Supplementary Table S1. L	ist of primers used in this study				
Primer name	Sequence (5'-3')	Restriction Enzyme			
ATM1 F	GGCCCATATGCGTACTCTCCACGGC	Ndel			
ATM1 IQ1 R	GGCCGGTCGACAATAGAAATTCCCCTTTTAAGTTC	Sall			
ATM1 IQ2 F	GGCCGGATCCATGTGTCTCCTGAAGGAACTTAAAAG	BamHI			
ATM1 IQ2 R	GGCCGGTCGACTTAAGTAGCAGCAGCCTTATGCC	Sall			
ATM1 IQ3 F	GGCCCATATGAAAGAGTTTGCTGAGTTACG	Ndel			
ATM1 IQ3 R	GGCCGGTCGACTTACACAACTGATGCATCG	Sall			
ATM1 IQ4 F	GGCCGGATCCATGATACAGTATAAGGGCATAG	BamHI			
ATM1 R	GGCCGGATCCTTAATCCCCTGAACATCTTC	BamHI			
ATM2 F	GGCCCATATGAGGAAAAAGGTTCTTCAAGG	Ndel			
ATM2 IQ1 R	GGCCGGTCGACAACCAATGTTACTTTCCGC	Sall			
ATM2 IQ2 F	GGCCGGATCCATGGCATACTTCCAAAATATGCGG	BamHI			
ATM2 IQ2 F	GGCCCATATGGCATACTTCCAAAATATGCGG	Ndel			
ATM2 IQ2 R	GGCCGGTCGACGTGTATGACTGCACTCAACTC	Sall			
ATM2 IQ3 F	GGCCGGATCCATGAGATTGTTTGACACTGAAGC	BamHI			
ATM2 IQ3 F	GGCCCATATGAGATTGTTTGACACTGAAGC	Ndel			
ATM2 R	GGCCGGTCGACTTATTTTTGTCTTTGCATACTATTG	Sall			
VIII-A IQs F	GGCCGGATCCATGAGGAATCGCACTCTGCATGGC	BamHI			
VIII-A IQs R	GGCCGGTCGACTTAACTAAGCCATCCAATATCCCC	Sall			
VIII-B IQs F	GGCCGGATCCATGCGTGGCATACTTGGATTACAG	BamHI			
VIII-B IQs R	GGCCGGTCGACTTAACTATTTAAGAGTTTACGAG	Sall			
LBb1.3	ATTTTGCCGATTTCGGAAC	N/A			
ATM1-4IQ F bacvirus	AAACCATGGCTCAGAAGGTTACTCC	Ncol			
ATM1-4IQ R bacvirus	TTTACCGGTTCCAATATCCCCTGAACATC	Agel			
ATM2-3IQ F bacvirus	AAACCATGGCATTATCGGCATCGCCG	Ncol			
ATM2-3IQ R bacvirus	TTTACCGGTTTGTCTTTGCATACTATTGAAATG	Agel			
ATM1 gF	GGGAAACGTTTCCTTCACCG	Genotyping			
ATM1 gR	AATTTATACAGAACTGCTCGAAG	Genotyping			
ATM2 gF	CCCGGAGTTCCAGTAGACATCG	Genotyping			
ATM2 gR	TCCGTGGTCACTTGTCTCGTG	Genotyping			
VIII-A gF	ctctgactatgagctagatcg	Genotyping			
VIII-A gR	GTTACCGTTTGGAAGTTGGAC	Genotyping			
VIII-B gF	TGCACAGCTGAGCCACTCTTG	Genotyping			
VIII-B gR	TGCAGTGGCAGATGCAGCTTA	Genotyping			

Supplementary Table S2	. Description of plasmid constructs used in this study		
Construct Name	461	Amino Acide	Vectors
ATM1 IO1	At3G19960	848-878	Nluc
ATM1 IQ1+2	At3G19960	848-904	Nuc pGEX-4-T3
ATM1 IQ1	At3G19960	867-904	Nuc
ATM1 IQ3	At3G19960	890-927	Nluc
ATM1 IQ3+4	At3G19960	890-943	Nuc pGEX-4-T3
ATM1 IQ4	At3G19960	916-943	Nuc
ATM1 Neck	At3G19960	848-943	Nluc, pET28b-GB1
ATM2 IQ1	At5G54280	878-910	Nluc
ATM2 IQ1+2	At5G54280	878-948	Nluc, pGEX-4-T3
ATM2 IQ2	At5G54280	899-948	Nluc
ATM2 IQ3	At5G54280	922-967	Nluc, pGEX-4-T3
ATM2 Neck	At5G54280	878-967	Nluc, pET28b
CaM81	M80836 (Genbank Accession)	Full	Cluc, pET5a
CML13	At1G12310	Full	Cluc, pET30a
CML14	At1G62820	Full	Cluc, pET30a
CML15	At1G18530	Full	Cluc
CML19	At4G37010	Full	Cluc
CML24	At5G37770	Full	Cluc
CML35	At2G41410	Full	Cluc
CML38	At1G76650	Full	Cluc
CML42	At4G20780	Full	Cluc, pET5a
CML6	At4G03290	Full	Cluc
CML8	At4G14640	Full	Cluc
VIII-A Neck	At1G50360	828-930	Nluc
VIII-B Neck	At4G27370	834-913	Nluc
See Methods for the vector	ors used for microscopy		



Supplemental Figure S1. Comparison of Arabidopsis CML13 and CML14 with predicted orthologs. (A) Protein alignment of CML13 and CML14 with orthologs from various plant species. Amino acid residues were shaded based on their percent identity, dark grey if identical, and progressively lighter grey until white as unconserved. Clustal Ω was used for alignment (Sievers and Higgens, 2014) and images were generated using Jalview Version 2.11.2.6. (B) Phylogenetic tree showing relatedness among the proteinss compared in panel A. SeaView (v 5.0, Gouy et al., 2021) was used to generate the tree using neighbour-joining with bootstrapping analysis (1000 reiterations).

Α		-61	-51	-41	-31	-21	-11	-1	10	20	30	40	49
	A.thaliana_CML13/1-148	1						MG K DG L	SDDQVSSMKE	AFMLFDTDGD	SK I A P S E L G I	LMRSLGGN	- PTQAQLKS 52
	A.thaliana_CML14/1-148	1						MSKDGL	SNDOVSSMKE	AFMLFDTDGD	SKIAPSELG I	LMRSLGGN	-PTESQLKS 52
	S.pombe_MLR4/1-141	1 MADEKKKVKKK	TVEECCTCET	ACEAACEAAT			COCERCERVE	KRACESVESVE	SOVOTAFEVE			LERACGON	ANDVELDA 119
	H saniens MYI 7/1-175	1		ASLAASLAAI	FAFAATFAF	MASRKAGT	RGKVAATKOA	ORGSSNVESME	FOADIOEEKE	AFSCIDONRDO	TICKADI RE	TYSOLGKY	SVPEEELDA 76
	H.sapiens MYL9/1-172	1				MSSK RA	K-AKTTKKRP	ORATSNVFAME	DOSOIOEFKE	AFNMIDONRDO	GFIDKEDLHD	MLASLGKN	-PTDEYLEG 72
	M.musculus_MYL9/1-172	1				MSSK RA	K-AKTTKKRP	QRATSNVFAMF	DQSQIQEFKE	AFNMIDQNRD	GFIDKEDLHD	MLASLGKN	-PTDEYLEG 72
	M.musculus_ML12B/1-172	1				MSSK KA	K-TKTTKKRP	QRATSNVFAMF	DQSQIQEFKE	AFNMIDQNRD	GFIDKEDLHC	MLASLGKN	-PTDAYLDA 72
	D.melanogaster_SQH/1-174	1				MSSRKTAG	R - RATTKKRA	QRATSNVFAMF	DQAQIAEFKE	AFNMIDQNRD	GF <u>vek</u> edlhd	MLASLGKN	-PTDDYLDG 74
	C.elegans_MLRH/1-172	1				MASRK	TVNRRQRP	QRATSNVFAMF	DQAQIQEFKE	AFNMIDQNRDO	SF I DQ E DL K C	MFASLGKE	-V∎EQFIDS 70
	S.pombe_MLR1/1-184	1	MF	SSKENSLG -	A K R A P	FSSNTT	SSQRVAAQAA	KRASSGAFAQL	TSSQ IQELKE	AFALLOKDOD	SNIGREDVKT	MLTSLNQD	-ASEDSINH 88
	S caravisiaa MLC2/1-163	1				<u>M</u> A	SIKR <u>R</u> L	DHSESLTENOL	TODY INKLED	AFELFORDEDED	SE IKKDALKI	TUXATLONT	-VMEDQLDA 63
	D.rerio MIRSA/1-169	1				МАРККА	KRRAAG	GEGSSNVESME	FOSDIOEYKE	AFTIIDONRDO	TISKODLEG	VLASMGOL	NVKNEELEA 70
		-							- No - No In Solari - Maria				
		58	63	73	81	90	100	110	120	130	136	146	+8
	A.thaliana_CML13/1-148	53 I I A S E N	- L S S P F D F N R F	LDLMAKHL -	-KTEPF - DRQ	LRDAFKVLDK	EGTGFVAVAD	LRHILTSIGEK	LEPNEFDEWI	KEVDVGSD	GKIRYEDF	IARMVAK -	148
	A.thaliana_CML14/1-148	53 I ITTE N	LSSPFDENRF	LDLMAKHL -	KTEPF - DRQ	LRDAFKVLDK	EGTGFVAVAD	LRHILTSIGEK	LQPSEFDEWI	KEVDVGSD	GKIRYEDF	IARMVAK -	148
	S.pombe_MLR4/1-141	4/ 1ES	-LPAEVDMEQE	LQVLNRPNGF	DMPGD-PEE	FVKGFQVFDK	DATEMIGVGE	LRYVLISLGEK	LSNEEMDELL	KGVPV-KD		VQMILAN-	141
	H capiens MYL7/1-175	77 MLOEGKG		LTLEGEKING	SGANDEDEV	I SAERMEDD	SCKOVVNKDE	FREMEMOREDK	ESPAEVEOME	ALTRMDIA		CYLITHOD	EVEE 179
	H.sapiens_MYL9/1-172	73 MMSEAPG	PINETME	LTMFGEKLNO	TOPEDV	IRNAFACEDE	EASGFIHEDH	LRELLTTMGDR	FTDEEVDEMY	REAPIDKK	GNENYVEE	TRILKHGA	KDKDD 172
	M.musculus_MYL9/1-172	73 MMNEAPG	PINETME	LTMFGEKLNO	TDPEDV	IRNAFACEDE	EASGFIHEDH	LRELLTTMGDR	FTDEEVDEMY	REAPIDKK	GNFNYVEF	TRILKHGA	KDKDD 172
	M.musculus_ML12B/1-172	73 MMNEAPG	PINFTMF	LTMFGEKLNG	G T D P E D V	IRNAFACFDE	EATGTIQEDY	LRELLTTMGDR	FTDEEVDELY	REAPIDKK	G N F N Y I E F	TRILKHGA	KDKDD 172
	D.melanogaster_SQH/1-174	75 MMNEAPG	PINFTMF	LTLFGERLQC	6 T D P E D V	IKNAFGCFDE	ENMGVLPEDR	LRELLTTMGDR	FTDEDVDEMY	REAPIK-N	G L F DY L E F	TRILKHGA	KDKDEQ 174
	C.elegans_MLRH/1-172	71 M I NEAPG	AQPINETMF	LTLFGEKLTO	G T D P E E V	IRNAFQCFDE	DNSGKLNEEH	LRELLTTMGER	YSEEQVDELF	RDAPIK-G	GQFDYVEF	TRMLKHGT	KDKDEA 172
	S.pombe_MLR1/1-184	89 MFESINP	PINLAAF	LTAMOGSMLCF	R ISPRND	LLEAFSTFDD	TQSGKIPIST	MRDALSSMGDR	MDPQEVESIL	RSYTS H	GVEYYEKF	VDAIAGSK	DSN 184
	D.discoideum_MLR/1-161	64 MFAEADII	CEECVCEPIE		2ISNEQI		CHOLNVOLNE	VIDCLKEACEE	NDEEEEAKIE	SISENE-Q		VNILFSKK	161
	D rerio MIRSA/1-169	71 MIKEASG	PINETVE	LTMEGEKLKO	ADPEDV	IVSAEKVIDP	EGTGSIKKEE	LEELLTTOCDR	ETAFEMKNLV	AAFPPDVA		CYVITHGE	EKEE 163
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Supplemental Fig S2. Comparison of Arabidopsis CML13/14 with regulatory myosin light-chains (RLCs) from various species of eukaryotes. (A) Protein alignment of CML13 and CML14 with myosin RLCs from multiple species. Amino acid residues were shaded based on their percent identity, dark grey if identical, and progressively lighter grey until white as unconserved. Clustal Ω was used for alignment (Sievers and Higgens, 2014) and images were generated using Jalview Version 2.11.2.6. (B) Phylogenetic tree showing relatedness among myosin RLCs compared in panel A. SeaView (v 5.0, Gouy et al., 2021) was used to generate the tree using neighbour-joining with bootstrapping analysis (1000 reiterations).



Supplemental Fig S3. Immunoblot showing expression of C-Luc-CaM/CML fusion proteins in N. benthamiana. Leaves of N. benthamiana were infiltrated with Agrobacteria expressing C-Luc (bait) vectors and N=Luc (prey) vectors as described in Materials and Methods. Immunoblots (upper panel) using anti-C-terminal luciferase antisera, or Coomassie-stained gels showing equal lane loading, following SDS-PAGE of samples of total, clarified protein extracts (~25ug) from leaves used in the split-luciferase assays.





CML14

Supplemental Figure S4. Whole-leaf image of split-luciferase protein-protein interaction bioluminescent assays using transiently transformed N. benthamiana. A representative image is shown for interaction analysis of CML13, CML14, CaM, and CML42 (negative control) as baits (as C-Luc fusion proteins) with the neck region of ATM1 (as N-luc fusion) prey protein.



Supplementary Fig. S5. *In vitro* protein-interaction overlay assays of CaM, CML13, CML14, and CML42 with IQ domains of ATM1 and ATM2. Triplicate samples (200 ng) of pure, recombinant proteins ATM1 or ATM2 full-neck region (ATM1, ATM2), paired IQ domains (ATM1-IQ1+2, -IQ3+4, ATM2-IQ1+2), or the isolated IQ domain of ATM2 (ATM2-IQ3), were spotted onto nitrocellulose, blocked with 5% casein in TBST, and incubated with 200 nM of CaM, CML13, CML14, or CML42, as indicated, each of which was covalently labeled with the infra-red dye, 680RD-NHS as described in Materials and Methods. Recombinant GB1 was tested as a negative control. Protein-protein interaction was assayed in the presence of 2 mM CaCl₂ (Ca) or 5 mM EGTA (Apo) and detected using the LI-COR Odyssey-XF infra-red imager. Representative Coomassie-stained blots are presented along the top row. Data are representative of a minimum of three independent experiments. See Supplementary Table 2 for a description of the primary sequence from the neck regions of ATM1 and ATM2 that were tested for binding.







Supplementary Figure S7. Representative phasor plots are shown corresponding to the FRET-FLIM analysis presented in Figure 3. This figure highlights the distinctions in the fluorescence decay of GFP (donor) fused to CMLs or CaM alone and when coexisting with RFP (acceptor) fused to the different class VIII myosin members. Each image represents one cell. Quantitative analysis of the whole experiment is shown in figure 3. CaM81 refers to the conserved CaM isoform (see Materials and Methods).