

Research

Analysis of EF-hand-containing proteins in *Arabidopsis*

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Abstract

Background: In plants, calcium (Ca^{2+}) has emerged as an important messenger mediating the action of many hormonal and environmental signals, including biotic and abiotic stresses. Many different signals raise cytosolic calcium concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$), which in turn is thought to regulate cellular and developmental processes via Ca^{2+} -binding proteins. Three out of the four classes of Ca^{2+} -binding proteins in plants contain Ca^{2+} -binding EF-hand motif(s). This motif is a conserved helix-loop-helix structure that can bind a single Ca^{2+} ion. To identify all EF-hand-containing proteins in *Arabidopsis*, we analyzed its completed genome sequence for genes encoding EF-hand-containing proteins.

Results: A maximum of 250 proteins possibly having EF-hands were identified. Diverse proteins, including enzymes, proteins involved in transcription and translation, protein- and nucleic-acid-binding proteins and a large number of unknown proteins, have one or more putative EF-hands. Phylogenetic analysis identified six major groups that contain some families of proteins.

Conclusions: The presence of EF-hand motif(s) in a diversity of proteins is consistent with the involvement of Ca^{2+} in regulating many cellular and developmental processes. Thus far, only 47 of the possible 250 EF-hand proteins have been reported in the literature. Various domains that we identified in many of the uncharacterized EF-hand-containing proteins should help in elucidating their cellular role(s). Our analyses suggest that the Ca^{2+} messenger system is widely used in plants and that EF-hand-containing proteins are likely to be the key transducers mediating Ca^{2+} action.

Background

Organisms must be able to respond to their environment to survive. In plants, mechanisms have evolved for sensing and responding to hormonal and environmental signals, both biotic (for example, pathogens) and abiotic (for example, heat, cold, light and salt/drought stresses). To elicit a response, the perceived signal must be conveyed to the cellular machinery. Messengers such as Ca^{2+} , cyclic nucleotides (cAMP and cGMP), hydrogen peroxide (H_2O_2) and nitric oxide transduce the perceived stimulus to proteins that

initiate a response. Ca^{2+} is one of the important messengers that mediate plant responses to hormones, developmental cues and external stimuli [1-3]. It is implicated in regulating such diverse and fundamental cellular process as cytoplasmic streaming, thigmotropism, gravitropism, cell division, cell elongation, cell differentiation, cell polarity, photomorphogenesis and plant defense and stress responses [1,3,4]. The Ca^{2+} concentration in the cytoplasm ($[\text{Ca}^{2+}]_{\text{cyt}}$) is maintained in the nanomolar range (approximately 100-200 nM), whereas the concentration in organelles and cell wall is in

the millimolar range [1,3,5,6]. Several signals (hormonal, abiotic and biotic) have been shown to cause transient elevation of $[Ca^{2+}]_{\text{cyt}}$ [1,2,5-7]. This transient increase in $[Ca^{2+}]_{\text{cyt}}$ is sensed by Ca^{2+} -binding proteins [4,8,9]. The conformation of the Ca^{2+} -binding protein changes on binding Ca^{2+} , resulting in modulation of its activity or its ability to interact with other proteins or nucleic acids and modulate their function or activity. The Ca^{2+} sensors in plants can be broadly divided into four major classes [2,9]: calmodulin (CAM) (class A), CAM-like and other EF-hand-containing Ca^{2+} -binding proteins (class B), Ca^{2+} -regulated protein kinases (class C) and Ca^{2+} -binding proteins without EF-hand motifs (class D).

Three classes (A, B, and C) contain proteins with EF-hand motifs. This motif is a helix-loop-helix structure that binds a single Ca^{2+} ion [10]. The loop consists of 12 residues with the pattern $X^*Y^*Z^*-Y^*-X^{**}-Z$. The residues X, Y, Z, -Y, -X, -Z participate in binding Ca^{2+} and the intervening residues are represented by asterisks (*). Asp or Asn is usually found at X and Y; Asp, Asn, or Ser at Z; a variety of residues at -Y; usually Asp, Asn, or Ser at -X, but this position is more variable; and usually Glu at -Z [11]. The helix-loop-helix is only 29 residues long, the E α -helix being residues 1-10, the loop 10-21, and the F α -helix 19-29 [11]. Residue 1 is often Glu (E), and a Gly at residue 15 is highly conserved, as is Ile at residue 17. It has been reported that some of the EF-hand domains do not bind Ca^{2+} [11].

In quiescent cells, proteins with EF-hands are in an apo-protein form; when $[Ca^{2+}]_{\text{cyt}}$ increases they bind Ca^{2+} and change their conformation. Some EF-hands can also bind Mg^{2+} (for example, the third and fourth EF-hands of troponin C bind Ca^{2+}/Mg^{2+} , whereas the first and second EF-hands are Ca^{2+} specific [12]). Ca^{2+}/Mg^{2+} discrimination relies on the affinities of the EF-hands for these cations, which is dependent on the types of amino-acid residues in the binding loop [11,13].

EF-hands can be present in proteins with no other known domains, as is the case for CAM, or in proteins with other domains such as a protein kinase. In most cases EF-hand motifs are found in pairs, and proteins with four EF-hands usually have two domains with a pair of EF-hands in each. Calpain is an exception to the pairing rule. It comprises a large subunit with five EF-hands at the carboxyl terminus and a small subunit that also has five EF-hands. The two unpaired hands in these subunits pair to form a heterodimer [14]. The large superfamily of EF-hand proteins has been divided into 66 subfamilies on the basis of differences in number and organization of EF-hand pairs, amino-acid sequences within or outside the motifs, affinity for Ca^{2+} and/or selectivity and affinity for target proteins [11]. Of the subfamilies, 28 consist of a unique single member. The EF-hand proteins used in the classification by Nakayama *et al.* [11] include proteins from animals, plants, fungi and protists, with plants represented in only nine of the 66 subfamilies.

Several EF-hand proteins have been identified in the model plant *Arabidopsis thaliana*, including several CAMs [15-18], a Ca^{2+} -binding protein (CaBP-22)[19], touch-induced proteins TCH2 and TCH3 [20], centrin [21], Ca^{2+} -dependent protein kinases (CPKs) [22], calcineurin B-like proteins/salt-overly-sensitive3 family (CBLs/SOS3) [23,24], fimbrins [25], respiratory burst oxidase homologs (Rboh) [26,27], a phospholipase [28,29], channel proteins [30,31], a NAD(H)-dependent glutamate dehydrogenase [32], a protein phosphatase [33,34], a NaCl-inducible protein [35], and a Ca^{2+} -binding protein in pollen [36]. Some have been identified by screening with animal homologs whereas others have been identified by sequencing the genes induced in response to biotic and abiotic signals. The first method would miss plant-specific EF-hand proteins and the second method relies on comprehensive analysis of all genes that might be induced or activated by various signals. With the recent completion of the sequencing of the *A. thaliana* genome, the first plant genome to be sequenced, new methods of identifying genes encoding proteins with specific domains have become possible [37]. Insight into the function of the proteins can be gained by identifying and characterizing EF-hand proteins encoded in the *Arabidopsis* genome. Classification of the EF-hand-containing proteins can be a starting point in identification of Ca^{2+} -binding proteins that might be involved in a particular cell process. With this in mind, we searched the *Arabidopsis* genome for genes encoding proteins with EF-hand motifs. We used three approaches to identify EF-hand-containing proteins in *Arabidopsis*. First, we analyzed data from the Munich Information Center for Protein Sequences (MIPS) *A. thaliana* database (MAtdB) [38,39]; second, we carried out BLAST searches using different known EF-hand sequences (nucleotide and amino acid) against the *Arabidopsis* genome database and third, we searched the literature. We identified 250 EF-hand or putative EF-hand proteins. This estimate represents a maximum number of possible EF-hand-containing proteins in the *Arabidopsis* genome as our analysis was very inclusive. Of these 250 proteins, only 47 have been reported in the literature. Of the 250, 73 were identified by only one prediction program as having an EF-hand and the rest were identified by two or more programs. Further study is needed to verify Ca^{2+} -binding activity of many of these proteins. Each protein sequence was analyzed for domains other than EF-hands. Several have a variety of domains, which may be useful in determining protein function.

Results and discussion

Identification of EF-hand-containing proteins

To identify EF-hand-containing proteins in *Arabidopsis*, the protein sequences listed as having EF-hands in the InterPro Domain Table at MAtdB [39] were retrieved. Each protein sequence was then analyzed for the presence of an EF-hand motif and other domain(s) using InterProScan [40]. There are many databases for analyzing proteins using different

approaches to search for patterns, profiles and hidden Markov models [41]. InterProScan was chosen because it integrates SWISS-PROT, PROSITE, PRINTS, Pfam, ProDom, SMART and TIGRFAMs programs into a single comprehensive format. Therefore, scanning one site is the equivalent of scanning seven databases that use different approaches [40]. The InterPro Domain Table at MAtDB listed 219 proteins as having EF-hands. Eighteen sequences did not have EF-hands identifiable by InterProScan and so were eliminated from our analysis. We also did sequence-similarity searches using three different EF-hand proteins that have been characterized in *Arabidopsis*. The nucleotide and protein sequences of *Arabidopsis* CAM4, a protein containing four EF-hands, were used to do BLAST searches (TblastN, BlastP) against the *Arabidopsis* genome at MAtDB [39]. We also used the protein sequences of a Ca²⁺-dependent protein kinase (CPK1) and a small protein with one EF-hand domain (At2g46600). Proteins showing similarity to these proteins were checked for the presence of EF-hands using InterProScan as above. Additional EF-hand proteins were found that had not been included in the MAtDB InterPro domain table. We also searched the literature for reports of EF-hand-containing proteins in *Arabidopsis* that had been identified by various experimental approaches. Additional EF-hand proteins were identified from this search. Together, these searches resulted in identification of a possible total of 250 EF-hand-containing proteins (Tables 1-3). Seventy-three of the EF-hands were identified by only one of the seven prediction programs included in InterProScan. These proteins, which are indicated in bold in Table 1, could be false positives. Further studies are needed to verify the Ca²⁺-binding ability of these putative EF-hands. It is, however, worth noting that the activity of two of the proteins in this category (AtPLC1 and KCO1) has been shown to be dependent on Ca²⁺ [31,42]. All proteins are listed by their protein ID number except CAM6, which has not been assigned an ID number. CPKs and closely related CRKs (CPK-related protein kinases) are listed in Table 2, as this is a large family of proteins that has been relatively well studied in *Arabidopsis*.

The InterPro domain table also lists the EF-hand-containing proteins for *Saccharomyces cerevisiae* (29), *Caenorhabditis elegans* (139) and *Drosophila melanogaster* (132). The number of EF-hand proteins in the human genome was given as 83, with a note that the number may be an underestimate as a result of the stringent *E*-value cutoff used for the analysis [43]. Figure 1a shows a comparison of the number of EF-hand-containing proteins in sequenced eukaryotic organisms and the percentage of the total number of genes represented by genes encoding EF-hand proteins. Our analysis revealed that there is possibly a very large number of EF-hand proteins in *Arabidopsis*.

We used TargetP [44] to identify cellular targeting signals in all the EF-hand proteins. The results from this analysis show that EF-hand proteins are present in all major subcellular compartments (Tables 1-3).

Ca²⁺-binding proteins with no recognized EF-hand

Table 3 lists proteins that were reported in the literature as proteins containing EF-hand-like domains but where the InterProScan of these proteins did not identify any. However, the proteins were shown to bind Ca⁺. We included proteins with sequence similarity to these proteins in Table 3. We did not include these proteins in the total number of EF-hand proteins nor in the phylogenetic analysis.

Caleosins are proteins with similarity to a rice protein that was shown to bind Ca²⁺ [45]. A localization study in rapeseed, using an antibody to AtClo1, showed the presence of this protein in the ER and lipid bodies [46]. Clo3 was shown to be induced by abscisic acid and to bind Ca²⁺ [47].

The InterPro documentation IPR000308 for 14-3-3 proteins describes them as a large family of proteins that are primarily homo- or hetero-dimeric within all eukaryotic cells. They appear to effect intracellular signaling by regulating the catalytic activity of the bound protein, by regulating interactions between the bound protein and other proteins, or by controlling the localization of the bound protein. The 14-3-3 protein GF14 ω was shown to bind Ca²⁺ and the binding was localized to loop 8 of GF14 ω [48,49]. Seven other proteins showing strong similarity to GF14 ω have the exact sequence in the loop considered to be the EF-hand and so were included in Table 3. Several other proteins showing similarity were divergent in this loop and so were not included.

SUB1 was identified as a protein involved in the cryptochrome and phytochrome signaling pathways [50]. Guo *et al.* [50] identified two EF-hand-like domains and demonstrated binding of Ca²⁺ by SUB1. SUL1 and 2 are proteins showing similarity to SUB1 but their sequences diverge somewhat in the EF-hand domains.

Number of EF hands

The number of EF-hands in each protein varied from one to six. Figure 1b shows the number and percentage of proteins having a specific number of EF-hands. As stated above, most EF-hand proteins have pairs of EF-hands, which facilitate binding of Ca²⁺ [11]. There are a large number of proteins with an odd number of EF-hand motifs (1, 3 or 5). Several possibilities are suggested by this observation. The proteins with an odd number of EF-hand domains may function as homo- or heterodimers, they may bind Ca²⁺ in a weaker manner, there may be another 'cryptic' Ca²⁺-binding motif that is not identifiable, but is functional, or they may not bind Ca²⁺ at all. Many of the proteins containing a single EF-hand motif were identified by only one prediction program and could be false positives.

Examples of these possibilities can be seen in EF-hand proteins that have been isolated and characterized previously. The K⁺ channel protein (KCO1) has one identifiable EF-hand but another region within the protein also shows similarity

Table 1**EF-hand-containing proteins (excluding CPKs and CRKs) in *Arabidopsis***

ID number [§]	Size (amino acids)	Number of EF hands	Domains*/remarks [†]	Targeting [‡]	Published name	References
Group I						
At1g02150	638	1	PPR repeats, NLS	sc (.616)		
At1g06220	987	2	Elongation factor aEF-2			
At5g25230	973	1	ATP_GTP_A, EFG_C, GTP_EFTU(_D2)			
At1g17090	93	1	-	sc (.944)		
At1g55040	849	1	Zn-finger in Ranbp and others	ch (.823)		
At1g74430	271	1	Myb DNA-binding domain			
At2g03150	1276	1	-	mt (.870)		
At5g40690	210	1	-	mt (.656)		
At3g13500	110	1	-			
At1g69030	320	1	-			
At3g59820	755	1	-	mt (.666)		
At1g73440	254	2	Josephin, UIM	ch (.941)		
At3g07740	548	1	Zinc finger, Myb DNA-binding, GatC			
At5g06260	424	1	-			
At4g00140	257	1	-	ch (.792)		
At1g20760	1019	2	EPS15 repeat, pfkB			
At1g21630	1181	4	EPS15 repeat			
At3g20290	485	2	ATP_GTP_A, EPS15, NLS_BP			
At4g05520	514	1	EPS15 repeat, dynamin family			
At1g47550	861	1	NLS_BP			
At1g47560	1564	1	NLS_BP			
At3g59270	335	1	-			
At5g41840	540	1	F-box domain			
Group II						
At1g02270	484	1	-			
At5g54130	232	1	-			
At5g46830	511	1	bHLH			
At1g05150	808	1	Zinc finger, TPR/put O-GlcNAc transferase			
At2g32450	802	1	Zinc finger, TPR/put O-GlcNAc transferase			
At2g35380	336	1	Plant peroxidase	sc (.960)		
At1g20490	530	1	AMP-dependent synthetase and ligase			
At1g20510	546	1	-	ch (.747)		
At1g21540	550	1	AMP-dependent synthetase and ligase			
At1g23160	578	1	-			
At3g04860	289	1	-	ch (.666)		
At5g28150	289	1	-	ch (.651)		
At1g44780	471	1	NLS_BP			
At3g01780	1192	1	-			
At3g02270	676	1	eIF4-gamma/eIF			
At5g64060	356	1	No apical meristem protein			
At4g05110	394	1	Nucleoside_tran			
At4g05120	418	1	Nucleoside_tran			
At4g05140	419	1	Nucleoside_tran			
At5g22840	538	1	Eukaryotic protein kinase			
At5g12120	619	1	Ubiquitin-associated domain	ch (.811)		
At1g64850	162	1	-			
At3g46220	804	1	Biotin operon repressor			
At3g44330	565	1	-	sc (.697)		
At5g21130	281	1	ATP synthase alpha and beta subunit			
At3g14270	1791	1	FYVE, PIP5K			
At3g32010	603	1	ATHILA			
At3g32880	503	1	ATHILA			

Table 1 (continued)

D number ^s	Size (amino acids)	Number of EF hands	Domains*/remarks [†]	Targeting [‡]	Published name	References
At3g32970	289	1	ATHILA			
At4g08080	561	1	-			
At4g08050	1428	1	-			
At4g03790	1064	1	ATHILA			
At1g80680	1032	1	Aminoacyl-transfer RNA syn class II			
At2g30110	1080	1	UBACT, ThiF_family			
At5g46360	260	1	Potassium channel/KCO1-like			
At5g46370	443	1	Potassium channel/KCO1-like			
At5g55630	363	1 [¶]	Potassium channel	ch (.772)	KCO1	[31]
At2g35800	844	1	Mitoch_carrier			
At5g07320	479	4	Mitoch_carrier, Graves disease			
At5g51050	487	2	Mitoch_carrier, Adptrnscase	ch (.621)		
At5g61810	476	4	Mitoch_carrier			
At1g33790	745	1	Jacalin-related lectin domain			
At5g28340	390	2	PPR repeats			
At5g28380	283	2	PPR repeats			
At5g58670	559	1	PIPLC		AtPLC1	[29,42]
At4g26700	687	1	Calponin homology		ATFIMI	[25]
At5g55400	714	1	Calponin homology			
Group III						
At1g03960	534	1	/protein phosphatase 2A group			
At5g44090	538	2	/protein phosphatase 2A group			[58]
At1g54450	535	2	/protein phosphatase 2A group			
At5g28850	536	3	/protein phosphatase 2A group			
At5g28900	536	3	/protein phosphatase 2A group			
At5g18580	464	3	NLS_BP			
At5g49480	160	3	/NaCl-inducible Ca ²⁺ binding protein-like		AtCPI	[35]
At1g32410	1048	1	PPR repeats	sc (.813)		
At3g18430	175	1	-			
At1g64480	214	3	Recoverin		CBL8	[13]
At5g24270	222	3	-		CBL4	[51]
At4g33000	246	3	Recoverin/CBL-like	sc (.931)		
At4g16350	227 [¶]	3 [#]	-		CBL6	[13]
At5g55990	226	3	-		CBL3	[23]
At4g26560	214	3	Recoverin		CBL7	[13]
At4g26570	226	3	-		CBL2	[23]
At4g17615	252	3	Recoverin		CBL1	[23]
At5g47100	213	2	Recoverin		CBL9	[13]
At4g01420	192	3	-		CBL5	[13]
At1g54530	127	1	-			
At2g44310	142	2	-			
At5g28830	324	2	-			
At5g22760	1516	1	PHD-finger, DDT domain			
At4g38810	375	2	-			
At4g13440	154	2	-			
At4g32060	498	4	-			
At4g39420	781	1	-			
At1g29020	1062	4	-	sc (.939)		
At2g34020	462	2	-	sc (.965)		
At2g34030	423	2	-	mt (.648)		
At1g53210	574	2	-	sc (.907)		
At4g25970	628	2	PS_Dcrbxylase, Porpobil_deam	ch (.849)		
At5g57190	615	4	PS_Dcrbxylase, Porpobil_deam			
At2g46600	135 ^f	1	-		KIC	Unpublished
At4g27280	130	1	-			

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Table 1 (continued)

D number ^s	Size (amino acids)	Number of EF hands	Domains*/remarks [†]	Targeting [‡]	Published name	References
At5g54490	127	1	-			
At3g17470	570	2	-			
At5g62250	549	1	-			
At4g27790	345	6	ER_TARGET	sc (.941)		
At5g08580	391	4	-	sc (.631)		
Group IV						
At1g05990	150	4	-			
At4g03290	154	4	-			
At2g43290	169	4	-			
At3g59440	195	4	-	sc (.844)		
At3g59450	148	1	-			
At3g07490	153	4	-			
At4g12860	152	4	-			
At1g18210	170	4	-			
At1g73630	163	4	-			
At1g66400	157	4	Calflagin			
At5g37770	161	4	-		TCH2	[20]
At2g36180	146	4	-			
At5g17470	146	4	Calflagin			
At3g03400	137	3	-			
At3g03410	131	4	-			
At2g15680	187	4	Recoverin	ch (.725)		
At1g21550	155	3	-			
At4g20780	191	3	-			
At5g44460	181	3	-			
At1g12310	148	3	-			
At1g62820	148	3	-			
At1g66410	149	4	EPS15 repeat		CaM4	[17]
At5g37780	149	4	-		CaM1	[16]
At2g27030	149	4	EPS15 repeat		CaM5	[17]
At2g41110	149	4	EPS15 repeat		CaM2	[16]
At3g56800	149	4	EPS15 repeat		CaM3	[15]
At3g43810	149	4	EPS15 repeat		CaM7	[18]
N/A	149	4	EPS15 repeat		CaM6	[17]
At2g41090	191	4	-		CaBP-22	[17]
At3g22930	173	4	-	mt (.702)		
At4g14640	151	4	-		CaM8	[18]
At2g41100	324	6	-		TCH-3	[20]
At3g51920	151	4	-		CaM9	[18]
At3g50360	169	4	-	mt (.609)	Centrin	[21]
At4g37010	167	4	/caltractin(centrin)-like	mt (.809)		
At1g18530	157	4	Calflagin			
At3g25600	161	4	Calflagin			
At1g32250	166	4	Calflagin, recoverin			
At3g03000	165	4	Calflagin			
At2g41410	216	4	-	ch (.770)	PMI29	[64]
At3g10190	209	4	-			
At3g03430	83	2	Recoverin/pollen allergenBra r II			
At5g17480	83	2	/Calcium-binding protein in pollen		APC1	[36]
At1g76640	159	4	-			
At1g76650	177	3	-			
At3g01830	146	2	-			
At3g50770	205	4	-	ch (.970)		
At5g42380	185	3	-	ch (.742)		

Table 1 (continued)

D number [§]	Size (amino acids)	Number of EF hands	Domains*/remarks [†]	Targeting [‡]	Published name	References
At3g29000	194	2	S-100/ICaBP type CBP	sc (.954)		
At5g39670	193	2	-	sc (.877)		
At3g47480	183	2	Recoverin	sc (.956)		
At1g24620	186	4	Calflagin			
At3g24110	229	2	-			
At4g26470	248	4	-			
Group V						
At2g27480	186	2	-			
At3g10300	330	2	-			
At5g04170	354	2	-			
Group VI						
At1g09090	838	2	Cytb-245 hc, Ferric_reduct/rboh-like		RbohB	[26]
At4g25090	863	2	Cytb-245 hc, Ferric_reduct/rboh-like			
At5g51060	905	1	Cytb-245 hc, Ferric_reduct/rboh-like		RbohC	[26]
At5g07390	902	1	Cytb-245 hc, Ferric_reduct/rboh-like		RbohA	[26]
At5g47910	921	2	Cytb-245 hc, Ferric_reduct/rboh-like		RbohD	[26]
At1g64060	929	1	Cytb-245 hc, Ferric_reduct/rboh-like		RbohF	[26,27]
At1g19230	926	1	Cytb-245 hc, Ferric_reduct/rboh-like		RbohE	[26]
At3g45810	835	1	Cytb-245 hc, Ferric_reduct/rboh-like			
At5g60010	839	2	Cytb-245 hc, Ferric_reduct/rboh-like			
At2g47860	635	1	PTM, NPH3	ch (.809)		
At3g44820	661	1	BTB/POZ domain			
At5g05180	432	1	-			
At5g13260	576	1	-	ch (.666)		
At5g13960	624	1	SET-domain of transcriptional regulators			
At3g05310	648	2	Small GTPase			
At5g27540	648	2	Small GTPase			
At3g63150	676	1	Small GTPase			
At2g20800	582	1	Pyr_redux			
At4g05020	583	1	FAD_pyr_redux			
At4g28220	559	1	Pyr_redux	mt (.880)		
At4g08000	609	1	En/Spm-like transposon proteins			
At4g03560	724	2	Cation (ca,na) pore region, HLH_Myc			
At1g02960	553	1	PTS_HPR_SER, NLS			
At5g59230	186	1	-			
At4g39560	343	1	F-box, Kelch repeat			
At4g26080	434	1	Protein phosphatase 2C subfamily		ABI1	[34,78]
At5g07440	411	1	NAD-binding site, GLFV_dehydrog	mt (.680)	GDH2	[32]
At1g76250	434	1	-	sc (.648)		
At5g44620	519	1	Cytochrome P450 enzyme, HLH_Myc			
At1g01280	510	1	Cytochrome P450 enzyme	sc (.725)		

The table is arranged by groups. *Domains as identified by InterProScan [40]. †Remarks are from MAtdB [39] or the reference. ‡Based on TargetP predictions [44]. Only signals with scores of 0.600 or above were included. mt, mitochondria; ch, chloroplast; sc, secretory pathway. §EF-hand motifs identified by only one database in InterProScan are in bold. ¶Only one EF-hand found by InterProScan but two identified by Czempinski *et al.* [31]. *Kudla *et al.* [23] report different protein sequence whose size and number of EF-hand domains varies from the MIPS database information. #There is a discrepancy between the MIPS database sequence and the experimentally determined size. N/A, not available; Adptrnscase, adenine nucleotide translocator 1; ATHILA, *Arabidopsis* retrotransposon (ATHILA) ORF-1; ATP_GTP_A, ATP/GTP-binding site motif A; bHLH, basic helix-loop-helix dimerization domain; Calflagin, flagellar calcium-binding protein; Cytb-245 hc, cytochrome B-245 heavy chain; EFG_C, elongation factor G, carboxyl terminus; ER_TARGET, endoplasmic reticulum targeting seq; FAD_pyr_redux, FAD-dependent pyridine nucleotide-disulfide oxidoreductase; Ferric_reduct, ferric reductase-like transmembrane component; FYVE, FYVE An-finger, rabphilin/VPS27/FABI type; GatC, glutamyl-tRNA amidotransferase C subunit; GLFV_dehydrog, glutamate/leucine/phenylalanine/valine dehydrogenase; GTP_EFTU_D2, elongation factor Tu domain 2; HLH_Myc, Myc-type helix-loop-helix dimerization domain; Mitoch_carrier, mitochondrial energy transfer proteins; NLS_BP, nuclear localization signal, bipartite; Nucleoside_tran, delayed-early response protein/equilibrative nucleoside transporter; PIP5K, phosphatidylinositol-4-phosphate-5-kinase; PIPLC, phosphatidylinositol-specific phospholipase; pfkB, pfkB family of carbohydrate kinases; Porpobil_deam, porphobilinogen deaminase; PS_Dcrbxylase, phosphatidylserine decarboxylase; PTM, phosphopantetheine attachment site; PTS_HPR_SER, serine phosphorylation site in HPR protein; Pyr_redux, pyridine nucleotide-disulfide oxidoreductase; TM, transmembrane; ThiF_family, UBA/THIF-type NAD/FAD-binding fold; TPR, tetratricopeptide repeat; UBACT, ubiquitin-activating enzyme; UIM, ubiquitin-interacting motif.

Table 2**Summary of the CPKs and CRKs in the *Arabidopsis* genome**

Gene ID	Size (amino acids)	Number of EF hands	Targeting signal	Other names	References
CDPKs					
At1g18890	545	4	ch (.877)	CPK10, AtCDPK1	[71,74]
At1g35670	495	4		CPK11 *, AtCDPK2	
At1g50700	521	4		CPK33	
At1g61950	547	4		CPK19 *	
At1g74740	567	4		CPK30, AtCDPK1a	
At1g76040	534	4		CPK29 *	
At2g17290	540	4		CPK6, CDPK3	[66,71]
At2g17890	571	4	ch (.93)	CPK16	
At2g31500	582	4	ch (.93)	CPK24	
At2g35890	520	1		CPK25	
At2g38910	583	4		CPK20	
At2g41860	530	4		CPK14	
At3g10660	646	4		CPK2, CDPK2	
At3g20410	541	4		CPK9	[66,82]
At3g51850	503	4		CPK13	
At3g57530	560	4		CPK32	
At4g04700	494	4		CPK27 *	
At4g04710	575	5		CPK22 *	
At4g04720	531	4		CPK21	
At4g04740	520	4		CPK23 *	
At4g09570	501	4		CPK4	[66]
At4g21940	554	4	ch (.731)	CPK15	
At4g23650	529	4	ch (.949)	CPK3, CDPK6	
At4g35310	556	4		CPK5	
At4g36070	536	4		CPK18 *	
At4g38230	484	4		CPK26	
T19J18 7-m	453	4		CPK31 *, g5732059	
At5g04870	610	4		CPK1, AK1	[83]
At5g12180	528	4		CPK17	
At5g12480	535	4		CPK7	[66]
At5g19360	523	4		CPK34	
At5g19450	533	4		CPK8, CDPK19	[66,69]
At5g23580	490	4	mt (.662)	CPK12, CDPK9	[66,69]
At5g66210	523	4	ch (.78)	CPK28	
CRKs					
At1g49580	606	1		CRK8	
At2g46700	595	1		CRK3	
At3g50530	601	1		CRK5	

The EF-hands were analyzed using InterProScan. Target signals are according to Target P (ch, chloroplast; mt, mitochondria). Only signals with scores of 0.600 or above were included. All CPKs and CRKs have variable numbers of transmembrane domains (2-7) and *N*-myristoylation site(s). The asterisks (*) indicate size incorrectly annotated in MAtdB; the corrected sizes are available at [65].

to an EF-hand. Although Ca²⁺ binding of KCO1 was not tested, the activity of the channel was shown to be Ca²⁺-dependent [31]. AtPLC1, one of a small family of phosphatidylinositol-specific phospholipase Cs (PLCs), has a putative EF-hand but Ca²⁺ binding was not evaluated [42]. No other AtPLC has an EF-hand domain but the amino-terminal

sequences of several other family members have two sets of α helices that may correspond to EF-hand domains [29]. The putative EF-hand loop of AtPLC1 lies between two of the α helices. The actin-binding activity of most fimbrins is inhibited by Ca²⁺ [51]. AtFIM1 was shown to be Ca²⁺-independent, suggesting this single-EF-hand protein does

Table 3**Plant proteins not identified as EF-hand-containing proteins using InterProScan but known to bind Ca²⁺**

ID number	Size (amino acids)	Number of EF-hands*	Other domains	Targeting [†]	Published name	References
Caleosins						
At1g70670	195	1	-		Clo4	[46]
At1g70680	192	1	-			
At1g23240	184	1	-		Clo5	[46]
At2g33380	236	1	-		Clo3	[46]
At4g26740	245	1	-		Clo1	[46]
At5g55240	243	1	-		Clo2	[46,47]
14-3-3s						
At1g78300	259	1	14-3-3		GFI4 _w	[48,49]
At1g35160	267	1	14-3-3			
At4g09000	267	1	14-3-3			
At3g02520	265	1	14-3-3			
At5g38480	255	1	14-3-3			
At5g16050	268	1	14-3-3			
At5g10450	273	1	14-3-3			
At5g65430	246	1	14-3-3			
SUBs[‡]						
At4g08810	552	2	Serpin		SUB1	[50]
At2g04280	568	2		mt(0.838)	SUL2	[50]
At4g12700	561	2		mt(0.749)	SUL1	[50]

*Number of EF-hands reported; [†]TargetP prediction; [‡]short under blue light.

not bind Ca²⁺ [25,51]. The respiratory burst oxidase family (see Table 1) has nine members in *Arabidopsis* that have either one or two EF-hands [26,27]. An alignment (data not shown) of these proteins, however, shows the presence of EF-hand like sequences for the missing EF-hand domain in the one-EF-hand proteins. Keller *et al.* [27] identified two EF-hand domains in RbohA which both bind Ca²⁺ *in vitro* (RbohF in this report and Torres *et al.* [26]) although only one is recognized by InterProScan. The ability to bind Ca²⁺ was not addressed for single-EF-hand proteins ABI1 or GDH2 [32,33]. The CBL/SOS3 family of proteins (see Table 1) shows the presence of three EF-hand domains [23,52]. Kudla *et al.* [23], however, identified a sequence that represents a variation of the EF-hand domain that may be a fourth EF-hand. AtCP1, a protein with three EF-hand domains, also has a fourth EF-hand-like sequence at the end of the protein but it is truncated and may not be functional [35].

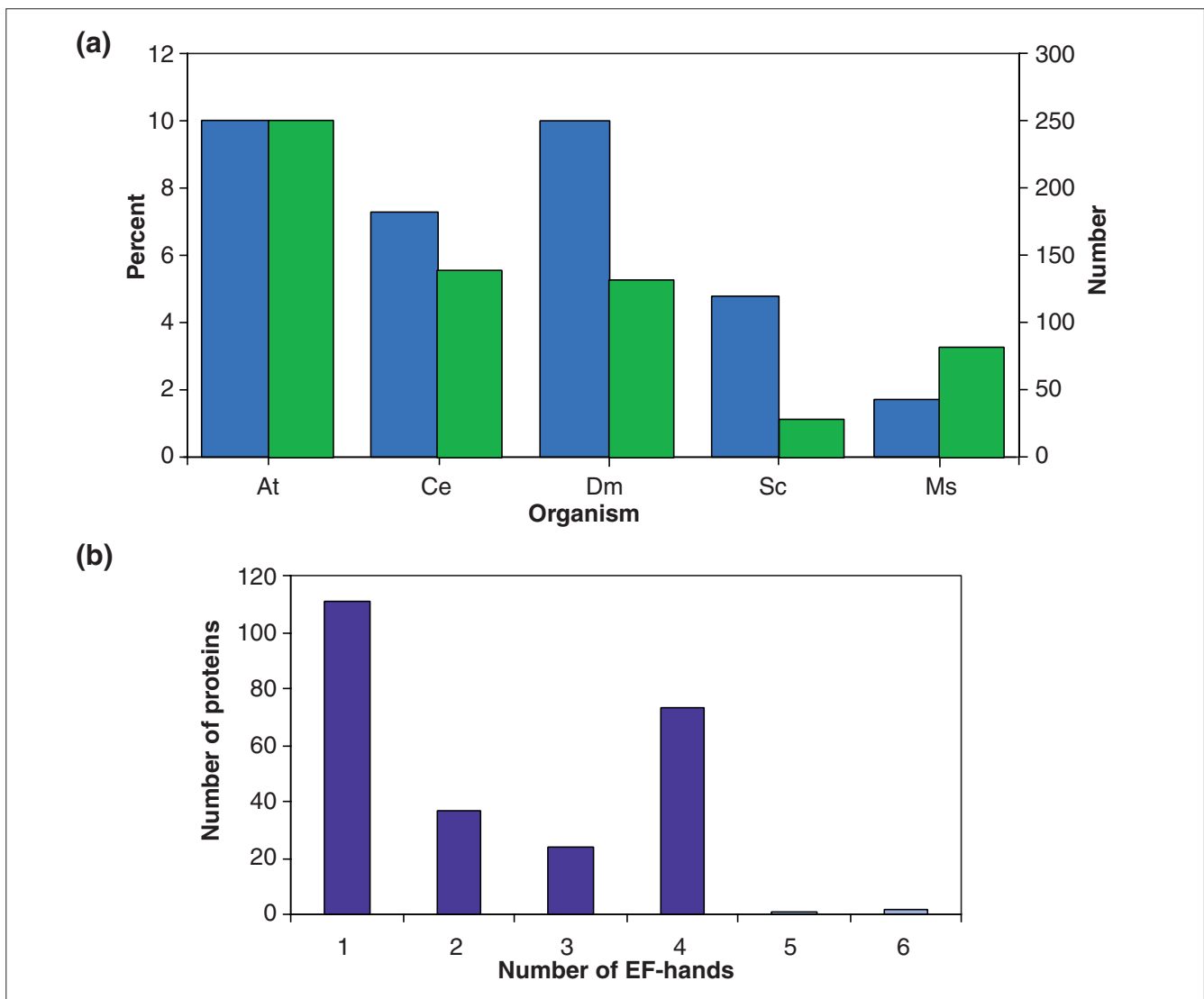
The reported proteins with two EF-hand domains include two pollen-associated proteins and the Rboh proteins (see Table 1). Three of the proteins with two possible EF-hands were identified by only one prediction program. The CAM family and proteins closely related to CAM - CaBP-22, PM129, TCH2 and centrin (Table 1) - and most of the CPKs (Table 2) have four EF-hand domains. The two proteins with

six EF-hands are TCH3 and an unknown protein (At4g27790) which are only 13% similar and thus are not likely to represent duplicate genes. Although the significance of the number of EF-hand domains in various proteins is not known, they may differ in their affinity for Ca²⁺ and, thereby, function to fine tune Ca²⁺-mediated cellular activities.

Identification of other domains in EF-hand-containing proteins

Table 4 lists the other domains found in the EF-hand-containing proteins, their InterProScan accession numbers and general type of protein or domain. As shown in Table 1, some of the proteins predicted to have other domains have putative EF-hand motifs identified by only one prediction program (shown in bold in Table 1). Schematic diagrams of representative EF-hand proteins are shown in Figure 2. As can be seen, the calmodulin-like proteins have the EF-hands distributed throughout the protein and no other domain is present. Other proteins have the EF-hands at one end or the other, or in the middle of the protein with enzymatic or regulatory domains preceding or following the EF-hands.

Some of the domains listed in Table 4 either contain an EF-hand within the domain or are a specific type of EF-hand. These include EPS15 repeats, calflagin, recoverin and S100/IcaBP. EPS15 repeats are protein-protein interaction

**Figure 1**

(a) A comparison of the number of genes encoding putative EF-hand proteins (green) in different species and their percentage of the total number of genes (blue). *At*, *Arabidopsis thaliana*; *Ce*, *Caenorhabditis elegans*; *Dm*, *Drosophila melanogaster*; *Sc*, *Saccharomyces cerevisiae*; *Hs*, *Homo sapiens*. **(b)** The number of *Arabidopsis* proteins having 1, 2, 3, 4, 5 or 6 EF-hands.

modules of about 95 residues that were first identified in tyrosine kinase substrates EPS15 and 15R. The first of three subdomains in EPS15 may include a Ca^{2+} -binding domain of the EF-hand type. Calflagins are flagellar Ca^{2+} -binding proteins found in *Trypanosoma cruzi* and *T. brucei* that have motifs similar to EF-hands. Recoverin is a retinal Ca^{2+} -binding protein that belongs in the EF-hand family of proteins.

Some of the EF-hand proteins contain an *Arabidopsis* retrotransposon (ATHILA) ORF-1 protein domain and one has an En/Spm-like transposon protein domain. As shown in Table 4, domains found in various enzymes are present in many of the EF-hand proteins. Because of the presence of EF-hand motifs in these proteins, regulation of these putative

enzymes is likely to be Ca^{2+} -dependent. The diversity of enzymes that contain EF-hand(s) indicates that a wide range of cellular processes is likely to be regulated by Ca^{2+} . It is also of interest that some proteins in a family have EF-hands and others do not, suggesting differential regulation of protein family members.

Several identified domains indicate that some EF-hand proteins interact with other proteins (or themselves) or with nucleic acids. Table 4 lists domains that are involved in interaction with protein or DNA. Cell processes that EF-hand proteins may be involved in are suggested by domains found in transcription or translation proteins including elongation factors and the bHLH domain found in transcription

Table 4

Summary of various domains present in *Arabidopsis* EF-hand-containing proteins and their InterPro accession numbers

Domain*	Accession number	Protein type
ATP_GTP_A	IPR001687	Binding site motif
Calfagin	IPR003299	Calcium-binding
Recoverin	IPR002048	Calcium-binding
S-100/ICaBP type CBP	IPR001751	Calcium-binding
Adptrnslcase	IPR000213	Carrier
Cation (ca,na) pore region	IPR001682	Carrier
Graves disease	IPR002167	Carrier
Mitoch_carrier	IPR002067	Carrier
Potassium channel	IPR001622	Carrier
PTM	IPR003880	Carrier
bHLH	IPR001092	Dimerization
BTB/POZ domain	IPR000210	Dimerization
Biotin operon repressor	IPR004209	DNA interaction
DDT domain	IPR004022	DNA binding
HLH_Myc	IPR003015	DNA binding
Myb DNA binding domain	IPR001005	DNA binding
Elongation factor aEF-2	IPR004543	Elongation factor
EFG_C	IPR000640	Elongation factor
GTP_EFTU(_D2)	IPR004161	Elongation factor
pfkB	IPR002173	Enzyme
Aminoacyl-transfer RNA syn class II	IPR002106	Enzyme
AMP-dependent synthetase and ligase	IPR000873	Enzyme
ATP synthase alpha and beta subunit	IPR000194	Enzyme
Cytb-245 hc	IPR000778	Enzyme
Cytochrome P450 enzyme	IPR001128	Enzyme
Euk protein kinase	IPR000719	Enzyme
FAD_pyr_redox	IPR001237	Enzyme
Ferric_reduct	IPR002916	Enzyme
GatC	IPR004415	Enzyme
GLFV_dehydrog	IPR001625	Enzyme
PIP5K	IPR002498	Enzyme
PIPLC	IPR001992	Enzyme
Plant peroxidase	IPR000823	Enzyme
Protein phosphatase 2C subfamily	IPR000222	Enzyme
PS_Darbxylase, Porpobil_deam	IPR003817	Enzyme
Pyr_redox	IPR001237	Enzyme
Small GTPase	IPR001806	Enzyme
ThiF_family	IPR000594	Enzyme
UBACT	IPR000127	Enzyme
Jacalin-related lectin domain	IPR001229	Lectin
Dynamain family	IPR001401	Microtubule associated
NAD-binding site	IPR000205	NAD binding
No apical meristem protein	IPR003441	Plant development

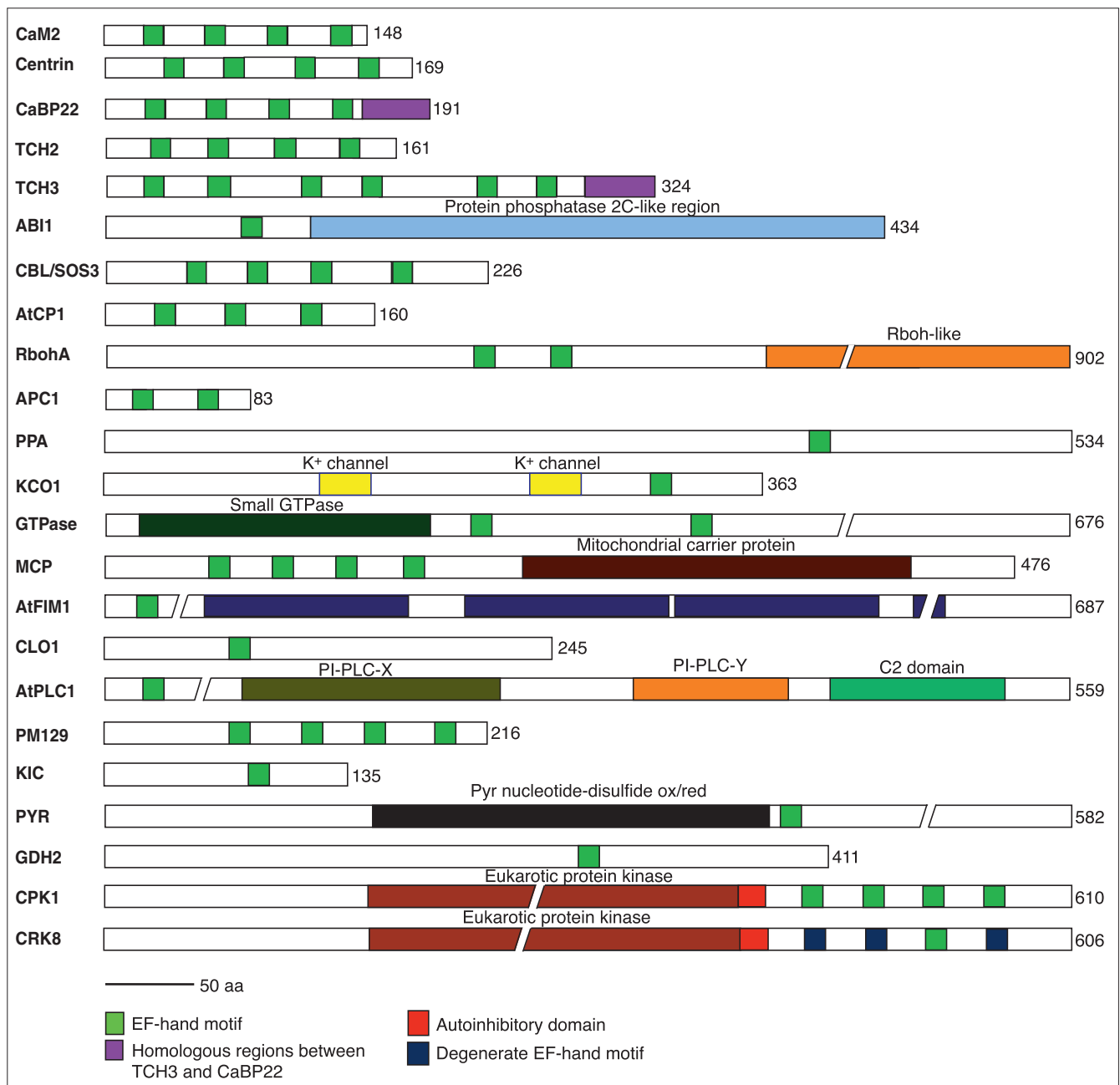
Table 4 (continued)

Domain*	Accession number	Protein type
Serpin	IPR000215	Protease inhibitor
I4-3-3	IPR000308	Protein interaction
Calponin homology	IPR001715	Protein interaction
EPS15 repeat	IPR000261	Protein interaction
F-box domain	IPR001810	Protein interaction
SET-domain of transcriptional regulators	IPR001214	Protein interaction
Ubiquitin-associated domain	IPR003903	Protein interaction
Zn-finger-ZZ type	IPR000433	Protein interaction
Zn-finger-FYVE	IPR000306	Protein interaction
Zn-finger-Ra-binding	IPR001876	Protein interaction
PPR repeats	IPR000285	Repeat
TPR	IPR001440	Repeat
Josephin, UIM	IPR002950	Repeat
Kelch repeat	IPR001798	Repeat
ATHILA	IPR004312	Retrotransposon
NPH3	IPR004249	Signal transduction
ER_TARGET	IPR000886	Targeting
NLS_BP	IPR001472	Targeting
PHD-finger	IPR001965	Transcription
eIF4-gamma/eIF	IPR003307	Translation
PTS_HPR_SER	IPR002114	Transport
Nucleoside_tran	IPR002259	Transport
En/Spm-like transposon proteins	IPR004242	Transposon

*See Table 1 for explanation of domain abbreviations.

factors. Domains such as potassium channels, pollen allergen Bra r II, mitochondrial carrier proteins, the cation (Ca²⁺ and Na⁺) pore region and nucleoside transporters suggest possible functions. One EF-hand protein has a domain found in NPH3 protein, a photoreceptor-interacting protein that is essential for phototropism. Another EF-hand protein has a jacalin domain found in lectins. These domains in EF-hand proteins should help in evaluating the function of these proteins. For instance, the EF-hand protein identified as having a pollen allergen Bra r II domain is similar to an EF-hand protein from pollen (APC1) isolated by Rozwadowski *et al.* [36]. They showed APC1's affinity for Ca²⁺ and the potential for a Ca²⁺-dependent conformational change.

Motifs such as the ATP/GTP-binding region suggest that the proteins containing them interact with or bind to certain molecules. PTM is the site for attachment of phosphopantetheine (the prosthetic group of acyl carrier proteins in some multienzyme complexes), PTS_HPR_SER is a serine phosphorylation site found in HPr (a protein in the phosphoenolpyruvate-dependent sugar phosphotransferase system in bacteria) and UIM (ubiquitin interaction motif) is a receptor for polyubiquitination of polypeptide chains.

**Figure 2**

Schematic diagrams of representative EF-hand proteins. The number of amino acids is given at the end of each diagram. Domain names are written above the domain except as given in the key. PI-PLC-X(Y) and C2, phosphatidylinositol-specific phospholipase C subdomains. ABI1, ABA-insensitive I; APC1, *Arabidopsis* pollen Ca²⁺-binding protein; AtCP1, *Arabidopsis thaliana* Ca²⁺-binding protein; AtFIM1, *Arabidopsis thaliana* fimbrin I; AtPLC1, *Arabidopsis thaliana* phosphatidylinositol-specific phospholipase C; CAM2, calmodulin 2; CaBP22, 22 kd Ca²⁺-binding protein; CBL/SOS3, calcineurinB-like, salt-overly-sensitive protein; CH, calponin homology; CLO1, caleosin I; CPK, Ca²⁺-dependent protein kinase; CRK, CPK-related kinase; GDH2, NAD(H)-dependent glutamate dehydrogenase; GTPase, small GTPase-like protein (At3g63150); KCO1, potassium channel outwardly rectifying protein I; KIC, KCBP-interacting CCD-I-like protein; MCP, mitochondrial carrier protein (At5g61810); PM129, protein isolated from plasma-membrane enriched library; PPA, protein phosphatase 2A-like protein (At1g03960); PYR, pyridine nucleotide-disulfide oxidoreductase (At2g20800); RbohA, respiratory burst oxidase homology; TCH2 and TCH3, touch-induced proteins. // indicates a break in the protein.

Phylogenetic analysis of EF-hand-containing proteins

The full-length sequences of all proteins identified by InterProScan as containing an EF-hand (including those identified

by only one database) were aligned using MEGALIGN (DNASTar). Phylogenetic analysis was carried out by PAUP 4.08a using a heuristic search method. A consensus tree was

generated from all saved trees. This tree was used to identify groups of EF-hand proteins and closely related proteins. Five major groups of proteins could be identified. Figure 3 shows the overall tree with a few representative members of each group. A sixth group includes members that did not fall into the other five groups. Figures 4-9 are the expanded trees for each group.

Group I proteins

None of the proteins in group I (Figure 4) has been reported in the literature. Some of them contain domains that give clues to their function, including elongation factors, DNA-, protein- or ATP/GTP-binding proteins and others (Tables 1 and 4).

Group II proteins

Group II includes KCO1, AtPLC1 and the two fimbrins (Figure 5) that have been reported in the literature (Table 1). Two other proteins show similarity to KCO1 and may also be Ca²⁺-regulated K⁺ channels. A family of phosphatidylinositol-specific phospholipase Cs have been isolated, but only one of them, AtPLC1, has an EF-hand domain (Figure 2, and see also [29,42]). AtPLC1, a protein isolated as a dehydration and salt stress-induced gene, was able to hydrolyze phosphatidylinositol-4,5-bisphosphate and the activity was completely dependent on Ca²⁺ [42]. AtFIM1 was identified as an EF-hand-containing protein (Figure 2), however, Kovar *et al.* [51] found that AtFIM1 was Ca²⁺-independent and so this may be a non-functional EF-hand as pointed out

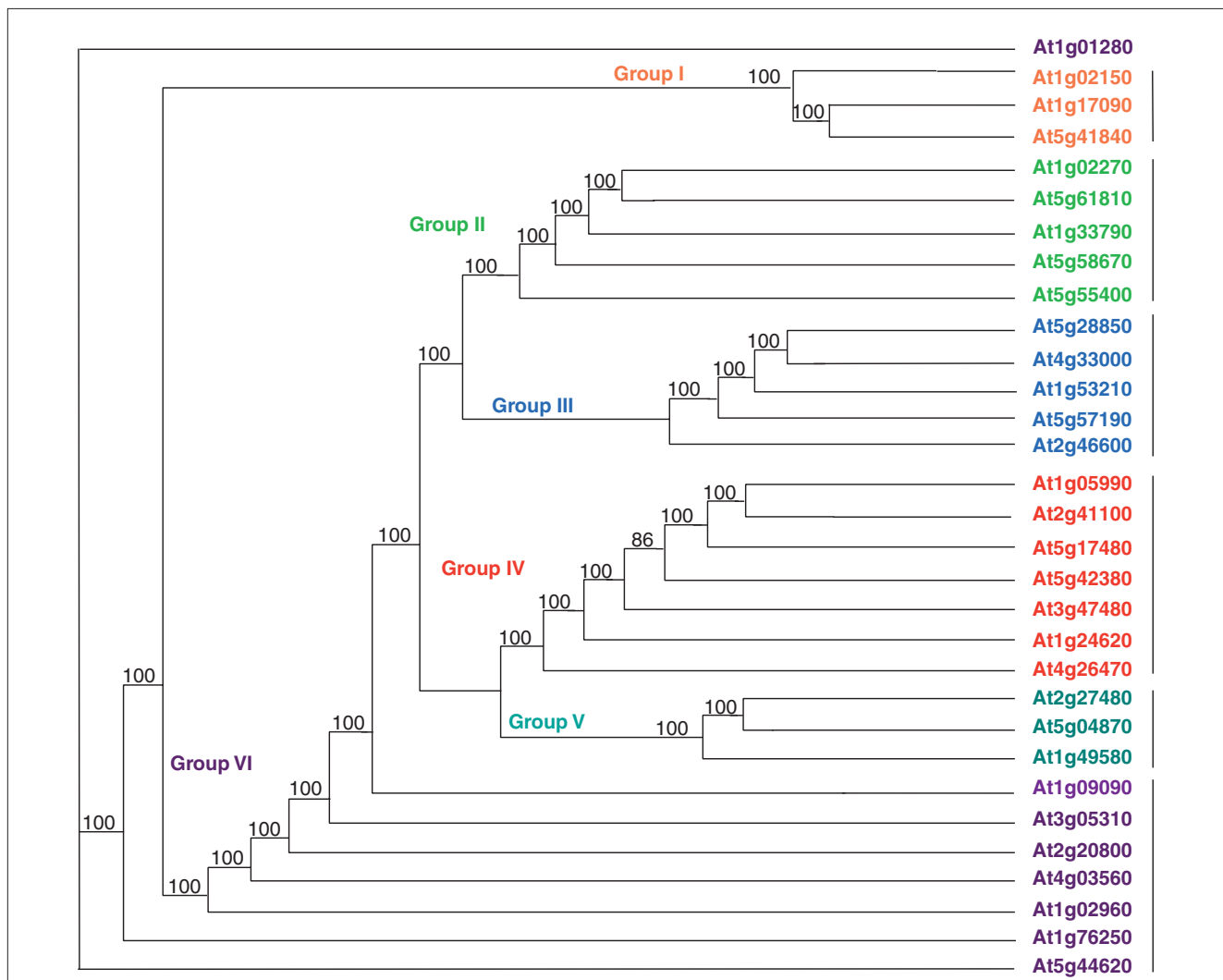


Figure 3 Phylogenetic tree showing the overall relatedness of the EF-hand proteins. All EF-hand proteins were aligned using MEGALIGN (DNASTar) and analyzed using a heuristic method in PAUP 4.08a. Numbers represent the number of times the branch appeared in 100 saved trees. The tree was reduced by hand to show a few representative proteins for each major group. The expanded groups are shown in Figures 4-9.

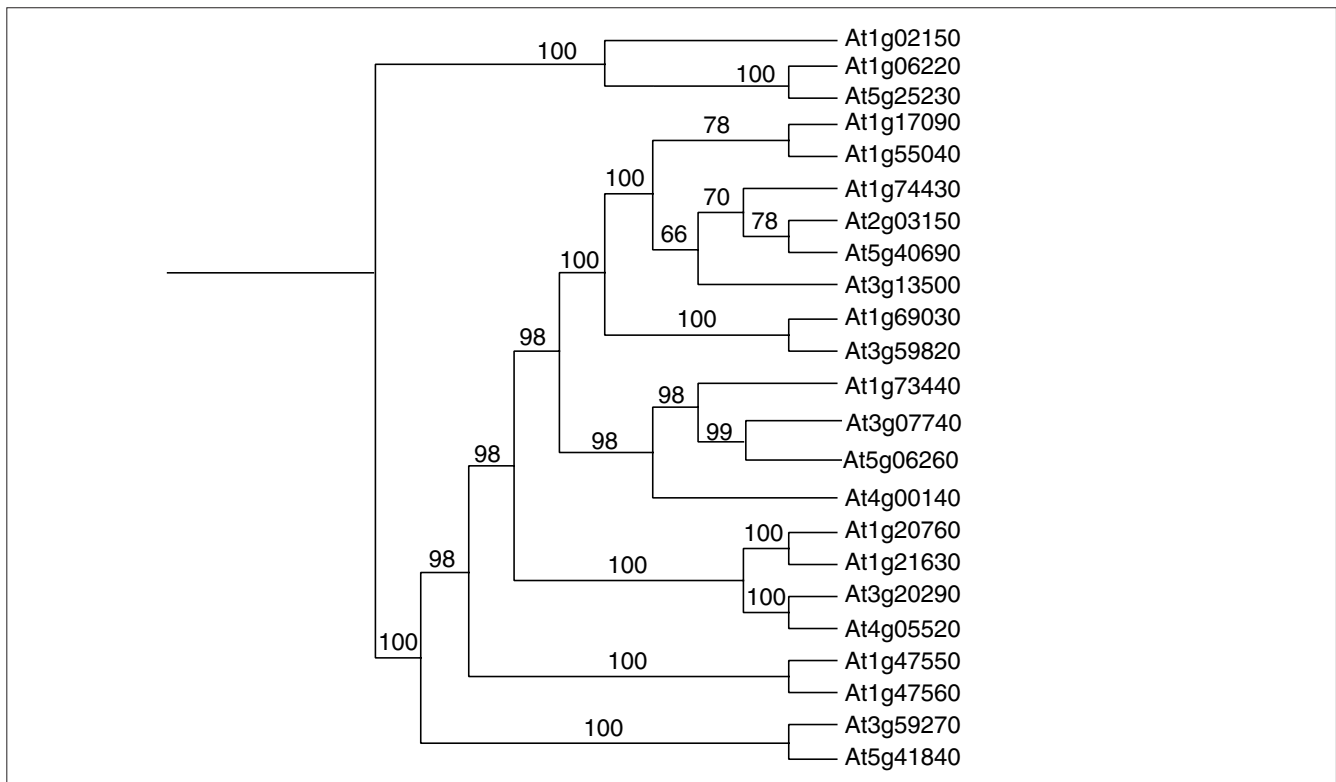


Figure 4
Group I tree showing all proteins included in this group. None has been published in the literature.

by McCurdy and Kim [25]. Four other proteins in the MatDB are similar to AtFIM1 but only one has an EF-hand motif.

Two small subgroups, nucleoside transporters and mitochondrial carrier proteins, also fell into group II (Figures 2, 5). As far as we know, Ca^{2+} -binding studies have not been done for these proteins.

Group III proteins

CBL/SOS3s fall into group III (Figure 6). The first CBL/SOS3 was isolated as a protein involved in salt stress (SOS3) and as a calcineurin B-like protein (CBL1) [23,24]. Ten CBL/SOS3s have been identified [13]. Expression of CBL4 is induced by drought, cold and wounding stress. In animals, calcineurin is a heterodimer composed of a regulatory B subunit and a protein phosphatase catalytic A subunit. CBL/SOS3s show similarity to the B subunit (Figure 2). SOS3 was, however, shown to interact with a protein kinase [53] and a family of interacting protein kinases has been identified [54-56]. Both Albrecht *et al.* [55] and Kim *et al.* [54] have identified the domain in the kinases required for the interactions of protein kinase with CBL/SOS3.

AtCP1, which contains three EF-hands (Figure 2), is another NaCl-stress-induced protein that has been shown

to bind Ca^{2+} [35]. A bean homolog of AtCP1 has been shown to be associated with the hypersensitive response [57]. A subgroup of proteins identified in this search show similarity to the protein phosphatase 2A regulatory B subunit. One of these, At5g44090 (AF165429), was reported in the literature [58]. They have one EF-hand domain but contain no other identifiable domains (Figure 2). One other protein of interest in this group, KIC (KCBP-interacting CCD-1-like protein), was identified as a protein that interacts with KCBP (kinesin-like calmodulin-binding protein), a protein known to interact with and be regulated by Ca^{2+} /calmodulin [59,60]. KIC has only one EF-hand (Figure 2) and is similar to a wheat Ca^{2+} -binding protein (CCD-1) [61].

Group IV proteins

Group IV contains the calmodulins (CAMs) and closely related proteins such as CaBP-22, centrin and the TCH gene proteins (Figures 7). CAMs are highly conserved small-molecular-weight acidic proteins of 148 amino acids (listed as 149, the starting Met is cleaved following translation). The four EF-hands (two pairs connected by a central helix) bind four molecules of Ca^{2+} [11,14]. Binding of Ca^{2+} to CAM results in a conformational change which then allows CAM to interact with target proteins to modulate their activity or function [3,18,62].

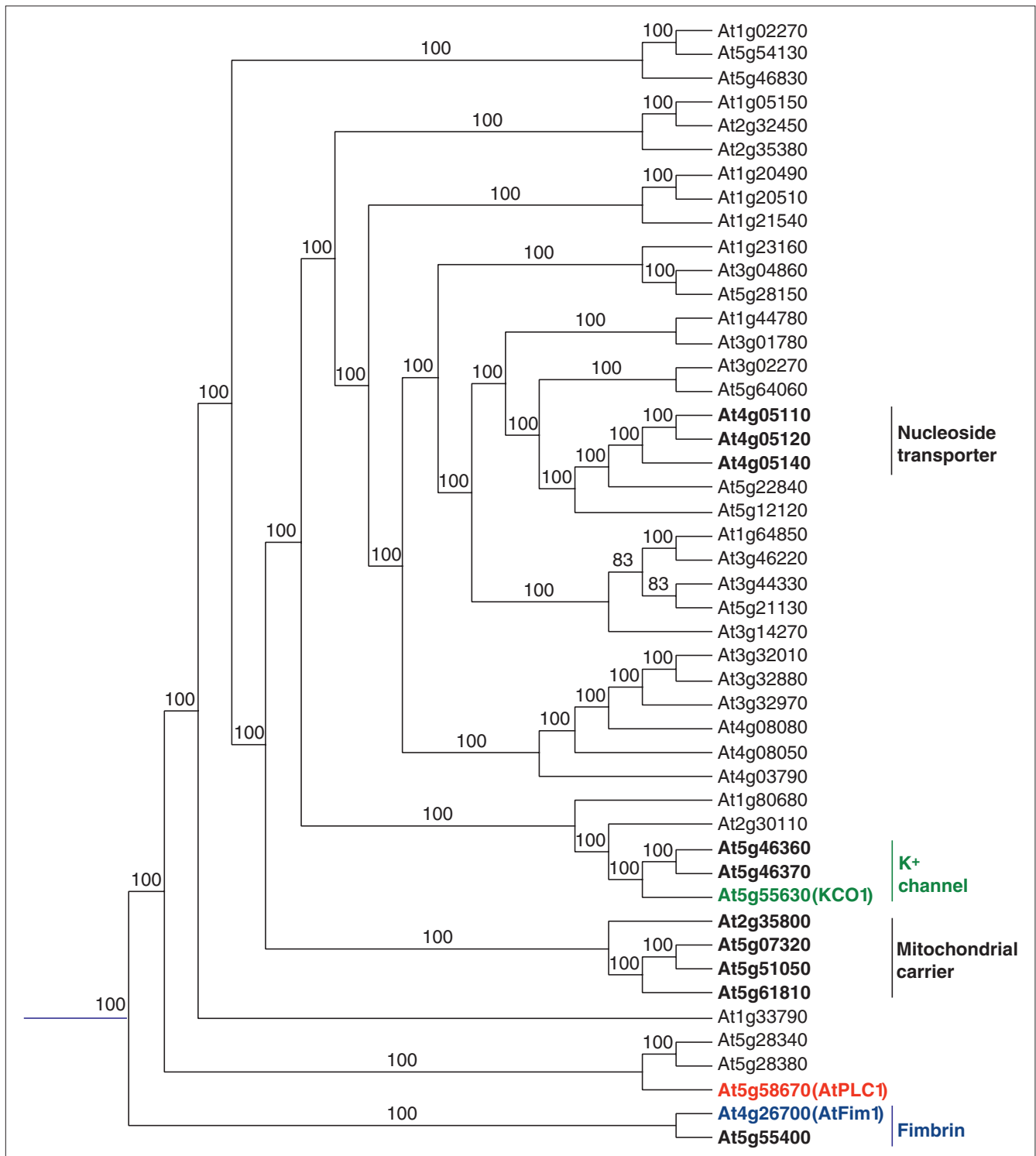
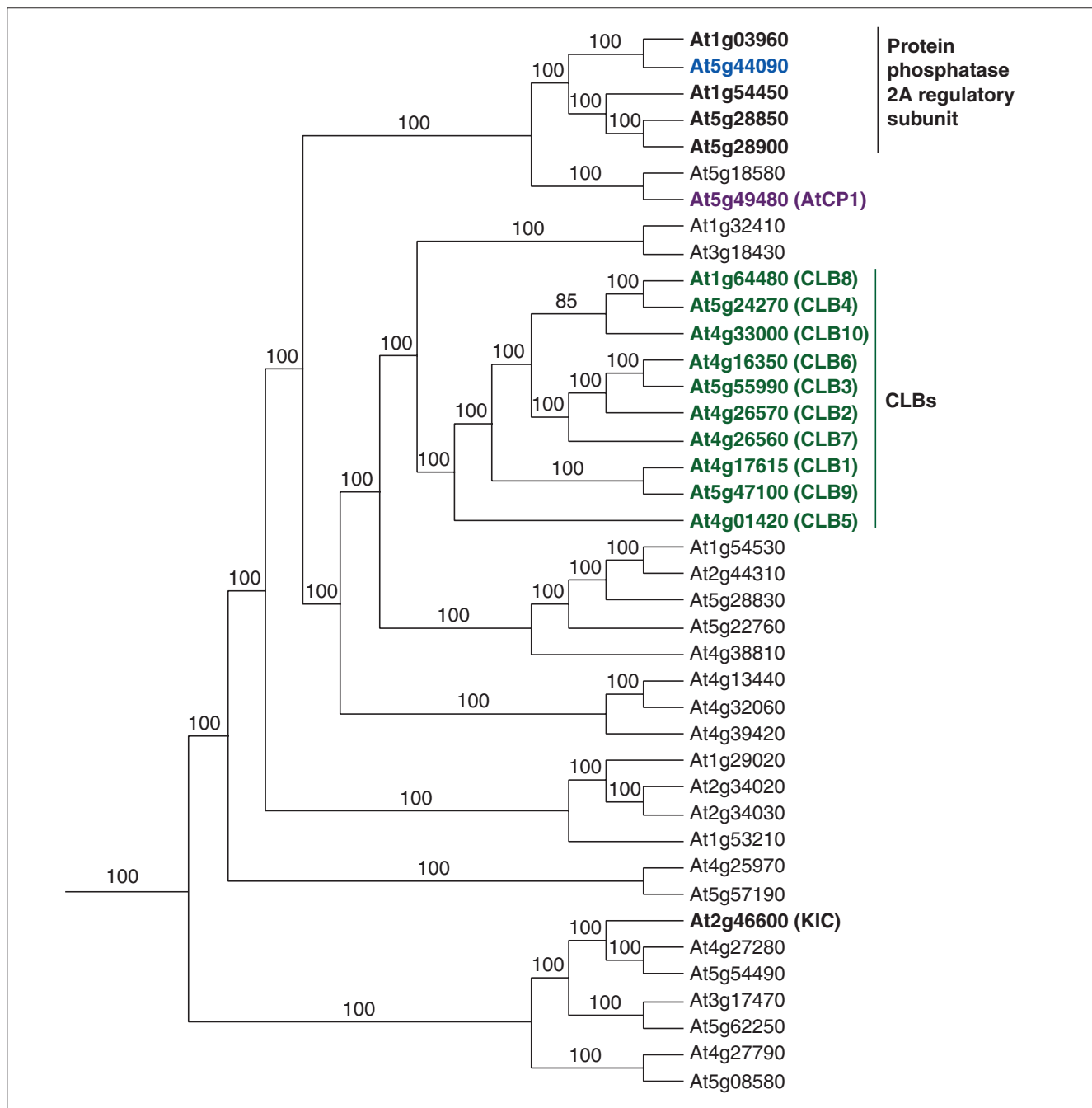


Figure 5
Group II tree showing all proteins included in this group. Proteins published in the literature are in color.

Nine *Arabidopsis* CAMs have been reported in the literature [15-18,20]. Seven of these are highly conserved, having 148 amino acids (CAMs 1-7) with only 1-4 amino-acid differences

between them. CAM6 has not been given a protein identification number. BLAST searches with CAM6 pick CAM7 as the closest sequence. However, at the nucleotide level they

**Figure 6**

Group III tree showing all proteins included in this group. Proteins published in the literature in color.

are only 86% identical. There are two ESTs that are 83 and 94% identical to *CAM6* but only 72 and 86% identical, respectively, to *CAM7*. *CAM8* and *CAM9* are divergent CAMs. They have 151 rather than 148 amino acids and vary considerably in the fourth EF-hand domain. Although they complemented a yeast calmodulin (*CMD1*) mutant, they did not form a complex with a basic amphiphilic helical peptide in the presence of Ca^{2+} , unlike conventional CAMs that do

[18]. As can be seen in Figure 7, they do not fall into the same group as the other CAMs, with *CAM9* being more divergent than *CAM8*. No other EF-hand proteins have 149 amino acids, although others do have a few more or less (Table 1). Expression studies of the *Arabidopsis* CAM genes show that they are differentially expressed in different tissues and circumstances. For *CAM1*, *CAM2*, and *CAM3*, *CAM1* was the only one expressed in roots and *CAM3* could

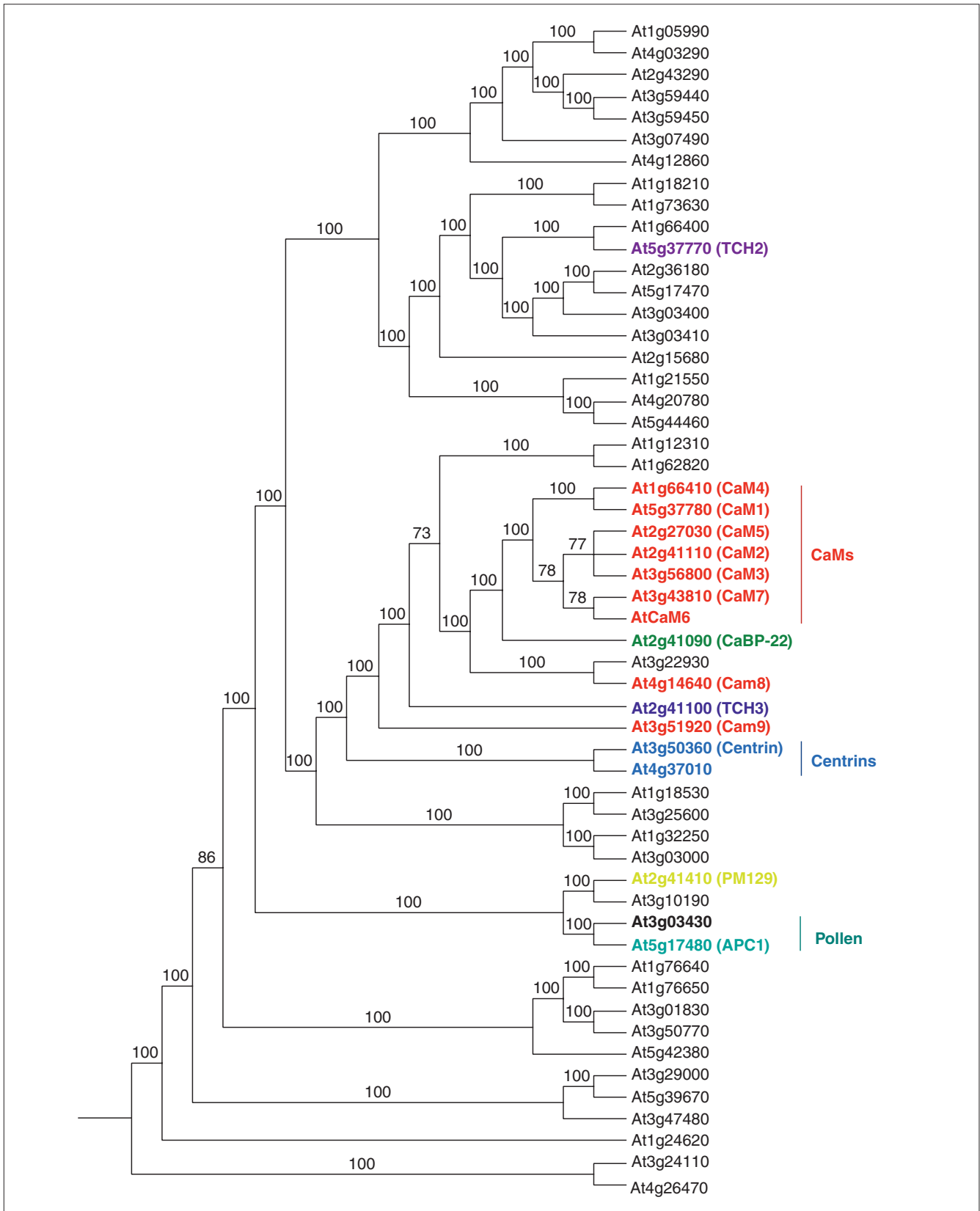


Figure 7
Group IV tree showing all proteins included in this group. Proteins published in the literature are in color.

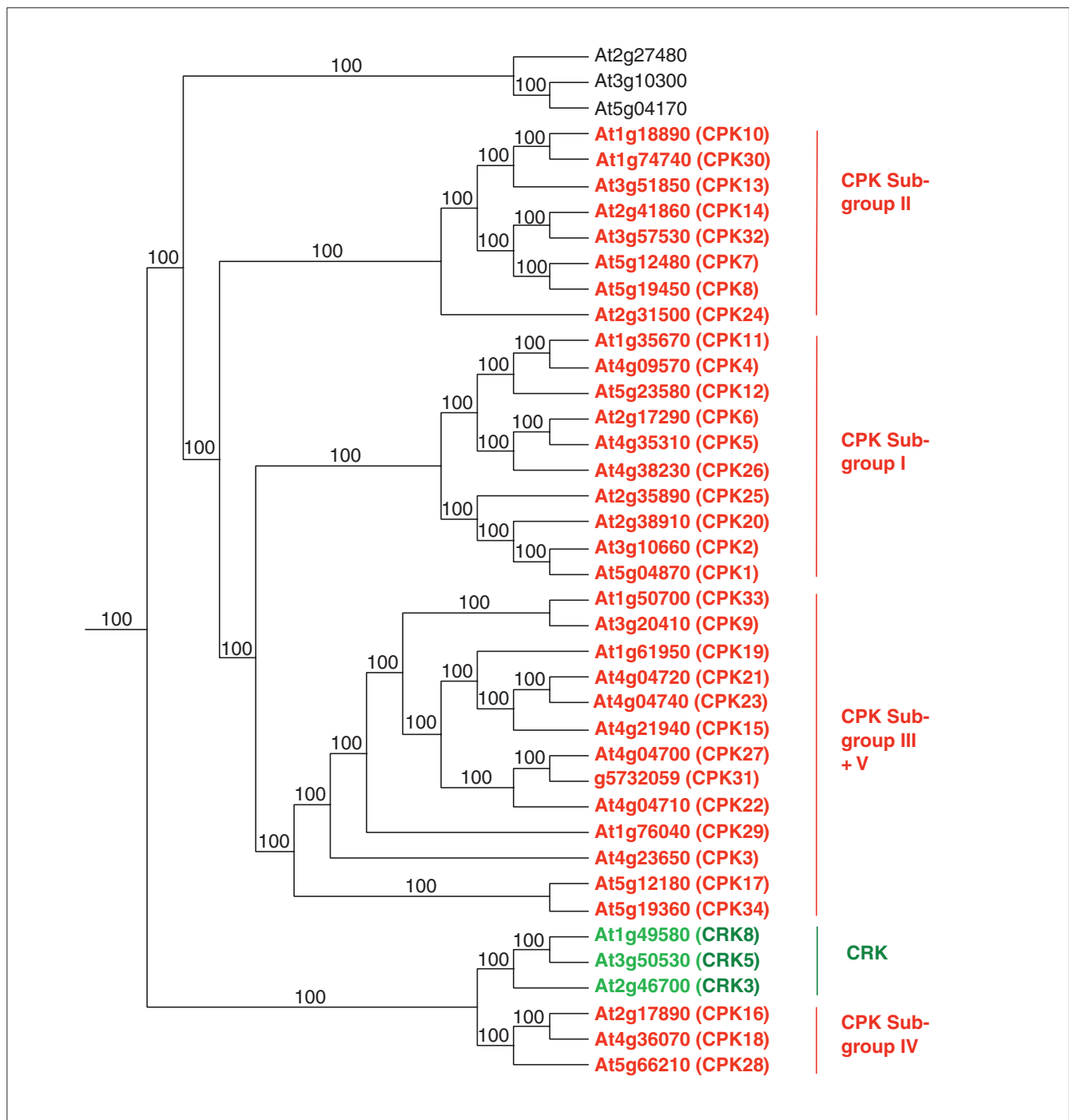


Figure 8
Group V tree showing all proteins included in this group. CPKs are in red and CRKs are in green. Subgroups are named as in Figure 10.

not be detected in floral stocks; *CAM1*, *CAM2*, and *CAM3* are inducible by touch stimulation but at different levels and with different kinetics [15]. *CAM4*, *CAM5* and *CAM6* were all expressed in leaves, but only *CAM4* and *CAM5* were detected in siliques [17]. Different *Arabidopsis* CAM isoforms also differ in their affinity for the same protein [60,63].

Two proteins induced by touch, rain, wind, wounding, and darkness, TCH2 and TCH3, are also in group IV and are related to the CAMs. TCH2 has 161 amino acids with four EF-hands and TCH3 has 324 amino acids with six EF-hands. Another CAM-like protein, CaBP-22, is closely related to the conventional CAMs (Figure 7). It has 191 amino acids, 66%

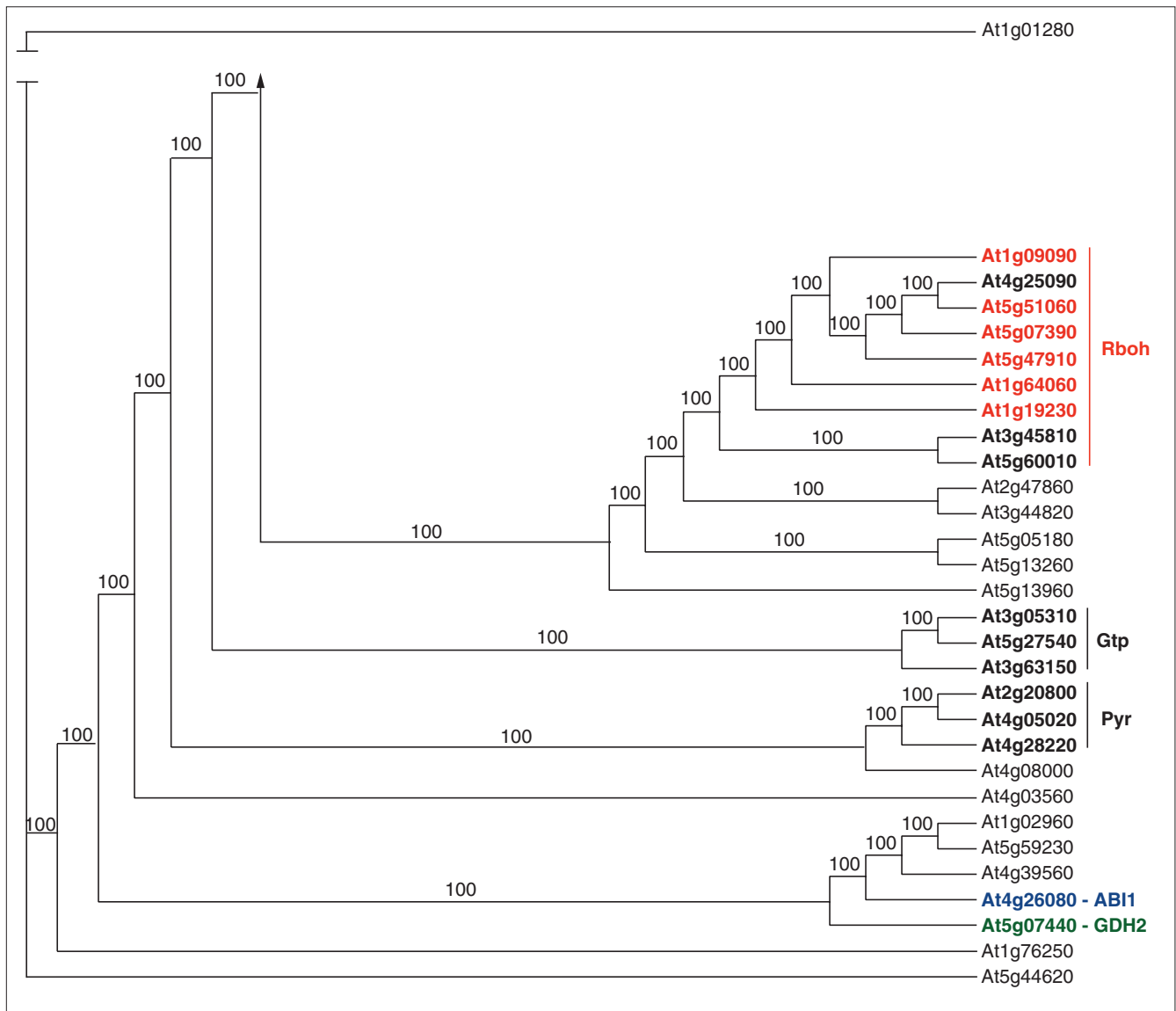


Figure 9
Group VI tree showing all proteins included in this group. Proteins published in the literature are in color.

amino-acid sequence identity with CAM (79% in the EF-hand domains) and has been shown to bind Ca^{2+} [19]. Centrins are a little more distantly related to CAMs (Figure 7). An *Arabidopsis* centrin gene (*ap3.3a*) was isolated as a gene rapidly induced after pathogen inoculation [21]. One other EF-hand protein is 65% similar to centrin, suggesting there are two centrin genes in *Arabidopsis*.

Two proteins reported in the literature fall into a subgroup of CAM-like proteins. A novel EF-hand protein (PM129, Figure 2) was isolated from a cDNA library that over-represents plasma-membrane-associated proteins [64]. The other protein, APC1, is a pollen Ca^{2+} -binding protein that is a member of the pollen allergen family [36]. Another protein,

At3g03430, is 89% similar to APC1. They are the smallest of the EF-hand proteins having only 83 amino acids (Figure 2).

Group V proteins

The 34 CPKs and three CPK-related protein kinases (CRKs) make up almost all of group V (Figure 8). The other three proteins in the group do not have any other identifiable domains. CPKs are serine/threonine protein kinases with a CAM-like domain (CLD) usually containing four EF-hands (with two exceptions) (Table 2, Figure 2). These kinases have been called CDPKs; however, we use the most recent designation, namely CPKs [22,65,66]. Three of the eight CRKs in *Arabidopsis* have one EF-hand domain. However, sequence alignment of the EF-hand regions of CPKs and

CRKs revealed that CRKs contain degenerate EF-hand motifs (Figure 2). CPKs are present only in plants and some protozoans. The PlantsP database [65] reports that most of the CPKs and CRKs contain transmembrane and *N*-myristoylation domains. TargetP predicts that some CPKs and CRKs are targeted to the chloroplast or mitochondria [44]. The cellular localization for most of these protein kinases needs to be confirmed experimentally.

CPKs range from 453 to 646 amino acids (Table 2) with four distinct domains; a variable region at the amino terminus (approximately 22-184 amino acids), a serine/threonine protein kinase domain (approximately 275 amino acids), an autoinhibitory domain (also called the junction region) (approximately 31 amino acids) and the regulatory CLD (approximately 165 amino acids) (Figure 2). The autoinhibitory domain is involved in inhibiting the enzyme activity in the absence of Ca²⁺ whereas the variable region at the amino terminus may account for their substrate specificity and/or localization [22]. CRKs have similar domain organization as compared to CPKs (Figure 2). Most of the CPKs contain fatty-acylation sites, including those for myristoylation and palmitoylation, which seem to be necessary for targeting to membranes and for protein-protein interactions [67]. The protein kinase domain in CRKs shows strong sequence similarity to the kinase domain in CPKs, but the autoinhibitory and CLDs in CRKs show weak sequence similarity to the corresponding domains in CPKs.

CPKs have basal activities in the absence of Ca²⁺ as a result of the presence of the autoinhibitory region. Ca²⁺ binds the EF-hands of the CLD, which results in intramolecular rearrangement and relief of autoinhibition [22,68]. Eight CPK isoforms have been shown to be activated by Ca²⁺ [66,69-71]. The presence of multiple isoforms of CPKs in the *Arabidopsis* genome implies that they may be involved in specific Ca²⁺-signaling networks, may respond differentially to changes in oscillation, frequency, magnitude and duration of Ca²⁺ signal, or may have temporal and spatial patterns of expression and localization. Little is known about the function and substrates for CPKs in *Arabidopsis*. CPK1 is known to interact with 14-3-3 proteins [72] and is involved in the inactivation of a Ca²⁺ pump [73]. Expression of CPK10 and 11 is inducible by cold and drought [70,74,75]. An *Arabidopsis* CPK phosphorylates tonoplast intrinsic protein, α -TIP, a putative water-channel protein [76]. Substrates of CPKs in other plants have been identified and can be used to deduce the function of homologs in *Arabidopsis*. The PlantsP website database [65] is a valuable source of information on CPKs.

Besides the inclusion of the full-length sequences of CPKs and CRKs in the overall phylogenetic analysis, a phylogenetic tree was constructed using the protein sequence of the CLD region of CPKs and CRKs (Figure 10). Similar trees were obtained using either full-length CPKs/CRKs (Figure 8) or the CLD region of the CPKs/CRKs (Figure 10). As shown in

Figure 10, the CPKs form five distinct subgroups (I-V). The CRKs are most closely related to subgroup IV. The tree made using full-length sequences (Figure 8) has four subgroups (members of subgroups III and V in Figure 10 fall into one subgroup in Figure 8).

Group VI

Figure 9 shows the remaining proteins that do not fall into one of the other five groups. It includes the respiratory burst oxidase homology proteins (Rbohs) ABI1, GDH2, and TPC1. Plant defense responses include production of reactive oxygen species (oxidative burst) [77]. Torres *et al.* and Keller *et al.* [26,27] isolated *Arabidopsis* homologs (Rbohs) to the *gp91^{phox}* subunit of the neutrophil NADPH oxidase, which generates a similar oxidative burst in neutrophils. Six Rbohs (A-F) have been isolated experimentally and three others have been identified in the *Arabidopsis* genome (Figures 2 and 9). RbohF (called RbohA in Keller *et al.* [27]), like animal Rboh enzymes, is an intrinsic plasma membrane protein but, unlike animal Rboh, it has EF-hands that were shown to bind Ca²⁺ [27]. Both Leung *et al.* and Meyer *et al.* isolated the *ABI1* gene [33,34]. *ABI1* is similar to serine/threonine phosphatase 2C, which in animals is Mg²⁺- or Mn²⁺- but not Ca²⁺-dependent (Figure 2). *ABI1* is induced by abscisic acid and was shown to regulate stomatal aperture in leaves and mitotic activity in root meristems [33,34]. A second *ABI* gene (*ABI2*) was isolated using *ABI1* as a probe [78]. The protein encoded by the cDNA had an eight-residue insertion in the EF-hand domain that does not conform to the EF-hand signature. A similar situation holds for glutamate dehydrogenases. Two genes were isolated; one coded for a dehydrogenase with an EF-hand (GDH2) (Figure 2) and the other one without (GDH1) [32]. Studies by Furuichi *et al.* [30] indicate that TPC1 is a two-pore channel that mediates Ca²⁺ influx. It has two EF-hand-like motifs located in a hydrophilic domain that connects the two transmembrane regions containing the pores [30]. Ca²⁺ binding was not shown experimentally for ABI1, GDH2, or TPC1.

Three proteins in this group have a domain present in a small GTPase protein (Figure 2 and Gtp in Figure 9). Another three-member group (Pyr in Figure 9) of proteins has a domain for FAD-dependent pyridine nucleotide-disulfide oxidoreductase (FAD_pyr_redox) (Figures 2 and 9).

Conclusions

A plant's adaptively variable behavior or plasticity during its lifetime has been described as 'plant intelligence' [79] and Ca²⁺ and its sensors are key players in this adaptive behavior. The large number of potential EF-hand-containing proteins indicates how [Ca²⁺]_{cyt} changes can profoundly affect a wide array of cellular processes. Plants seem to have a large number of EF-hand-containing proteins, some of which have homologs in non-plant systems whereas others are unique to plants. In addition, plants also have unique sets of

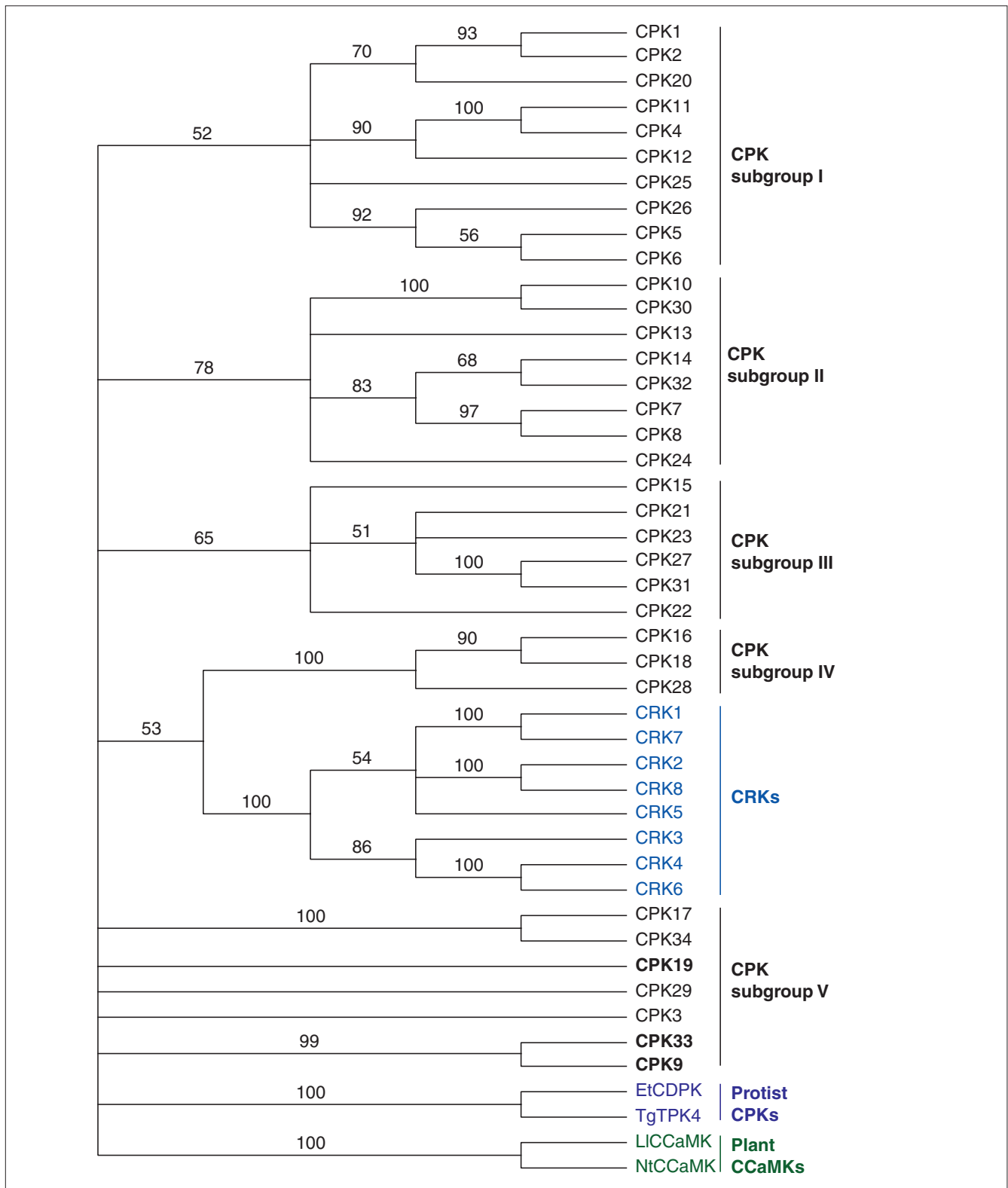


Figure 10

Phylogenetic analysis of CPK, CRK and CCaMK. The EF-hand domains of CPKs, CRKs and other plant and protist protein kinases were aligned in MEGALIGN (DNASTar) and analyzed using a bootstrap method. Numbers are the percentage of bootstrap replicates showing the branch. The accession numbers are listed here in brackets for EtCDPK (CAA96439; 332-487 amino acids), TgTPK4 (AAC02532; 355-501 amino acids), LiCCaMK (AAC49008; 339-520 amino acids) and NtCCaMK (AAD28791; 336-517 amino acids). Et, *Eimeria tenella*; Li, *Lilium longiflorum*; Nt, *Nicotiana tabacum*; Tg, *Toxoplasma gondii*.

proteins that interact with Ca²⁺ sensors [80]. Signals from stresses such as pathogens, drought, cold and salt are mediated by EF-hand proteins [20,23,3,42,57]. Hormones induce EF-hand proteins [46,78] as do pathogen attacks [21,26]. Some EF-hand proteins, such as small GTPases and potassium channels, are involved in signaling [31]. Others appear to be involved in developmental pathways. For example, the S-100 domain proteins are involved in cell growth and differentiation, cell-cycle regulation and metabolic control [40]. The wide variety of domains in EF-hand proteins also shows the diversity of the processes in which Ca²⁺ is involved. However, many of the EF-hand proteins give little clue as to their function and may lead us in many other directions as their functions are elucidated.

The complexity of the Ca²⁺ messenger system is increased through the existence of families of proteins. At the first level, calmodulin adds complexity first in the number of isoforms present in *Arabidopsis*. The regulation of expression and the kinetics of interaction of these isoforms with different proteins can lend complexity to cell signaling. A second level of complexity is the number of proteins identified that interact with calmodulin. At least 100 calmodulin-binding proteins (CBPs) have been identified in *Arabidopsis* [80]. Differential expression of these proteins in cell types, developmental periods and in response to signals adds more complexity to the pathways that can be regulated by CAM.

The CPKs, a very large family of protein kinases, also add complexity to Ca²⁺ regulation. Although the mechanism of regulation may be similar in each CPK, *Arabidopsis* CPKs may differ in their affinity for Ca²⁺ in general and in the presence of their specific substrate in particular. CPK's affinity for Ca²⁺ is many times higher in the presence of substrate than in its absence [22]. The involvement of only specific CPKs in stress responses has already been shown [74]. Identification of the substrates for each CPK and their temporal and spatial expression will be needed for elucidating the pathways of Ca²⁺ control in plants.

Other families, such as the phosphatases, Rbohs and CBL/SOS3s, also contribute to the complexity of Ca²⁺ involvement in the regulation of many processes. Characterization of more Ca²⁺-binding proteins will lead to further understanding of the roles of Ca²⁺ and cross-talk among various components of Ca²⁺-signaling and other messengers in plants.

Materials and methods

Identification of EF-hand-containing proteins

Proteins containing EF hands were first identified using the InterPro Domain Table at MIPS *A. thaliana* database (MATDB) [39]. The protein sequences of the 219 proteins listed at MATDB were obtained and analyzed for EF-hands and other domains using InterProScan [40]. Proteins not

showing EF-hand domains were eliminated from the list. To identify proteins not listed in the InterPro Domain Table at MATDB, BLAST searches were done with three different EF-hand-containing proteins: calmodulin, KIC, and a Ca²⁺-dependent protein kinase (CPK1). BlastP searches were done for KIC and CDPK and BlastP and TblastN for calmodulin [39]. Sequences for proteins showing sequence similarity to these proteins were also checked by InterProScan and any protein containing an EF-hand domain but not found on the InterPro Domain Table was added to the list. A literature search for *Arabidopsis* proteins containing EF-hands was also done using PubMed at NCBI (National Center for Biotechnology Information) [81].

Identification of domains and organellar targeting signals

Information about domains other than the EF-hand domain was collected from the InterProScan searches done for each protein sequence. Targeting information for each protein was obtained from the MATDB general report that includes the results of TargetP [39].

Phylogenetic analysis

The full-length sequences of all proteins identified by InterProScan as containing an EF-hand (including those that are identified by only one prediction program) were aligned using MEGALIGN. A heuristic method using PAUP 4.08a generated 100 trees. A majority-rules consensus tree was computed from the 100 trees. For the CPKs and CRKs, a second alignment was done using the CAM-like domains. A bootstrap method of PAUP.4.06a with 100 bootstraps was used to generate the tree.

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