Plant metacaspases type I (MCA-Is), the closest structural homologs of caspases, are key proteases in stress-induced regulated cell death processes in plants. However, no plant MCA-Is have been characterized in vitro to date. Here, we show that only plant MCA-Is contain a highly hydrophobic loop within the C terminus of their p10 domain. When removed, soluble and proteolytically active plant MCA-Is can be designed and recombinantly produced. We show that the activity of MCA-I depends on calcium ions and that removal of the hydrophobic loop does not affect cleavage and covalent binding to its inhibitor SERPIN. This novel approach will finally allow the development of tools to detect and manipulate the activity of these cysteine proteases in vivo and in planta.

Keywords: cysteine protease; green algae; programmed cell death; proteolysis; regulated cell death; trypsin-like
prolonged incubation in calcium [7,16]. It should be noted, however, that while several type II metacaspases have been successfully recombinantly expressed in bacteria to date [9,14,17–22], only type I metacaspases from yeasts [23,24] or protozoa [25–27], but none from any photosynthetic organism, have been heterologously expressed and characterized in vitro, despite intensive research in planta.

Of the two metacaspase types (type I and type II) found in green plants, increasing evidence suggests that type I metacaspases are specifically involved in stress-induced cell death [28]. AtMCA-Ia (before AtMC1) and AtMCA-Ib (before AtMC2), both type I metacaspases, antagonistically control cell death: while AtMCA-Ia is its positive regulator, AtMCA-Ib acts as a negative regulator of the pathogen-triggered hypersensitive cell death response in Arabidopsis thaliana [29]. Moreover, type I metacaspases have been linked to cell death mechanisms not only in land plants [28,30,31], but also in microalgae [32–34]. However, the development of specific tools for their further characterization has up to now been hampered by the inability to produce these proteases recombinantly.

Here, we show that type I metacaspases of green algae and land plants contain an additional highly hydrophobic region within the C terminus of their p10 domains. This motif is absent in type I metacaspases of prokaryotes, yeasts and protozoa and in the p10 domains of type II and III metacaspases. We therefore designed, cloned and recombinantly expressed the core p20-p10 sequence lacking this hydrophobic motif of the only type I metacaspase of the model green alga Chlamydomonas reinhardtii (CrMCA-I) in E. coli. Removal of the hydrophobic region resulted in the production of a soluble protease, which was expressed in high quantities. To demonstrate that removal of the hydrophobic region did not affect proteolysis, we analysed its catalytic properties and characterized its interaction with the putative native inhibitor CrSERPIN.

Methods

Analysis of metacaspase primary amino acid sequences

Metacaspase sequences were chosen based on the target type and organism from the study where all p20-containing proteins were recently identified [44]. A list of all sequences analysed in this study is presented in Table S1. Sequences were aligned using Promals [45], and Jalview [46] was used to present the alignments.

Cloning of CrMCA-I and CrSERPIN constructs

Genes encoding all four CrMCA-I variants and CrSERPIN were ordered as synthetic genes from Twist bioscience (South San Francisco, CA, USA) with codon usage optimized for expression in E. coli. CrMCA-I constructs contained NcoI and XhoI restriction sites while CrSERPIN contained XbaI and XhoI restriction sites together with the RBS at the 5′ end of the sequence. Both, synthetic genes and the destination pET28b(+) vector, were cut with respective restriction enzymes and ligated. The pET28_CrMCA-I_CL was subsequently used as a template to produce the catalytically inactive variant CrMCA-I_CL_C. The mutation was introduced using the Quick-Change Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer’s instructions. All nucleotide sequences were verified by DNA sequencing.

Expression and purification of CrMCA-I_CL and CrSERPIN constructs

Escherichia coli BL21(DE3) bacteria were transformed with the expression plasmids and grown in shaker cultures at 37 °C in autoexpression medium containing 50 μg·mL−1 kanamycin for 6 h and then moved to 25 °C, where they were left shaking overnight [47]. The cell pellet collected from 500 mL of bacterial culture was resuspended in 20 mL of resuspension buffer (20 mM HEPES, pH 7.5, 500 mM NaCl, 20 mM imidazole) and sonified 5 × 30 s (80% power) on ice. Following centrifugation at 25 000 g for 10 min to remove insoluble debris, the supernatant was applied to a preloaded nickel ion HisTrap column (Cytiva, Marlborough, MA, USA) connected to an AKTA FPLC system. After washing with resuspension buffer, bound proteins were eluted in the same buffer but with 250 mM imidazole. Peak fractions were collected, concentrated using an Amicon filtration unit equipped with a 10-kDa exclusion membrane, and applied to a Superdex 75 size-exclusion chromatography column (GE Healthcare Life Sciences, Chicago, IL, USA) connected to an AKTA FPLC system. The column was equilibrated in 20 mM HEPES, pH 7.5, 500 mM NaCl, and a flow rate of 0.5 mL·min−1 was used to separate proteins. Peak proteins were collected and stored at −80 °C until further use.

Analysis of protein interactions by size-exclusion chromatography

Purified proteins (final concentration of 2 mg·mL−1) were applied to a Superdex 75 size-exclusion chromatography column connected to AKTA FPLC system. The column was equilibrated in 20 mM HEPES pH 7.0, 150 mM NaCl, and 5 mM CaCl2 where specifically indicated. A flow rate of 0.5 mL·min−1 was used to separate all proteins.
Kinetic assays

Protease activity was measured by monitoring the release of the fluorescent group AMC (7-amino-4-methylcoumarin) from various substrates (all from PeptaNova, Sandhausen, Germany) at excitation and emission wavelengths of 383 nm and 455 nm, respectively, using a Jasco FP-6300 spectrophotometer (Silver Spring, MD, USA). Assays were performed in 20 mM HEPES (pH 7.0) containing 150 mM NaCl, 5 mM CaCl₂, and 5 mM DTT at 20 °C in a 1 x 1 cm² quartz cuvette. To determine the pH profile of CrMCA-I_core_loopless, the buffers used were 100 mM acetate (pH 4–pH 5.5), 100 mM MES (pH 6–pH 6.5), 100 mM HEPES (pH 7.0–pH 8.0), 100 mM Tris (pH 8.5–pH 9) and 100 mM CAPS (pH 9.5–pH 11). All assays were performed with 5 μM substrate and 10 nM enzyme unless otherwise stated. Inhibitors were used at the indicated concentrations. Data were analysed using GRAPHPAD PRISM 9.1.1 (GRAPHPAD Software Inc., La Jolla, CA, USA).

Intrinsic tryptophan fluorescence and determination of calcium-binding constants

The interaction of wild-type and mutant CrMCA-I_CL proteins with calcium was monitored by measuring intrinsic tryptophan fluorescence with a Jasco FP-6300 spectrophotometer using the same experimental set-up as previously described for GtMCA-III [7], with the sole exception of emission maxima, which were used to quantify the interaction between proteins and CaCl₂. The intensities of fluorescence at 332 nm were used for the proteolytically active and at 334 nm for the proteolytically inactive variant.

Results and discussion

Only type I metacaspases of the green lineage contain an additional hydrophobic region within the p10 domain

Type I metacaspases are the only metacaspase type with two resolved protein structures: one from Trypanosoma brucei, TbMCA-Ib (before TbMC2, Fig. 1A) [35], and the second from Saccharomyces cerevisiae, ScMCA-I (before Yca1, [36]). Recently, the first structure of an Arabidopsis type II metacaspase, AtMCA-IIa (before AtMC4), was also resolved [15]. However, despite intensive research over the past two decades, no recombinant and proteolytically active
proteases from any organism of the green lineage could be produced heterologously in *E. coli*.

To understand why certain type I and all type II and type III metacaspases are readily expressed as soluble proteases and those from the green lineage are not, we aligned and compared their primary amino acid sequences. The most obvious suspect for the solubility problems of type I metacaspases was the long N-terminal domain, found in representatives of yeasts, red and green algae, protozoa and plants and is absent in prokaryotes and in organisms formed by secondary endosymbiosis (Fig. 1B, in blue). However, since yeast [24] and protozoan [25,37] type I metacaspases containing it can be expressed as soluble, active proteases, the presence of the N-terminal domain does not seem to be the reason for insolubility.

We next performed a detailed analysis of the core sequence, p20-p10, and discovered that only in type I metacaspases from green algae and land plants, an additional sequence is present at the C terminus of the p10 domain. Consistent with the numbering in TbMCA-Ib, this region is located between helix 5 (\(a_5\)) and strand 8 (\(b_8\)) (Fig. 1C, highlighted by the dashed red line). According to the amino acid position of this motif, which starts at amino acid residue 363 according to the numbering of CrMCA-I, and because of its still unknown folding, we designated this hydrophobic region as a 360-loop, although protein structure prediction servers suggested that it partially forms a helix (Fig. 2A). This region is approximately 25 amino acid residues long and highly hydrophobic, being composed mainly of Ala, Gly, Leu and Ser residues. Moreover, in contrast to the diverse sequences of N-terminal domains, it shows high amino acid conservation from the earliest Bryophyta (*Marchantia polymorpha*, MpMCA-I) to angiosperms (*Zostera marina*, ZmMCA-I)

![Fig. 2. Removal of the N-terminal prodomain and the 360-loop enables expression of soluble, proteolytically active CrMCA-I in *E. coli*.](image) (A) The model of CrMCA-I is shown in three orientations. The core p20-p10 is coloured in sand, the N-terminal domain in blue and the 360-loop in red. The helices and strands are numbered according to the TbMCA-Ib structure. Structures were modelled using I-TASSER [38] and images were generated using PyMOL (DeLano Scientific; http://www.pymol.org). (B) Schematic representation of the four CrMCA-I variants. All expressed proteins contain a C-terminal hexahistidine tag, denoted with C-terminal black shading on all p10 domains. The red p10 domain denotes a p10 domain with the 360-loop and the sand-coloured p10 represents the p10 domain lacking it. The amino acid sequences of all four variants are shown and aligned in Fig. S2. (C) Lysate fractions before (lys-) or after (lys+) autoinduction and soluble (sol) or insoluble (insol) fractions of the lysates after induction were separated on 12% SDS/PAGE gels. The expected positions of both variants containing the N-terminal domain are denoted with a blue arrow. The expected position of CrMCA-I_core with a red arrow and CrMCA-I_core_loopless with a sand-coloured arrow. Rounded rectangles above the gel are coloured white where no proteolysis of a fluorogenic Z-FR-AMC was observed, whereas the green colour represents positive fluorescence.
and Arabidopsis thaliana, AtMCA-Ia, Table S1 and Fig. S1), which could also imply a conserved function. More importantly, this motif is absent in the p10 domains of all type II and III metacaspases, as well as in type I metacaspases present in organisms not included in the Viridiplanta clade (Fig. S1). The 360-loop is therefore most likely a derived trait in type I metacaspases of green algae and land plants.

The truncated variant of CrMCA-I, the only Chlamydomonas reinhardtii type I metacaspase, can be expressed as a soluble, active protease in E. coli

We aimed to test whether by removal of the N-terminal domain, or the 360-loop, or both, we could express a plant type I metacaspase as a soluble and active protease in E. coli. As a model protein, we chose the only type I metacaspase found in the model green alga Chlamydomonas reinhardtii, CrMCA-I.

For this reason, we constructed four protein variants: the wild-type, containing the full-length sequence of the protein (NCBI protein ID: XP_001696956.1), CrMCA-I, the variant containing the p20-p10 core sequence but lacking the N-terminal domain and starting with Thr119, CrMCA-I-core, the full-length sequence lacking the 360-loop (AMet360- Ser388), CrMCA-I-loopless, and the variant lacking the N-terminal domain and the 360-loop, CrMCA-I_core_loopless. The domain organization of the four CrMCA-I variants is shown in Fig. 2B, and their amino acid sequences are listed and aligned in Fig. S2.

All four constructs, designed as synthetic genes with codon-optimized sequences for expression in E. coli, were cloned into a pET28b (+) vector in frame with a C-terminal His6-tag. After overnight expression at 25 °C, all four proteins were detected using anti-His antibodies in cell lysates (Fig. S3). However, the two variants containing the N-terminal domain (CrMCA-I and CrMCA-I_loopless) were expressed in lower quantities than the two core variants, that is those lacking the N-terminal domain (CrMCA-I_core and CrMCA-I_core_loopless). The highest expression was observed for the CrMCA-I_core, with a visible protein band in the lysate after expression. However, CrMCA-I_core was mostly expressed in the insoluble fraction (Fig. 2C). In contrast, protein bands corresponding to CrMCA-I_core_loopless were visible in the soluble fraction and could be further affinity purified.

Since yeast ScMCA-I is involved in clearance of insoluble protein aggregates [23] and is recruited to the insoluble protein deposits [39] and Arabidopsis AtMCA-Ia partially localizes to insoluble aggregates [31], the deposition of CrMCA-I in the insoluble fraction did not necessarily imply misfolding and thus inactivity of the expressed protein. To assess whether metacaspase-like proteolytic activity could be detected in any of the obtained fractions, we added protein extracts from the soluble or insoluble fractions to the reaction mixture containing the fluorogenic substrate Z-FR-AMC, calcium ions and DTT. Fluorescence was detected only in the two fractions containing soluble CrMCA-I_core_loopless protein (lysate and the soluble fraction). This indicated not only its presence but also proteolytic functionality (Fig. 2C). For this reason, we used the CrMCA-I_core_loopless variant for all subsequent analyses and will henceforth use the term CrMCA-I_CL for this construct for simplicity.

Removal of the 360-loop does not affect the proteolytic activity of CrMCA-I_CL

Overexpression of CrMCA-I_CL led to the production of a soluble protein with an approximate size of 30 kDa in very high yields (approximately 30 mg·L⁻¹ overexpression culture). Due to high expression, CrMCA-I_CL was the predominant protein in the eluates after the nickel-chromatography purification (Fig. S4A). Furthermore, minor impurities were successfully removed by a size-exclusion chromatography on a Superdex 75 column (Fig. S4B). The protein could be concentrated to 20 mg·mL⁻¹ without precipitation and separation on size-exclusion chromatography revealed no multimeric states, as it eluted as a monomer with an elution volume corresponding to a protein of approximately 30 kDa (Fig. 3A). The activity of CrMCA-I_CL was proven to be calcium-dependent, as no detectable proteolysis against the fluorogenic substrate Z-FR-AMC was observed in the absence of calcium ions. Measuring proteolytic activity at increasing calcium concentrations revealed that the highest proteolysis is reached at low millimolar concentrations (Fig. 3B), consistent with the calcium-dependent properties of the type I metacaspase TbMCA-Ib [16]. Furthermore, titration with calcium revealed the presence of two calcium-binding sites: one with calculated dissociation constant at low and the second at high micromolar concentrations (Fig. 3C), consistent with those obtained for TbMCA-Ib and type III metacaspase GtMCA-III (before GtMC2, 7). Similar to other type I metacaspases, CrMCA-I_CL exhibited greatest proteolytic activity at neutral pH and retained more than 50% of it at pH values higher than 6.0 and lower than 8.0 (Fig. 3D).

To test its affinity for various substrates containing the Arg residue at the P1 position, we measured the
$K_M$ values for three fluorogenic substrates: FR-AMC, R-AMC or VRPR-AMC and plotted initial proteolytic velocities against substrate concentrations (Fig. 3E). For the three substrates tested, the highest affinity was measured for FR-AMC with the $K_M$ value of 38.4 $\mu$M, which is comparable to the $K_M$ value of the orthocaspase MaOC1 [3]. The lowest $K_M$ value and therefore the lowest affinity was observed for the VRPR-AMC substrate, which is approximately 4 times lower compared to AtMCA-IIa [9]. To test whether CrMCA-I_CL recognized substrates with Lys residue at the P1 position, K-AMC and VLK-AMC substrates were used together with K-AMC for comparison (Fig. 3F). No proteolysis was observed for the K-AMC substrate, whereas approximately 20-fold lower cleavage efficiency was measured for the VLK-AMC substrate compared with R-AMC. These results suggest that CrMCA-I_CL can cleave after Lys residue, but preferentially cleaves substrates with Arg residue at the P1 position. Lower preference for the VRPR tetrapeptide was reflected also in levels of inhibition, while the addition of 1 $\mu$M VRPR-CMK reduced the proteolytic activity of CrMCA-I_CL for approximately 60%, FFR-CMK almost abolished it, falling below 5% (Fig. 3G). In accordance with previous inhibition studies, no decrease in activity was observed when caspase...
inhibitor Z-VAD-FMK or papain-like cysteine inhibitor E-64 were used. On the contrary, addition of EDTA in excess over calcium ions completely abolished the activity of CrMCA-I_CL.

**CrMCA-I_CL binds calcium independently of its proteolytic ability**

Since type II metacaspases are readily degraded after initial activation with CaCl₂ ions, whereas type III metacaspases remain stable and unprocessed after several hours, we incubated CrMCA-I_CL in 5 mM CaCl₂ and subjected it to a reducing SDS/PAGE gel after certain time points. The p20-p10 core remained intact as no cleavage, indicating autoproteolytic events, was observed even after 24 h of incubation (Fig. 3H). These results are consistent with *in planta* experiments for AtMCA-Ia, which exists in cells in two forms: either as a full-length protein or as a core variant lacking the N-terminal domain [40]. Similarly, no cleavages within the core p20-p10 polypeptide were observed for TbMCA-Ib [35,41].

Against our expectations, size-exclusion chromatography in the presence of 5 mM CaCl₂ revealed that proteolytically active CrMCA-I_CL underwent large conformational changes compared to its nonactivated form, as it eluted at visibly lower elution volumes (Fig. 3I). While in the absence of calcium CrMCA-I_CL migrated as a monomer and eluted at approximately 12.5 mL, corresponding to a protein with an approximate molecular weight of 35 kDa, the elution peak shifted for almost 2.5 mL to 14.9 mL in the presence of 5 mM CaCl₂. Since the SDS/PAGE analysis confirmed lack of any intramolecular autoproteolysis even at long incubation times with calcium ions, we suggest that large conformational changes occur within the protein in the active form, largely reducing the protein’s hydrodynamic radius, resulting in increased retention volumes. To disprove the possibility that protein forms a dimer, which elutes at 12.5 mL, we conducted crosslinking experiments using 0 A crosslinker (formaldehyde) in the absence and presence of calcium ions at two different protein concentrations (10 and 100 µM). Separation of the proteins after the incubation with 50 µM formaldehyde on the SDS/PAGE is shown in Fig. S5. Only one band, corresponding to the monomeric protein, and no multimeric structures are visible in the absence or presence of calcium and the crosslinker, thus confirming that the observed shift on the...
size-exclusion chromatography can be attributed to the change in conformation and not size of the protein.

To assess whether CrMCA-I_CL can bind calcium ions in its proteolytically inactive state, we constructed a catalytically inactive variant with substituted catalytic Cys265 residue with Ala residue (CrMCA-I_CL_CA, Fig. S6A) and repeated the size-exclusion chromatography migration. The proteolytically inactive variant eluted at the same elution volumes as the proteolytically active variant in both the absence and the presence of calcium, despite being completely proteolytically inactive (Fig. S6B), suggesting that calcium ion binding is independent of proteolytic ability. However, intrinsic tryptophan fluorescence revealed that the proteolytically active and inactive variants had different calcium-binding affinities. Compared with the active variant, the proteolytically inactive CrMCA-I_CL_CA reached saturation at lower calcium concentrations (Fig. S6C). This observation is not consistent with the hypothesis that the second – the low-affinity – calcium-binding site is formed and active only in the simultaneous presence of the substrate and calcium ions [42]. It therefore remains to be further investigated in detail.

Interaction with CrSERPIN is dependent on proteolytic activity but not on the presence of the 360-loop in CrMCA-I_CL

Finally, to confirm that removal of the 360-loop does not affect the interaction of CrMCA-I_CL with known interaction partners, we assessed its binding to an inhibitor of metacaspases, SERPIN. Although these proteins were initially identified as inhibitors of serine proteases, they were shown to successfully bind and inhibit the activity of both type I [40] and type II [43] metacaspases. We therefore cloned and expressed the only gene encoding a SERPIN-like protein from Chlamydomonas, CrSERPIN, and used it to test its interaction with CrMCA-I_CL. We first analysed their putative comigration under physiological conditions using size-exclusion chromatography. In the absence of calcium ions, both proteins eluted at volumes, corresponding to their monomeric molecular mass sizes. No shifts were observed for either protein when mixed in equimolecular ratio (Fig. 4A). However, when the two proteins were mixed in the presence of calcium ions, a new peak appeared at approximately 10.7 mL, indicating the formation of a larger complex (Fig. 4B).

Fig. 5. Schematic representation of the conformational changes in CrMCA-I_CL and its proteolytically inactive variant CrMCA-I_CL_CA. In the absence of calcium ions, the protein is in an elongated conformation and is proteolytically inactive (His and Cys residues marked in red). Addition of calcium ions at low micromolar concentrations activates the high-affinity calcium-binding site formed by four Asp residues in the p20 domain (grey middle region). Higher calcium concentrations (in the low millimolar range) activate the low-affinity binding site (two Asp residues in the p20 and two in the p10 domain, green edges of both domains), resulting in a conformational change that largely reduces the hydrodynamic radius of the protein. This activates the active site (His and Cys residues marked in green), allowing proteolysis of substrates to occur. In the case of SERPIN, cleavage of SERPIN (marked with an asterisk) leads to the formation of a covalent complex between the protease and the inhibitor. No activity or cleavage occurs in proteolytically inactive CrMCA-I_CL-CA variant despite the conformational change of the protease.
To further determine whether a covalent bond is also formed between the protease and the inhibitor in the case of CrMCA-I_CL, we incubated the two proteins in increasing CrMCA-I_CL:CrSERPIN molar concentrations in the presence of calcium ions and analysed the reaction mixture under nonreducing or reducing conditions on the SDS/PAGE gel. Under nonreducing conditions, we observed cleavage of CrSERPIN, which was most evident at high CrMCA-I_CL:CrSERPIN ratios, as the band corresponding to the full-length CrSERPIN (denoted with a violet arrow) disappeared and a new one at approximately 38 kDa appeared (denoted with a violet asterisk) (Fig. 4C). Nevertheless, when the same samples were separated on the SDS/PAGE gel in the presence of a reducing agent, no complex formation was observed, confirming the formation of a covalent thioester bond between the inhibitor and active protease (Fig. 4D). Same set of experiments was performed also using the proteolytically inactive CrMCA-I_CL_CA variant, where no interaction between the two proteins occurred either in the absence or in the presence of calcium ions (Fig. 4E–F). This is explained by the inability of CrMCA-I_CL_CA to cleave CrSERPIN (Fig. 4G–H), thus not rendering it active to bind and inhibit the protease. Schematic representation of CrMCA-I_CL activation, activity, binding and cleavage of CrSERPIN is shown in Fig. 5.

Our results suggest that by removal of the highly hydrophobic 360-loop in the p10 domain and the N-terminal prodomain a highly soluble and proteolytically active plant type I metacaspase can be designed and produced. This finding therefore opens up new horizons for studies of proteolytic mechanisms of type I metacaspases as well as for the development of novel tools for their further in vitro and in planta characterization. Even though, according to our results, the removal of the 360-loop does not affect the proteolytic ability of the protein, the physiological role of this hydrophobic region remains to be elucidated. This not only incudes protein’s localization and interaction with other cellular macromolecules but also its proteolytic activation, activity and specificity.

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Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data accessibility

The data that support the findings of this study are available in presented figures and/or the supplementary material of this article.

Author contributions

Conceptualization, MK; Methodology, KPvM and TP; Investigation, KPvM, TP, and MK; Writing – Original Draft, MK; Writing –Review and Editing, KPvM, TP, and MK; Supervision, MK.

References

1 Rawlings ND, Barrett AJ, Thomas PD, Huang X, Bateman A and Finn RD (2018) The MEROPS database of proteolytic enzymes, their substrates and inhibitors in 2017 and a comparison with peptidases in the PANTHER database. Nucleic Acids Res 46, D624–D632.
2 Uren AG, O’Rourke K, Aravind LA, Pisabarro MT, Seshagiri S, Koonin EV and Dixit VM (2000) Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. Mol Cell 6, 961–967.
3 Klemenčič M, Novinec M and Dolinar M (2015) Orthocaspases are proteolytically active prokaryotic caspase homologues: the case of Microcystis aeruginosa. Mol Microbiol 98, 142–150.
4 Choi CJ and Berges JA (2013) New types of metacaspases in phytoplankton reveal diverse origins of cell death proteases. Cell Death Dis 4, e490.
5 McLuskey K and Mottram JC (2015) Comparative structural analysis of the caspase family with other clan CD cysteine peptidases. Biochem J 466, 219–232.
6 Acosta-Maspons A, Sepulveda-García E, Sánchez-Baldoquín L, Marrero-Gutiérrrez J, Pons T, Rocha-Sosa M and González L (2014) Two aspartate residues at the putative p10 subunit of a type II metacaspase from Nicotiana tabacum L. may contribute to the substrate-binding pocket. Planta 239, 147–160.
7 Klemenčič M and Funk C (2018) Type III metacaspases: calcium-dependent activity proposes new function for the p10 domain. New Phytol 218, 1179–1191.
8 Minina EA, Staal J, Álvarez VE, Berges JA, Berman-Frank I, Beyaert R, Bidle KD, Bornancin F, Casanova M et al. (2020) Classification and nomenclature of metacaspases and paracaspases: no more confusion with caspases. Mol Cell 77, 927–929.
9 Vercaemmen D, van de Cotte B, De Jaeger G, Eeckhout D, Casteels P, Vandepoele K, Vandenberge I, Van Beeumen J, Inzé D and Van Breusegem F (2004) Type II metacaspases Atmc4 and Atmc9 of Arabidopsis thaliana cleave substrates after arginine and lysine. J Biol Chem 279, 45329–45336.

10 Fortin J and Lam E (2018) Domain swap between two type-II metacaspases defines key elements for their biochemical properties. Plant J 96, 921–936.

11 Moss CX, Westrop GD, Juliano L, Coombs GH and Mottram JC (2007) Metacaspase 2 of Trypanosoma brucei is a calcium-dependent cysteine peptidase active without processing. FEBS Lett 581, 5635–5639.

12 Zhang Y and Lam E (2011) Sheathing the swords of death. Plant Signal Behav 6, 2051–2056.

13 Machado MFM, Marcondes MF, Juliano MA, McLuskey K, Mottram JC, Moss CX, Juliano L and Oliveira V (2013) Substrate specificity and the effect of calcium on Trypanosoma brucei metacaspase 2. FEBS J 280, 2608–2621.

14 Watanabe N and Lam E (2011) Calcium-dependent Activation and Autolysis of Arabidopsis Metacaspase 2d. J Biol Chem 286, 10027–10040.

15 Zhu P, Yu X-H, Wang C, Zhang Q, Liu W, McSweeney S, Shanklin J, Lam E and Liu Q (2020) Structural basis for Ca+2-dependent activation of a plant metacaspase. Nat Commun 11, 2249.

16 Gilio JM, Marcondes MF, Ferrari D, Juliano MA, Juliano L, Oliveira V and Machado MFM (2017) Processing of metacaspase 2 from Trypanosoma brucei (TbMCA2) broadens its substrate specificity. Biochimica et Biophysica Acta 1865, 388–394.

17 Bozhkov PV, Suarez MF, Filonova LH, Daniel G, Zamyatin AA, Rodriguez-Nieto S, Zhivotovsky B and Smertenko A (2005) Cysteine protease mcl-I-Pa executes programmed cell death during plant embryogenesis. PNAS 102, 14463–14468.

18 He R, Drury GE, Rotari VI, Gordon A, Willer M, Farzaneh T, Woltering EJ and Gallois P (2008) Metacaspase-8 modulates programmed cell death induced by ultraviolet light and H2O2 in arabidopsis. J Biol Chem 283, 774–783.

19 Piszczek E, Dudkiewicz M and Sobczak M (2011) Molecular cloning and phylogenetic analysis of cereal type II metacaspase cDNA from wheat. Biol Plant 55, 614.

20 Hander T, Fernández-Fernández AD, Kumpf RP, Willems P, Schatowitz H, Rombaut D, Staes A, Nolf J, Pottie R et al. (2019) Damage on plants activates Ca2+-dependent metacaspases for release of immunomodulatory peptides. Science [Internet]. Mar 22 [cited 2021 Apr 17]:363(6433). Available from: https://science.sciencemag.org/content/363/6433/eaar7486

21 Mata MT, Palma A, García-Gómez C, López-Parages M, Vázquez V, Cheng-Sánchez I, Sarabia F, López-Figueroa F, Jiménez C and Segovia M (2019) Type II-Metacaspases are involved in cell stress but not in cell death in the unicellular green alga Dunaliella tertiolecta. Microbial Cell 6, 494–508.

22 Shen W, Liu J and Li J-F (2019) Type-II Metacaspases mediate the processing of plant elicitor peptides in arabidopsis. Molecular Plant 12, 1524–1533.

23 Lee REC, Brunette S, Puente LG and Megeney LA (2010) Metacaspase Yc1 is required for clearance of insoluble protein aggregates. PNAS 107, 13348–13353.

24 Leang L, McDonald MC, Mineo CR, Jones B, Barker T, Gagliardi C and Fox KM Identification and characterization of Schizopyllum commune type I metacaspases. Biochem Biophys Res Commun 20, 100706. https://doi.org/10.1016/j.bbrep.2019.100706.

25 Lee N, Bertholet S, Debrabant A, Muller J, Duncan R and Nakhasi HL (2002) Programmed cell death in the unicellular protozoan parasite Leishmania. Cell Death Differ 9, 53.

26 Proto WR, Castanys-Munoz E, Black A, Tetley L, Moss CX, Juliano L, Coombs GH and Mottram JC (2011) Trypanosoma brucei Metacaspase 4 Is a Pseudopeptidase and a Virulence Factor. J Biol Chem 286, 39914–39925.

27 Li M, Wang H, Liu J, Hao P, Ma L and Liu Q (2016) The Apoptotic Role of Metacaspase in Toxoplasma gondii. Front Microbiol 6, https://doi.org/10.3389/fmicb.2015.01560

28 Berenguer E, Minina EA, Carneros E, Bárány I, Bozhkov PV and Testillano PS (2020) Suppression of metacaspase- and autophagy-dependent cell death improves stress-induced microspore embryogenesis in brassica napus. Plant Cell Physiol 61, 2097–2110.

29 Coll NS, Vercaemmen D, Smidler A, Clover C, Breusegem FV, Dangl JL and Epple P (2010) Arabidopsis Type I metacaspases control cell death. Science 330, 1393–1397.

30 Minina EA, Filonova LH, Fukada K, Savenkov EI, Gogvadze V, Clapham D, Sanchez-Vera V, Suarez MF, Zhivotovsky B et al. (2013) Autophagy and metacaspase determine the mode of cell death in plants. J Cell Biol 203, 917–927.

31 Coll NS, Smidler A, Puigvert M, Popa C, Valls M and Dangl JL (2014) The plant metacaspase AtMC1 in pathogen-triggered programmed cell death and aging: functional linkage with autophagy. Cell Death Differ 21, 1399–1408.

32 Bidle KD and Bender SJ (2008) Iron starvation and culture age activate metacaspases and programmed cell death in the marine diatom Thalassiosira pseudonana. Eukaryot Cell 7, 223–236.

33 Nedelcu AM (2009) Comparative genomics of phylogenetically diverse unicellular eukaryotes provide new insights into the genetic basis for the evolution of...
the programmed cell death machinery. *J Mol Evol* **68**, 256–268.

34 Murik O, Elboher A and Kaplan A (2014) Dehydroascorbate: a possible surveillance molecule of oxidative stress and programmed cell death in the green alga Chlamydomonas reinhardtii. *New Phytol* **202**, 471–484.

35 McLuskey K, Rudolf J, Proto WR, Isaacs NW, Coombs GH, Moss CX and Mottram JC (2012) Crystal structure of a *Trypanosoma brucei* metacaspase. *Proc Natl Acad Sci USA* **109**, 7469–7474.

36 Wong AH-H, Yan C and Shi Y (2012) Crystal structure of the yeast metacaspase Yca1. *J Biol Chem* **287**, 29251–29259.

37 Laverriè re M, Cazzulo JJ and Alvarez VE (2012) Antagonic activities of *Trypanosoma cruzi* metacaspases affect the balance between cell proliferation, death and differentiation. *Cell Death Differ* **19**, 1358–1369.

38 Yang J, Yan R, Roy A, Xu D, Poisson J and Zhang Y (2015) The I-TASSER Suite: protein structure and function prediction. *Nat Methods* **12**, 7–8.

39 Hill SM, Hao X, Liu B and Nyström T (2014) Life-span extension by a metacaspase in the yeast *Saccharomyces cerevisiae*. *Science* **344**, 1389–1392.

40 Asqui SL, Vercammen D, Serrano I, Valls M, Rivas S, Breusegem FV, Conlon FL, Dangl JL and Coll NS (2018) AtSERPIN1 is an inhibitor of the metacaspase AtMC1-mediated cell death and autocatalytic processing in planta. *New Phytol* **218**, 1156–1166.

41 Helms MJ, Ambit A, Appleton P, Tetley L, Coombs GH and Mottram JC (2006) Bloodstream form *Trypanosoma brucei* depend upon multiple metacaspases associated with RAB11-positive endosomes. *J Cell Sci* **119**, 1105–1117.

42 Klemenčič M and Funk C (2019) Evolution and structural diversity of metacaspases. *J Exp Bot* **70**, 2039–2047.

43 Vercammen D, Belenghi B, van de Cotte B, Beunens T, Gavigan J-A, De Rycke R, Brackenier A, Inzé D, Harris JL and Van Breusegem F (2006) Serpin1 of *Arabidopsis thaliana* is a Suicide Inhibitor for Metacaspase 9. *J Mol Biol* **364**, 625–636.

44 Klemenčič M, Asplund-Samuelsson J, Dolinar M and Funk C. Phylogenetic distribution and diversity of bacterial pseudo-orthocaspases underline their putative role in photosynthesis. *Front Plant Sci* **10**, https://doi.org/10.3389/fpls.2019.00293.

45 Pei J and Grishin NV (2007) PROMALS: towards accurate multiple sequence alignments of distantly related proteins. *Bioinformatics* **23**, 802–808.

46 Waterhouse AM, Procter JB, Martin DMA, Clamp M and Barton GJ (2009) Jalview Version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics* **25**, 1189–1191.

47 Studier FW (2005) Protein production by auto-induction in high density shaking cultures. *Protein Expr Purif* **41**, 207–234.

**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Representatives of type I metacaspases used in this study.

**Figure S1.** Alignment of p10 domains of representative type I, type II and type III metacaspases.

**Figure S2.** Aligned amino acid sequences of the four *CrMCA-I* variants used in this study as expressed from the pET28(b)+ vector.

**Figure S3.** PVDF membrane showing the detection of the His-tagged proteins using anti-His antibodies.

**Figure S4.** Coomassie stain of two 12.5% SDS/PAGE gels after purification of *CrMCA-I_CL*.

**Figure S5.** Coomassie stain of 12.5% SDS/PAGE gel after crosslinking with formaldehyde.

**Figure S6.** Mutation of the catalytic Cys265 residue (*CrMCA-I_CL_CA*) abolishes proteolytic activity but does not have an effect on binding of calcium ions.