*	*Name*	*Microarray*	*MPSS*
1	Os03g17310	ATPase2, IIA type	✓	✓
2	Os05g02940	ATPase2, IIA type	✓	
3	Os03g52090	ATPase3, IIA type		✓
4	Os04g51610	ATPase9, IIB type	✓	✓
5	Os12g39660	ATPase2, IIB type	✓	✓
6	Os10g28240	ATPase13, IIB type	✓	
7	Os02g08018	ATPase10, IIB type		✓
8	Os01g11414	Exchanger	✓	
9	Os03g08230	Exchanger	✓	
10	Os03g45370	Exchanger	✓	
11	Os04g55940	Cation/proton exchanger 3	✓	
12	Os01g37690	Cation/proton exchanger 1a		✓
13	Os11g05070	Sodium/potassium/ exchanger 6		✓

MPSS, massively parallel signature sequencing.

transporter genes (7 Ca^{2+} ATPases and 6 Ca^{2+} exchangers), common regulatory elements shared by Ca^{2+} ATPase and Ca^{2+} exchangers genes were identified and analyzed (this may be useful for some genes, though it is not available for all genes, as the annotation of most cis-elements is undefined). All identified *cis*-elements in the promoter region of 31 Ca^{2+} transporter genes and their relevant occurrence frequencies were calculated. After comparison of the average frequency of occurrence of each element in the promoters of all 31 Ca^{2+} transporter genes (1 Ca^{2+} channel, 14 Ca^{2+} ATPases, and 16 Ca^{2+} exchangers), the 10 most abundant cis-elements were detected (Table 5). AAAG, the core site required for binding of Dof proteins, was over-represented in all the Ca^{2+} transporter genes, as well as 13 seed-preferential Ca^{2+} transporter genes, with averaged frequencies of occurrence of 6.57 and 4.33 times in ATPases and exchangers, respectively. More than 14 copies of this element were detected in the promoter region of Os01g71240 (type IIB ATPases). Other cis-regulatory elements over-represented in the promoter region of calcium transporter genes are the ones involved in stress, seed, and embryo-specific expression.

Discussion

Calcium content in cereal grains varies from species to species. Rice grains contain 10 mg/100 g of calcium, whereas some genotypes of finger millet contain relatively high amounts of calcium in their seeds (376–515 mg/100 g), which is far above levels seen in other cereals and millets (Barbeau and Hilu, 1993; Panwar et al., 2010). Though calcium is important for maintaining human health, few research efforts have been directed toward understanding the mechanisms of calcium accumulation in developing cereal grains. Research carried out in other plants has indicated that the calcium content in seeds increases from anthesis to ripeness (Butowt et al., 1997; Cabanne and Doneche, 2003). It was thought that high concentrations of seed calcium were due to xylem translocation of a larger proportion of calcium from the tops to the seed (Moraghan and Grafton, 1997; White and Broadley, 2003). Recently, we assessed the spatial distribution of calcium in the developing seed of finger millet using the SEM-DEX technique, and found that calcium content in the aleurone layer and seed coat increases with advancing seed-developing stage (Nath et al., 2012). This indicates the existence of active machinery which pumps calcium into the aleurone layer cells. Since finger millet genomic information is not available at present, and rice shows co-linearity with finger millet, rice could be used as a model system to understand the mechanism of calcium accumulation during grain filling.

In silico *expression analysis and localization of seed-expressed calcium transporter genes*

Using publicly available rice whole genome microarray data and rice MPSS data, 31 rice calcium transporters genes reported in literature were analyzed, which were composed of 1 calcium channel, 14 calcium ATPases (further divided into 3 type IIA and 11 type IIB), and 16 calcium exchangers (further divided into 6 cation/proton exchangers and 10 sodium/calcium exchangers). The expression data of 28 calcium transporter genes screened using Z scores (>90% confidence) showed that 8 transporter genes are significantly expressed during the panicle development and seed development stages, 5 in the root, 3 in the mature leaf, and 2 each in young leaves and SAM. More specifically, based on the expression data only 5 calcium transporter genes, comprised of 3 ATPase and 2 calcium exchangers, are significantly expressed during the S1–S5 seed development stages. However, a credible confidence level of 80% increased the number of calcium

Table 5. The Accession Number (in the PLACE Database), Average Occurrence Frequency (Total Number of Occurred Cis-Elements Divided by That of Promoters), Core Sequence, Putative Function, and Reference of the 10 Most Abundant and Annotated Cis-Elements in Promoters of 31 Calcium Transporter Genes (14 ATPases and 16 Exchangers)

S. No.	*Accession number*	*Elements*	*DNA sequence*	*Average occurrence frequency in ATPases*	*Average occurrence frequency in exchangers*	*Putative function*
1	S000265	DOFCORE	AAAG	7.428571	4.75	Core site required for binding of Dof proteins
2	S000144	E BOX	CANNTG	4.214286	4.1875	Found in storage-protein promoter of seeds
3	S000407	MYCCONSENS	CANNTG	4.214286	4.1875	MYC recognition site found in the promoters of the dehydration-responsive gene
4	S000415	ACGTATERD1	ACGT	2.428571	2.1875	ACGT sequence required for etiolation-induced expression of erd1 (early response to dehydration)
5	S000501	CGCG BOX	VCGCGB	2.142857	2.1875	Ca^{2+}/calmodulin binding box
6	S000176	MYB CORE	CNGTTR	1.928571	1.375	Binding site for MYB
7	S000453	GT1GMSCAM4	GAAAAA	1.357143	0.8125	Found in the promoter of some CaM isoform
8	S000103	SEF4 MOTIF	RTTTTTR	1.142857	0.75	Soybean embryo factor binding site
9	S000292	DPBF CORE	ACACNNG	1.071429	0.8125	DPBF (Dc3 promoter-binding factor-1 and −2) binding core sequence, embryo-specific expression
10	S000453	ABRELATERD1	GAAAAA	0.642857	0.8125	ABRE-like sequence required for etiolation-induced expression of erd1 (early response to dehydration)

transporters expressed during the seed development to 9, now comprised of 4 Ca^{2+} exchangers, and 3 PM-type, and 2-ER type ATPases. Interestingly, the only channel gene present in the rice genome significantly expressed during the P2 stage of panicle development, but not during the seed development stages, indicates that it probably does not have any significant role in transporting calcium into the developing grains, but may have a role during flower development. Further, these Ca^{2+} ATPase and Ca^{2+} exchanger genes expressed during the S1–S5 seed development stages, which show stage-specific expression, indicate their specific roles during specific seed development stages. Interestingly, the exchangers are significantly expressed during the early stages of seed development, which declines as seed development advances to stage S5. Namely, exchanger I is expressed highly in S1 and S2, while exchanger II is expressed only in the S1 stage. Similarly to the expression patterns of the two Ca^{2+} exchanger genes discussed above, one of the three Ca^{2+} ATPases, a PM-type ATPase, is significantly expressed at the S1 stage, but the two ER-type ATPases are highly and significantly expressed throughout seed development stages (S1–S5), indicating that the ER-type ATPases have important roles up to the final seed maturation stage.

ANOVA and PCA analysis to test the seed-specific pattern of calcium transporters

Along with expression analysis of 28 calcium transporter genes, it was further investigated whether these transporter genes differentially express across the tissues, and if so, if their expression levels could be used to differentiate seed tissue from the rest of the tissues studied. This addressed the functional specificity of calcium transporters in contributing to the seed. Using ANOVA, we found that calcium transporters are indeed differentially expressed across the tissues. Of the 28 transporter genes represented on the Affymetrix array, 26 were found to be differentially expressed across the tissues. Interestingly, of the total differentially-expressed transporters, 9 (two IIA and three IIB type calcium ATPases and four calcium exchangers) were expressed highly in seed tissue, either solely or along with other tissues. This signifies that these transporters play a regulatory role in transporting Ca^{2+} in developing seeds, and hence show selective expression patterns. In tissue profiling, a set of genes with a distinctive expression pattern can categorize the concerned tissue when analyzed using classification techniques. We used PCA, a dimensionality reduction method, to test the classification of seed tissue given MPSS signature sequence counts. The first three principal components could explain $>50\%$ of the variance in the data. Though the cumulative amount of variance explained is not significantly high, the principal components could discernibly segregate the seed tissues on the cartesian coordinate space. This is more significant, as we were able to segregate the tissues on the basis of only 23 transporters. The rest were either not present in the MPSS database or had 0 values all across. From the biplot (Supplementary Material 4; see online supplementary material at http://www.liebertonline.com), it is seen that two transporters: Os03g52090, a type IIA calcium ATPase, and Os04g51610, a type IIB calcium ATPase, are most remarkably projected towards the group of seed tissues. This means that these two transporters are comparatively most expressed in seed as compared to other tissues in the MPSS data. Interestingly the microarray probes for both these ATPases (Os.33998.1.S1_at and Os.28422.1.S1_at, respectively) are found to be differentially expressed in ANOVA analysis. Keeping in mind the variability between experimental platforms, we find Os04g51610 to be differentially expressed in the Affymetrix data also.

A hypothetical model for defining the role of various calcium transporter genes involved in calcium accumulation in seeds

Based on the expression analysis of the calcium transporter genes and the data available in the literature, a hypothetical model for the accumulation of calcium in cereal grain seeds can be proposed (Fig. 4). In cereals, after fertilization the 3n endosperm cell initially divides repeatedly without forming a plasma membrane to give rise to the synctium. The nucleus spreads all along the periphery of a large vacuole. In the next stage, known as the cellularization stage, the plasma membrane is formed, enclosing each nucleus. The cells then divide periclinally to form aleurone layer cells on the outside, and endosperm cells to the inside, covering the whole vacuole. The endosperm cells thus formed before undergoing apoptosis actively express seed-specific genes, such as genes of transcription factors, regulatory proteins, and genes responsible for the accumulation of seed storage protein, fatty acid, and carbohydrate. The aleurone cells (also called the aleurone layer) cover the endosperm by forming layers of cells, and stays alive until germination. From the expression data it can be seen that most of the calcium transporter genes expressed in seed are expressed during the initial stages of seed development and decline later, indicating that most of the calcium through the transporters are mobilized in the initial stages of seed development. Members of all three transporters (type IIA and type IIB Ca^{2+} ATPases and calcium exchangers) were present at the S1 stage of seed development, and expression of all the genes declined except the two ER type ATPases, which had high levels of expression until the S5 stage. Since transport cells (TC) at the basal part of the developing endosperm act as an entry point for solutes from the maternal plant to the developing endosperm, we presumed that there must be channels present in the membranes of the TCs. However, since there are no calcium channel genes expressed at this stage, this function may be carried out by either PM-type Ca^{2+} ATPases or exchangers. At the S1 stage 3 PM-type Ca^{2+} ATPases and 4 Ca^{2+} exchangers are expressed, and calcium enters the TCs through some of these transporters present in the plasma membrane of TCs located on the maternal tissue side. ATPases and exchangers present in the PM of TCs on the other side (i.e., towards the syncytium) are responsible for pumping the incoming calcium into the developing coenocyte. When the calcium concentration in the coenocytes increases beyond a certain level, and since calcium cannot move back into the maternal plant, it is pumped into the large vacuole by the action of ER-type ATPases located in the vacuolar membrane. Further, the calcium exchangers may also transport calcium to the developing embryo from the syncytium. In addition to this, the other ER-type ATPases expressed at high levels might also pump calcium into the vacuoles of the developing embryonic cells. Also there may be Ca^{2+} ATPases that have a similar function. At the S1 and S2 stages, one of the PM-type ATPases is expressed at high levels, along with two expressed

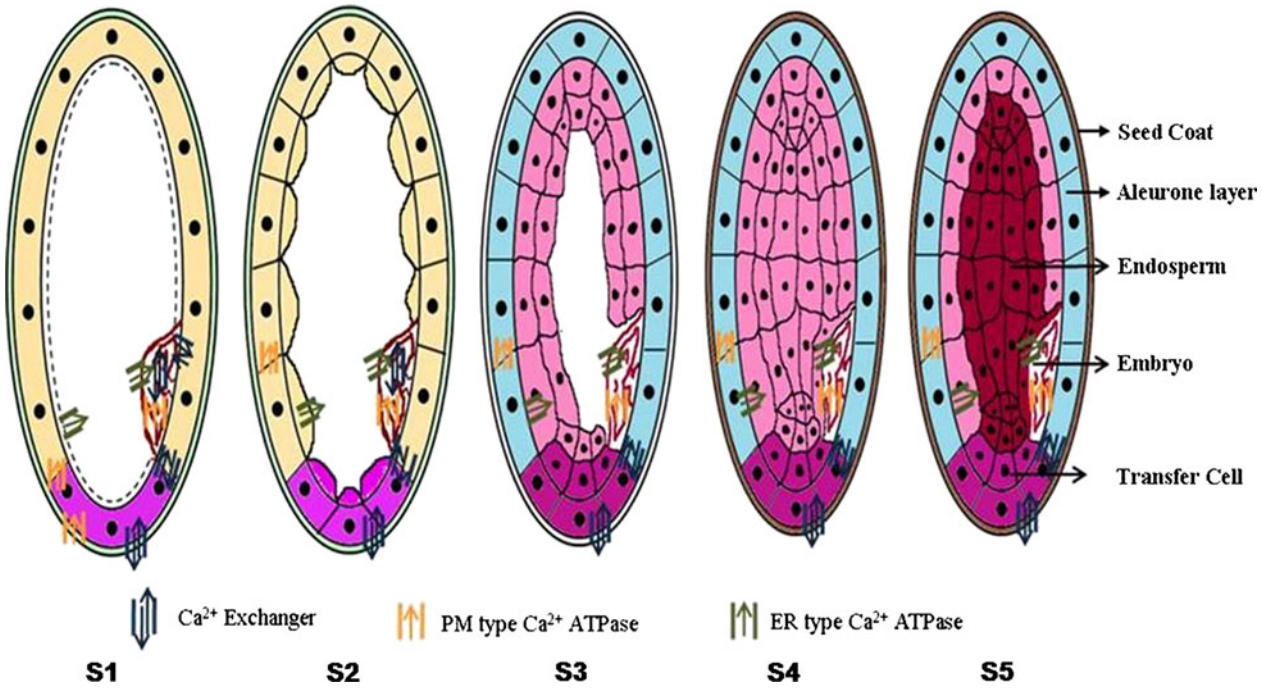

FIG 4. A hypothetical model to show the possible presence of Ca^{2+} transporter genes that may be responsible for calcium accumulation in cereal grains during grain filling.

at lower levels. Since at the S1 stage there is no aleurone layer, higher-expressing ATPase cells at the S1 stage are present in the TCs, and are involved in transporting calcium into the developing coenocytes. The other two ATPases, expressing constantly but at lower levels up to the S5 stage, are probably located on the plasma membrane side towards the aleurone layer, where they pump calcium into the aleurone layer from the beginning of the S3 stage to the end of the S5 stage or until the seed maturation stage. However, further experimentation is needed to confirm whether the increase in calcium content seen in the seed coat is due to transporters present in the aleurone layer towards the seed coat, or from the maternal tissue side.

Transcriptional regulation of seed-expressed calcium transporter genes as deduced from analysis of cis-elements

Transcriptional regulation acts as a key switch during plant developmental processes, and the *cis*-elements of the genes contain crucial information for such regulatory mechanisms. *Cis*-elements of genes provide a predominant mechanism for the generation of novel phenotypes (Doebley and Lukens, 1998), implying unique gene expression specific to one genetic system. Based on the identification of over-represented *cis*-elements in promoter regions of all 31 calcium transporter genes (1 Ca^{2+} channel, 14 Ca^{2+} ATPases, and 16 Ca^{2+} exchangers), as well as the 13 calcium transporter genes (3 type IIA and 4 type IIB Ca^{2+}ATPases and 6 Ca^{2+} exchangers) found to be highly expressed during seed development stages, we found some clues for their transcriptional regulation underlying the coordinated developmental processes of accumulation of calcium in rice seed. The most represented *cis*-element for all 31 calcium transporter genes, as well as the seed-preferential 13 calcium transporter genes, was found to be AAAG, the core site required for binding of unique plant-specific Dof proteins (first found in maize), suggesting an essential and most remarkable role of Dof TFs in regulatory networks controlling calcium accumulation during rice seed development. Interestingly, our observation is quite consistent with our recent finding that as many as 17 Dof transcription factor genes are differentially expressed during the seed development stages, indicating that most of the seed-expressed Ca^{2+} exchangers and Ca^{2+} ATPase genes identified in this study might be the putative targets of Dof transcription factors (Gaur, 2010). Since other Dof transcription factors have been reported to act like master regulators, it is tempting to postulate that Dof transcription factors might control the expression of seed-expressed calcium transporter genes.

Cis-regulatory element analysis also revealed the presence of stress-responsive (such as MYC CONSENSUS, MYBCORE, ACGTATERD1, ABRELATERD1, and CGCG BOX), as well as seed- and embryo-specific regulatory elements (such as E BOX, DPBF CORE, and SEF-4 motif). The presence of these motifs indicates their role in regulating the expression of seed-expressed calcium transporter genes during seed development, and various stresses including desiccation during seed maturity. Embryo-specific regulatory elements present in the promoters indicates that these genes probably also express in the embryo, strengthening our hypothesis that there are ATPases and exchangers responsible for pumping calcium into the developing embryo.

Conclusion

This study presents a hypothetical model aimed at understanding the accumulation of calcium during grain filling in cereal grains using *in silico* analyses of rice expression data

and upstream *cis*-regulatory elements. Since calcium is transported across membranes through calcium transporters, the calcium transporter genes expressed during the grain filling stages must be responsible for calcium accumulation in grains. Gene expression analysis revealed 13 seed-preferential calcium transporter genes (7 ATPases and 6 exchangers) expressed during grain filling. Based on our analysis and the available literature, how and where these ATPases and exchangers express and function in transporting calcium into the developing grain is presented in our hypothetical model. However, the present model is probabilistic, and experimental evidence like gain- or loss-of-function studies are required to validate the hypothesis. Keeping in mind the question of high calcium content, parallel studies on rice, sorghum, and finger millet, have been undertaken to study the expression pattern of calcium transporters across different tissues including seed. Such a combined approach of *in silico* analyses and experimental follow-up will lead to comprehensive understanding of the crucial events of seed development in cereal grains.

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