

Review

Metacaspases

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Metacaspases are cysteine-dependent proteases found in protozoa, fungi and plants and are distantly related to metazoan caspases. Although metacaspases share structural properties with those of caspases, they lack Asp specificity and cleave their targets after Arg or Lys residues. Studies performed over the past 10 years have demonstrated that metacaspases are multifunctional proteases essential for normal physiology of non-metazoan organisms. This article provides a comprehensive overview of the metacaspase function and molecular regulation during programmed cell death, stress and cell proliferation, as well as an analysis of the first metacaspase-mediated proteolytic pathway. To prevent further misapplication of caspase-specific molecular probes for measuring and inhibiting metacaspase activity, we provide a list of probes suitable for metacaspases.

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More than a decade ago, an end was put to the until-then frustrating hunt for the presence of caspase orthologues in plants and other non-metazoan organisms. Based on predicted structural homologies with the catalytic domain of caspases, metacaspase sequences were identified in protozoa, fungi and plants.¹ Phylogenetic analysis indicated that eukaryotic caspases, metacaspases and paracaspases are about equally distant from each other and together with the legumains, separases and the bacterial clostripains and gingipains, they are classified within the clan CD of Cys proteases.² A common structural feature of this clan is the presence of a caspase/hemoglobinase fold.³

Two types of metacaspases can be distinguished. Type I metacaspases have a N-terminal prodomain containing a proline-rich repeat motif and, in plant members, also a zinc-finger motif. Type II metacaspases lack such a prodomain but harbor a linker region between the putative large (p20) and small (p10) subunits. In protozoa and fungi only type I metacaspases are found, whereas plant genomes encode both types with frequent prevalence of type I over type II by gene numbers (Figure 1).

Besides a few exceptions having a catalytic Ser residue instead of a Cys,⁴ metacaspases, like caspases have a His-Cys catalytic dyad in their predicted active site, with the Cys residue acting as a nucleophile for substrate peptide bond hydrolysis. For most metacaspases studied so far, enzyme maturation involves autocatalytic processing of the zymogen;^{5–8} however, in some cases this step is not necessarily required for proteolytic activation.^{9–11} The most striking biochemical feature of all metacaspases that

distinguishes them from caspases is a strict Arg and Lys substrate specificity.^{5,6,10,12,13}

In *Arabidopsis* studies, metacaspases have been described with two distinct nomenclatures. The Van Breusegem lab used the five-character name AtMC1–AtMC9 (*Arabidopsis thaliana* Metacaspase) whereas the Lam lab used the seven-character name AtMCP1a–AtMCP1c (*A. thaliana* Metacaspase) for the type I and AtMCP2a–AtMCP2f for the type II metacaspases. In order to avoid confusion and for the reasons of simplicity we have decided to establish a uniform nomenclature of *Arabidopsis* metacaspases, namely AtMC1–AtMC9, which we urge to be adopted by other researchers in future publications and discussions (Table 1).

The first decade of metacaspase research documented by more than 130 publications is over now. It has brought exciting information about biological functions of metacaspases and the first attempts to uncover molecular mechanisms of metacaspase action. This period was also marked by vast contradictions within the field, mainly because of different opinions about biochemical and functional relatedness between caspases and metacaspases.^{2,14–16} Here we review the essential information related to the biochemistry and function of metacaspases. In addition, we detail some of the important tools that are necessary to specifically dissect the role and activity of this highly conserved group of proteases.

Multifunctionality, Functional Specialization or Redundancy of Metacaspases

As the metacaspase Yca1 (also termed Mca1) was found to be a positive regulator of oxidative stress- and senescence-associated

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Abbreviations: AMC, 7-amino-4-methylcoumarin; ER, endoplasmic reticulum; FB1, fumonisin B-1; fmk, fluoromethylketone; MCA, 4-methylcoumarin-7-amide; 2NA, β -naphthylamide; PCD, programmed cell death; SN, staphylococcal nuclease; TSN, Tudor staphylococcal nuclease

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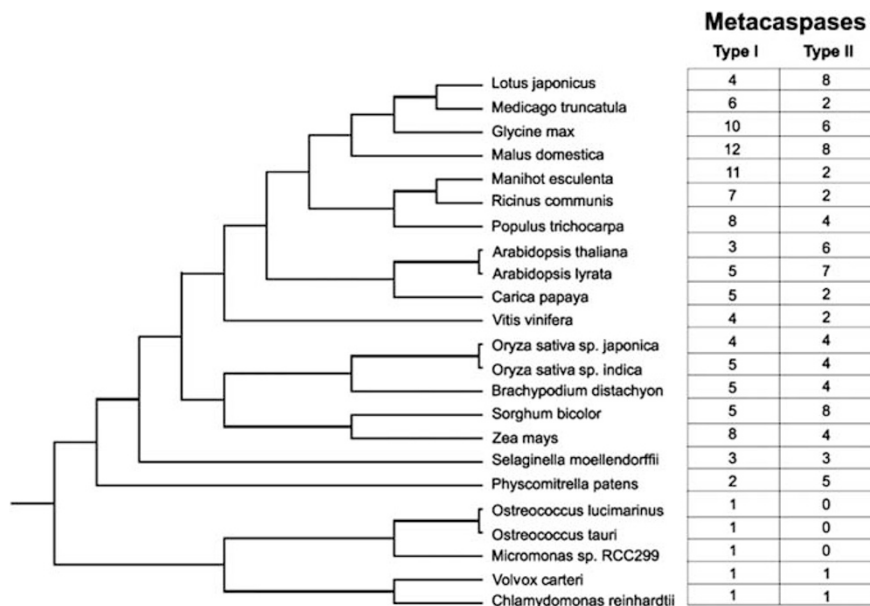


Figure 1 Distribution of type I and II metacaspases within plant genomes

Table 1 The two different *Arabidopsis* metacaspase nomenclatures and the unified one

Gene AGI	Metacaspase type	Van Breusegem lab ⁵	Lam lab ¹²	Unified
At1g02170	I	AtMC1	AtMCP1b	AtMC1
At4g25110	I	AtMC2	AtMCP1c	AtMC2
At5g64240	I	AtMC3	AtMCP1a	AtMC3
At1g79340	II	AtMC4	AtMCP2d	AtMC4
At1g79330	II	AtMC5	AtMCP2b	AtMC5
At1g79320	II	AtMC6	AtMCP2c	AtMC6
At1g79310	II	AtMC7	AtMCP2a	AtMC7
At1g16420	II	AtMC8	AtMCP2e	AtMC8
At5g04200	II	AtMC9	AtMCP2f	AtMC9

cell deaths in the budding yeast (*Saccharomyces cerevisiae*),¹⁷ a great deal of reverse genetic analysis has been directed to functionally characterize metacaspases in various organisms. This analysis has revealed that cell death regulation is just one of the multifaceted abilities of metacaspases, which can also control other biological processes, either related or unrelated to cell death (Table 2).

The number of metacaspase genes in the genomes of different organisms varies considerably (Figure 1; Table 2; <http://merops.sanger.ac.uk/>). Such an unequal distribution of metacaspases between different phyla is a good paradigm to study multifunctionality and functional specialization of metacaspases.

A single metacaspase Yca1 of the budding yeast provides a striking example of a multifunctional protein, as apart from cell death activated under various settings, it is also required for cell cycle regulation and clearance of protein aggregates (Table 2).¹⁴ Deletion of the single metacaspase gene *Pca1* of the fission yeast (*Schizosaccharomyces pombe*) suppresses lipotoxic and inositol starvation-induced death.^{18,19} LmjMCA, a single metacaspase of *Leishmania major*, is involved in cell

cycle regulation²⁰ and, when overexpressed, stimulates oxidative stress-induced cell death.²¹ Further studies using corresponding deletion and inactivation mutants are required to determine whether the single metacaspases present in *L. major* and fission yeast are multifunctional proteins comparable to Yca1.

Most organisms have two or more metacaspase genes (Table 2). There is no evidence yet to indicate significant degree of either specialization or redundancy in the physiological functions of different metacaspase genes expressed in the same organism. Partial redundancy has, however, been demonstrated for three *Trypanosoma brucei* metacaspases during the development of a bloodstream-specific form of the parasite,²² for two *Podospira anserina* metacaspases in the regulation of senescence-associated death²³ and for two *Aspergillus fumigatus* metacaspases in the resistance to endoplasmic reticulum (ER) stress.²⁴ Redundancy may not always be the case, as two *Aspergillus nidulans* metacaspases antagonistically control ER stress-induced cell death, one metacaspase (CasA) acting as positive regulator and another as a negative one (CasB).²⁵ Similarly, a recent study also documented the antagonistic functions of two *Arabidopsis* type I metacaspases, AtMC1 and AtMC2, in mediating hypersensitive response (HR)-associated cell death (Table 2).²⁶

Compared with genomes of protozoa and fungi with a single or a few type I metacaspase genes and no type II metacaspase genes, genomes of higher plants encode larger metacaspase families including both type I and type II members; for example, there are nine and twelve metacaspase genes in *A. thaliana* and *Populus trichocarpa*, respectively (Figure 1; <http://merops.sanger.ac.uk/>). Although the complete range of the roles that metacaspases may serve in plant biology remains to be understood, there is growing evidence that they are required for PCD to take place under diverse settings (Table 2; see following section).

Table 2 Metacaspase functions identified by reverse genetics

Kingdom	Species	Total number of metacaspase genes	Genes with known physiological function	Method	Function	Reference
Protozoa	<i>Leishmania donovani</i>	2	<i>LdMc1</i> , <i>LdMc2</i>	OE	Positive regulators of oxidative stress-induced cell death	Lee <i>et al.</i> ¹⁰
	<i>Leishmania major</i>	1	<i>LmjMCA</i>	KO, OE OE	Cell cycle regulation Positive regulator of oxidative stress-induced cell death	Ambit <i>et al.</i> ²⁰ Zalila <i>et al.</i> ²¹
	<i>Trypanosoma brucei</i>	5	<i>TbMCA2</i> , <i>TbMCA3</i> , <i>TbMCA5</i>	RNAi, KO	Required for bloodstream form	Helms <i>et al.</i> ²²
	<i>Trypanosoma cruzi</i>	2	<i>TcMCA5</i>	OE	Positive regulator of fresh human serum-induced cell death	Kosec <i>et al.</i> ³⁴
Plantae	<i>Arabidopsis thaliana</i>	9	<i>AtMC8</i> , <i>AtMC1</i> , <i>AtMC2</i> , <i>AtMC4</i>	KO KO, OE, IM KO, OE, IM	Required for UVC stress-induced cell death Antagonistically control HR cell death activated by intracellular immune receptors Mediates PCD activation by the fungal toxin FB1 and abiotic stress inducers	He <i>et al.</i> ³⁰ Coll <i>et al.</i> ²⁶ Watanabe and Lam ³²
	<i>Nicotiana benthamiana</i>	?	<i>NbMC1</i>	VIGS	Confers resistance to fungal necrotroph <i>Colletotrichum destructivum</i>	Hao <i>et al.</i> ⁶⁶
	<i>Picea abies</i>	?	<i>MclI-Pa</i>	RNAi	Required for embryogenesis and associated PCD	Suarez <i>et al.</i> ²⁹
Fungi	<i>Aspergillus fumigatus</i>	2	<i>CasA</i> , <i>CasB</i>	KO	Confer cytoprotection under ER stress	Richie <i>et al.</i> ²⁴
	<i>Aspergillus nidulans</i>	2	<i>CasA</i> , <i>CasB</i>	KO	Antagonistically control ER stress-associated cell death	Colabardini <i>et al.</i> ²⁵
	<i>Candida albicans</i>	1	<i>CaMCA1</i>	KO	Required for oxidative stress-induced cell death	Cao <i>et al.</i> ²⁸
	<i>Podospira anserina</i>	2	<i>PaMca1</i> , <i>PaMca2</i>	KO	Required for senescence-associated cell death	Hamann <i>et al.</i> ²³
	<i>Saccharomyces cerevisiae</i>	1	<i>Yca1</i>	KO, OE	Required for oxidative, salt, propolis and osmotic stress-induced cell death	Madeo <i>et al.</i> , ²⁷ Chahomchuen <i>et al.</i> ⁶⁷ and de Castro <i>et al.</i> ⁶⁸
				KO, OE	Required for cell death associated with defects in ubiquitination, DNA replication, mRNA stability and mitochondrial metabolism	Madeo <i>et al.</i> ²⁷ and Walter <i>et al.</i> ⁶⁹
				KO	Required for virus-induced cell death	Ivanovska and Hardwick ⁷⁰
				KO	Required for chronological aging-associated death	Herker <i>et al.</i> ⁷¹
				KO	Mediates carnitine-dependent lifespan extension	Palermo <i>et al.</i> ⁷²
				KO, IM KO, IM KO	Required for clearance of protein aggregates Cell cycle regulation Required for lipotoxic cell death in minimal medium and cell death induced by inositol starvation	Lee <i>et al.</i> ³⁸ Lee <i>et al.</i> ³⁹ Low <i>et al.</i> ¹⁸ and Guerin <i>et al.</i> ¹⁹
	<i>Schizosaccharomyces pombe</i>	1	<i>Pca1</i>			

Abbreviations: FBI, fumonisin B-1; IM, inactivation mutant; KO, knock out; OE, overexpression; PCD, programmed cell death; RNAi, RNA interference; VIGS, virus-induced gene silencing

Metacaspases in Cell Death

Madeo *et al.*¹⁷ first reported metacaspase-dependent cell death using yeast *Yca1* mutant strains subjected to oxidative stress. Since then, a pro-cell death role for *Yca1* has been demonstrated in budding yeast cells in response to viral toxins and different types of abiotic and metabolic defect-associated stresses and chronological aging (Table 2).²⁷ A similar requirement for a metacaspase has been reported in the fission yeast, albeit for a more limited number of cell death conditions, including lipotoxic stress¹⁸ and inositol starvation¹⁹ (Table 2). Further in the fungi kingdom it has been established that both *PaMca1* and *PaMca2* are required for senescence-associated cell death in *P. anserina*,²³ a single *Candida albicans* metacaspase, *CaMCA1*, mediates oxidative

stress-induced cell death in this pathogenic yeast,²⁸ whereas one of the two metacaspases, *CasA*, promotes ER stress-associated cell death in *A. nidulans*²⁵ (Table 2).

A pro-cell death role of metacaspases has now been extended to protozoa and plants. In *Leishmania* species metacaspases are required for oxidative stress-induced cell death (Table 2).^{10,21} In the gymnosperm plant, Norway spruce (*Picea abies*), gene silencing of a type II metacaspase, *mclI-Pa*, suppressed terminal cell differentiation and PCD in the embryo-suspensor causing developmental arrest at the early stage of embryogenesis.²⁹ Unexpectedly, PCD phenotypes in metacaspase knockout lines of the model plant *Arabidopsis* have been less forthcoming, possibly due to gene redundancy. In *Arabidopsis*, all nine metacaspase genes are expressed at different levels in various parts of the plant

(our unpublished results). Among these genes, a type II metacaspase gene *AtMC8* is strongly upregulated during oxidative stress. This upregulation requires the gene *Radical-Induced Cell Death1*,³⁰ which is thought to be a mediator of oxidative stress responses in *Arabidopsis*.³¹ Consistent with these observations, *atmc8* knockout lines exhibited reduced cell death triggered by UVC radiation or hydrogen peroxide.³⁰ Oxidative stress-induced PCD represents a dramatic example of a metacaspase-dependent process that is conserved throughout a long evolutionary distance, from protozoa to plants (Table 2). Loss of the predominant type II metacaspase in *Arabidopsis*, *AtMC4*, has been found recently to compromise cell death induction by the fungal toxin fumonisin B-1 (FB1), an incompatible bacterial pathogen, and chemical inducers of oxidative stress in seedlings.³² This work has further shown that *AtMC4* processing from its zymogen is accelerated during activation of cell death by these agents, consistent with its deduced role in orchestrating PCD under these conditions. Lastly, a type I metacaspase *AtMC1* was shown to be a positive regulator of the HR cell death in *Arabidopsis*, whereas another type I metacaspase, *AtMC2*, was found to counteract this pro-cell death effect of *AtMC1* (see also review by Coll *et al.*, in this issue).²⁶ In the case of *AtMC2* as antideath regulator, it is interesting to note that its proteolytic activity is apparently not required for this function in *Arabidopsis*. Genetic manipulation of the two metacaspases could nearly eliminate HR activated by plant intracellular immune receptors.²⁶ To conclude, trans-kingdom conservation of a role for metacaspases in PCD regulation emphasizes that these proteases are a fundamental part of the non-metazoan cell death machinery.

Although several molecular components of PCD pathways have been recently identified in non-metazoan organisms, metacaspase position(s) in these pathways remain elusive. The identification of Tudor staphylococcal nuclease (TSN) as a common substrate for both Norway spruce metacaspase mCII-Pa and human caspase-3 (see below)³³ suggests that metacaspases can execute PCD like effector caspases. Nuclear translocation of metacaspases during cell disassembly in the Norway spruce embryo-suspensors⁶ and in the dying epimastigotes of *Trypanosoma cruzi*³⁴ indirectly supports this scenario. By contrast, the antagonistic control of cell death by two type I metacaspases, such as the one shown for the *Arabidopsis* HR²⁶ and *A. nidulans* ER stress²⁵ suggests a possible role in the decision phase.

Noteworthy, in budding yeast approximately 60% of the examples of cell death reported so far are Yca1-independent.²⁷ For example, Yca1 is not required for PCD during mating,³⁵ in response to ammonia³⁶ or induced by human BAX expression.³⁷ Therefore, it can be expected that further understanding of PCD mechanisms in plants, which have both type I and type II metacaspases, should facilitate a better definition of metacaspase-dependent and -independent pathways.

Metacaspases in Protein Aggregation and ER Stress

Proteome analysis of yeast cells deleted for *Yca1* has revealed a role for this metacaspase in the clearance of insoluble protein aggregates (Table 2).³⁸ The ablation of *Yca1*

was found to direct an accumulation of insoluble protein aggregates during physiological growth conditions, correlated with an increased abundance of vacuolar peptidases and stress-response chaperones. This accumulation of protein aggregates induced the autophagic pathway. Moreover, the normally cytosolic Yca1-GFP was observed to colocalize with the protein aggregation marker Hsp104-mRFP during heat stress and in aged cultures. Deletion of the poly-Q (QQXX) motif in the Yca1 prodomain demonstrated that it was largely responsible for the metacaspase localization in the protein aggregates. Interestingly, catalytic activity of Yca1 was not required for its aggregate-specific localization, representing the first indication that metacaspase could participate in the formation of protease-activation scaffold analogous to signaling platforms such as the apoptosome.^{14,38}

These data indicate that metacaspases can function to promote the dissipation of protein aggregates under normal and stress conditions in addition to their role in PCD regulation. It is possible to argue that $\Delta yca1$ yeast cells have a reduced level of PCD under stress because of an increased expression of molecular chaperones and of the activation of autophagy, a pro-survival mechanism.³⁸ This interpretation suggests a possible indirect effect of metacaspase function on PCD regulation, at least in some conditions. However, the poly-Q motif required for aggregate localization is only present in one of the nine *Arabidopsis* metacaspases (*AtMC3*) and is absent in the other metacaspases shown to regulate PCD, suggesting that this proposition based on observation in yeast might not hold true when investigated in other organisms.

In the human fungal pathogen *A. fumigatus*, deletion of the two metacaspase-encoding genes, *CasA* and *CasB*, leads to hypersensitivity to agents that induce ER stress (Table 2).²⁴ In contrast, no effect on growth was observed with the $\Delta casA/\Delta casB$ mutant under conditions of a variety of agents that induce oxidative stress. These results indicate that in *A. fumigatus* the metacaspases may be important mediators of survival under ER stress with one possible scenario in their involvement in clearing aggregated proteins in the ER, similar to yeast Yca1. Alternatively, these proteases may be important for cleavage of specific cellular targets that have an important role in the management of ER stress. It would be important to test whether proteolytic activity of *CasA* and *CasB* is required for their role for survival under ER stress.

Metacaspases in Cell Proliferation

Ambit *et al.*²⁰ found that the metacaspase in *L. major*, LmjMCA, exhibits specific subcellular localization. Although it is relatively dispersed throughout the cell during interphase, it tends to colocalize with the kinetoplast during mitochondria segregation and it also translocates to the nucleus and mitotic spindle of the parasite during mitosis. Attempts to manipulate LmjMCA levels resulted either in a growth defect with suppression of cytokinesis frequency (overexpression) or inviability (deletion), suggesting that this protein may have an essential function(s) during cell division.²⁰ However, whether the protease activity of LmjMCA is essential for its role in cell cycle progression in this parasite remains to be clearly demonstrated.

In the budding yeast, loss of Yca1 or its replacement with a point mutation variant in its active site Cys resulted in a delay of the doubling time that correlates with a slower G1/S phase transition.³⁹ Even more dramatically, loss of Yca1 activity results in the uncoupling of cell cycle arrest at the G2/M checkpoint to nocodazole treatment, which disrupts microtubules important for cytokinesis. Interestingly, the desensitization of cell cycle progression to nocodazole treatments has also been found to result from a loss of caspase-3 activities in human hepatoma cells.⁴⁰ These studies thus underscore that metacaspase and caspase could have a common, conserved role in cell cycle progression of eukaryotes.

Measurement and Inhibition of Metacaspase Activities

Owing to the presence of the C14 caspase domain, many efforts initially aimed to demonstrate caspase-like proteolytic activity (namely, proteolysis after an Asp residue) of various metacaspases. Detection of specific proteolytic activity is usually performed by using synthetic tri- or tetrapeptides C-terminally coupled to fluorogenic moieties such as 7-amino-4-methylcoumarin (AMC), 4-methylcoumarin-7-amide (MCA) or β -naphthylamide (2NA). Initial reports using caspase substrates VEID-AMC and IETD-AMC monitored differential caspase activity levels in lysates from wild-type, $\Delta yca1$ and overexpression yeast strains upon treatment with cell-death promoting agents. The observed activities were abrogated by the broad-range caspase inhibitor zVAD-fluoromethylketone (fmk).¹⁷ Furthermore, metacaspase involvement in the yeast cell death models was investigated after *in vivo* staining for caspase activity by flow cytometry with FITC-labeled VAD-fmk (FITC-VAD-fmk).^{17,41–44} Likewise, in the fungus *C. albicans*, it was suggested that the antifungal compound Plagiochin E activated metacaspases based on the appearance of FITC-VAD-fmk labeled cells.⁴⁵ In the phytoplankton *Chlamydomonas reinhardtii* and other marine species, such as the diatom *Thalassiosira pseudonana* and the unicellular coccolithore *Emiliania huxleyi*, increased caspase-like activity was correlated with increased accumulation of metacaspase mRNA and protein, thereby providing indirect evidence that metacaspases had caspase-like activity.^{46–48} Also in plants,

silencing of *mclI-Pa* in the Norway spruce embryos reduced the level of the VEIDase caspase-like activity.²⁹ Overall, these reports suggested that metacaspases might be directly responsible for cellular caspase-like activities in fungi and plants. However, a horde of biochemical studies using recombinant metacaspases or protein extracts from loss- or gain-of-function mutants have now clearly demonstrated that metacaspases are highly specific for Arg or Lys at the P1 position.^{4,6,7,9–13,22,23,30,33,49,50} This implies that conclusions, mainly based on a genetic perturbation of metacaspase-encoding genes, that metacaspases have caspase-like proteolytic activities were premature.

To accurately monitor metacaspase activities in cell lysates it is, therefore, essential to use specific metacaspase activity assays that are rooted in substrate specificity and other biochemical characteristics of metacaspases. The determination of the *Arabidopsis* AtMC9 autoprocessing site ITSR(183) (Table 3) was the first indication for a basic amino-acid cleavage preference⁵ and steered successful testing of fluorogenic tri- and tetrapeptides with an Arg or Lys residue at the P1 position for cleavage by metacaspases. Based on the autocatalysis of *mclI-Pa* after KFKV(269) and FESR(188) (Table 3),⁶ an equivalent substrate analogue was employed to detect metacaspase activity in embryonic cell extracts of Norway spruce.³³ A tetrapeptide positional scanning synthetic combinatorial library screen indicated that VRPR-AMC (when Arg was fixed at P1) and IISK-AMC (when Lys was fixed at P1) were the preferred synthetic substrates for recombinant *Arabidopsis* AtMC9.¹³ Recombinant *T. brucei* TbMCA2 was shown to autocleave at RDAK(55) and ADVK(268) (Table 3) and diverse substrates with Arg or Lys at P1 (GGR, GRR, VRPR, IKLR, IKLK) were efficiently cleaved.⁹ Similarly, *Allomyces arbuscula* AMca2 was autoprocessed at GKVR(15) and DDTR(27) (Table 3).¹¹ Furthermore, peptidyl substrates with Arg at P1 such as GRR-AMC and GGR-AMC were preferentially proteolysed by recombinant metacaspases AtMC1, AtMC4, AtMC5, AtMC8, AtMC9 and TbMCA2,^{5,8,9,12,30} by immunoprecipitated metacaspases LdMC1, LdMC2 and LmjMCA from *Leishmania* species,^{7,10} and through ectopic overexpression of AtMC1, AtMC4, AtMC5 and Yca1.^{8,12}

Table 3 Experimentally shown metacaspase autoprocessing sites and cleavage sites in the substrate proteins

	Metacaspase	Cleaved protein	Cleavage site sequence ^a	P1 position	Reference
Autoprocessing	TbMCA2		RDAKGLHG	55	Moss <i>et al.</i> ⁹
			ADVKNAT	268	Moss <i>et al.</i> ⁹
	AMca2		GKVRDLYG	15	Ojha <i>et al.</i> ¹¹
			DDTRSTSS	27	Ojha <i>et al.</i> ¹¹
	AtMC9		ITSRALPF	183	Vercammen <i>et al.</i> ⁵
	AtMC4		AKDKSLPL	225	Watanabe and Lam ⁸
Substrates	mclI-Pa		FESRGIHL	188	Bozhkov <i>et al.</i> ⁶
			KFKVVLVT	269	Bozhkov <i>et al.</i> ⁶
	AtMC9	AtSerp	IKLRGLLM	351	Vercammen <i>et al.</i> ¹³
	mclI-Pa	TSN	ASIRDLP	158	Sundström <i>et al.</i> ³³
			VLNRDVRI	287	Sundström <i>et al.</i> ³³
			SNSKAIRD	371	Sundström <i>et al.</i> ³³
TbMCA2		EF-Tu	HSARESPV	558	Sundström <i>et al.</i> ³³
			PIVRHGSA	172	Moss <i>et al.</i> ⁹
			SALKALEG	177	Moss <i>et al.</i> ⁹

^aP1 residue is indicated in bold

The substrate specificity of metacaspases towards Arg or Lys at the P1 position in peptide substrates was also consolidated through the identification of a first set of natural substrates and inhibitors. A recombinant serpin-like protein (AtSerpin1) was cleaved within its reactive center loop by recombinant AtMC9 at IKLR(351) (Table 3).¹³ During expression of TbMCA2 in *E. coli* cells, the bacterial elongation factor EF-Tu was proteolyzed at TPIVR(172) and GSALK(177) (Table 3).⁹ Finally, endogenous TSN was cleaved by mclI-Pa at ASIR(158), VLNR(287), SNSK(371) and HSAR(558) during both developmental and oxidative stress-induced PCD in Norway spruce embryos (Table 3).³³

Arginal protease inhibitors leupeptin and antipain, as well as tosyl-lysyl-chloromethylketone have proven to be potent inhibitors of *Arabidopsis*, *Leishmania* and *Trypanosoma* metacaspases^{5,6,9,12} in addition to inhibitory substrate analogues such as FK-trimethylbenzoyloxymethyl ketone, EGR-chloromethyl ketone and GVR-chloromethyl trifluoro acetate.^{5,6,11}

Biochemical studies of metacaspases from *Arabidopsis* (AtMC4 and AtMC8), spruce (mclI-Pa), *Leishmania* (LdMC1 and LdMC2) and *Trypanosoma* (TbMCA2) indicate that the majority prefers neutral to slightly basic (7.0–8.5) pH optima for *in vitro* activity assays.^{5,6,9,10,30} Until now, AtMC9 is the only reported metacaspase that requires acidic conditions before activation.⁵ Noteworthy *in vitro* activities of AtMC4, AtMC5, mclI-Pa, TbMCA2 and the *A. arbuscula* AMca2 depend on high Ca²⁺ concentrations.^{5,6,9,12} More recent biochemical studies with recombinant AtMC4 demonstrated that these high Ca²⁺ levels are required to activate the protease activity through induced cleavage at a highly conserved site AKDK(225) (Table 3) that has also been shown to be cleaved in AtMC9.⁸ This cleavage was demonstrated to be critical for *in vitro* protease activity of metacaspases^{5,8} as well as *in vivo* function of AtMC4 to complement Δ yc1 mutant yeast strains in cell death activation.⁸

To conclude, caspase-specific substrates such as YVAD-AMC, DEVD-AMC, VEID-AMC and IETD-AMC (with Asp at P1) are not indicative of metacaspase catalytic activity. This implies that conclusions drawn on the involvement of metacaspases in specific processes from reports based on caspase-specific peptide substrates, should be revisited and it is advised that activity assays tailored towards the specific catalytic characteristics of metacaspases should be used instead. As the level of VEIDase activity was reported to be decreased along with either the loss of *Yca1* in yeast¹⁷ or by suppression of *mclI-Pa* in spruce cells,²⁹ these results would indicate that cryptic caspase-like proteases may be activated downstream of the metacaspase regulation point. The suppressing effect of pan-caspase inhibitor such as zVAD-fmk and zVEID-fmk on cell death-related phenomena in yeast and spruce cells would support the role for such caspase-like proteases in orchestrating cell death downstream of metacaspases in some of the cell death pathways.^{12,17,51} We urge the use of fluorogenic tri- or tetrapeptide substrates (e.g. VRPR-AMC, IISK-AMC, EGR-AMC, GRR-AMC, GGR-AMC and FESR-AMC) with Arg or Lys residues at the P1 position to detect metacaspase activities in cellular extracts or of recombinant metacaspases. Accordingly, the use of caspase inhibitors against metacaspases is inappropriate and should

be replaced by arginal protease inhibitors. It should be noted, however, that in addition to metacaspases, these inhibitors can suppress other arginine-specific proteases present in the cells or test tube. Given the variability of metacaspase pH optima and possible Ca²⁺ dependence, it is advisable to screen for proteolytic activity at different levels of pH and different concentrations of Ca²⁺ as well.

In Vivo Metacaspase Degradome: Tudor Staphylococcal Nuclease

Unraveling the identity of natural substrates of metacaspases will give an insight into molecular mechanisms of metacaspase-dependent processes. At present, TSN is the only protein shown to be cleaved by a metacaspase *in vivo*.³³ TSN is an evolutionarily and structurally conserved protein found in all eukaryotes (except for budding yeast). It has an invariant domain architecture including a tandem of four staphylococcal nuclease-like domains (SN), followed by a Tudor domain and the fifth SN domain (Figure 2a). Owing to its complex domain structure, TSN takes part in several fundamental mechanisms of gene regulation in animal cells, including transcription,⁵² mRNA splicing⁵³ and RNA silencing.^{54,55} Decrease of *TSN* expression triggers cell death^{33,56} and impairs plant viability and stress tolerance.^{33,57}

During both developmental and oxidative stress-induced cell death in Norway spruce embryos active metacaspase mclI-Pa cleaves endogenous TSN at four sites. Three sites are situated between SN domains and the fourth site inside SN2 domain (Figure 2a).³³ All cleavage sites contain either Arg or Lys at P1 position, but there was no strict specificity beyond P1 residue (P4-P3-P2 and P1') (Table 3). In the same study caspase-3-mediated cleavage of human TSN at the canonical cleavage site DAVD/S (Pop and Salvesen⁵⁸) situated between the Tudor and SN5 domains has been revealed in apoptotic cells.³³ A shared *in vivo* target of caspases and metacaspases suggests that plants and animals may have conserved some key proteolytic pathways to compromise cell viability, despite the large differences in morphology and biochemistry of cell death between the two kingdoms. In addition, cleavage of TSN by an effector caspase and a type II metacaspase provides a good model system for comparative analysis of the biochemical properties of caspases and metacaspases *in vivo*.¹⁴ One conclusion from such analysis is that metacaspases appear to be far more liberal than caspases in regards to P4-P3-P2 and P1' substrate residues (Figure 2a). This conclusion is consistent with earlier results of high-throughput screening of a combinatorial tetrapeptide substrate library with recombinant AtMC9.¹³ Future studies of *in vivo* metacaspase degradome might, however, identify more prominent metacaspase specificity towards the aforementioned residues.

The crystal structure of the Tudor-SN5 part of human TSN has been determined to high resolution (Figure 2b).⁵⁹ To complete the 3D model of the protein, each of the SN1-4 domains can be reliably reconstructed based on the available crystal structures of staphylococcal nuclease (PDB 1SNC) and human SN5 (PDB 2O4X), for example, the SN2 domain of spruce TSN shown in Figure 2c. The analysis of the metacaspase mclI-Pa cleavage sites in this model reveals

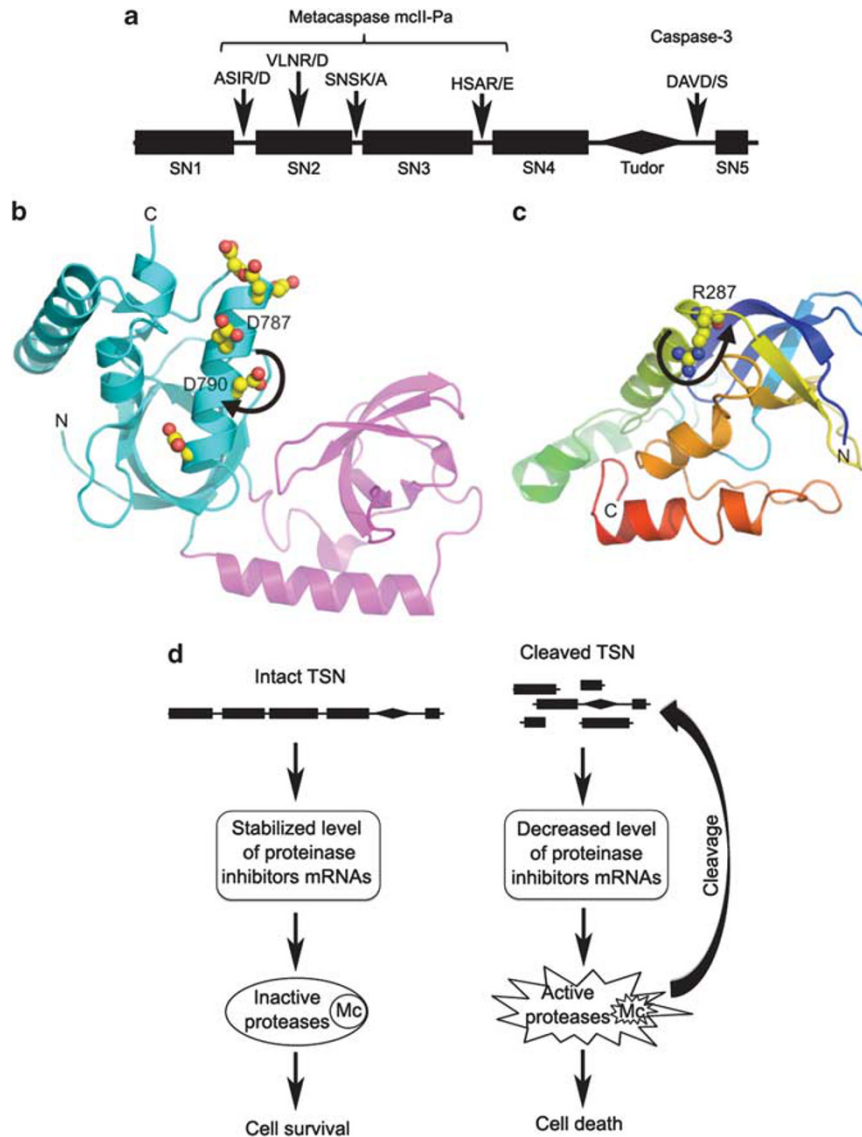


Figure 2 The mechanisms of TSN cleavage during cell death. **(a)** Domain structure of TSN, and location of metacaspase and caspase cleavage sites. **(b and c)** Structural preferences for the cleavage of TSN by caspase-3 and metacaspase mclI-Pa. **(b)** Caspase-3 cleaves helix $\alpha 2$ of human TSN. Tudor-SN5 part of human TSN is shown as cartoon with the SN5 and Tudor domains painted in cyan and pink, respectively. The caspase-3 cleavage motif (787-DAVD-790) is located in the middle of helix $\alpha 2$. Three of four residues of the motif, Asp 787 and 790 and Val 789 are accessible to the solvent. The $\alpha 2$ helix exposes six Asp residues (including two Asp residue of the DAVD motif; shown in spheres), creating a highly negatively charged patch on the surface of the helix. The abundance of Asp residues around the cleavage site might facilitate its recognition by caspase-3. **(c)** Metacaspase mclI-Pa cleaves a structured but solvent accessible loop ($\alpha 2$ - $\beta 8$) of the SN2 domain (cartoon painted in rainbow) of spruce TSN. The loop is cleaved after Arg 287 (shown in spheres). The structure of the SN2 domain of spruce TSN was modeled with SwissModel using staphylococcal nuclease (PDB 1SNC) and Tudor-SN5 part of human TSN (PDB 2O4X) as template structures. Arrows in **b** and **c** indicate cleavage sites. Graphics was generated using PyMol. **(d)** A proposed model for pro-cell death role of metacaspase-mediated cleavage of TSN. Mc, metacaspases

that all of them are located in loop regions: three in the loops situated between SN1-4 domains and one in the middle of a structured but solvent accessible loop ($\alpha 2$ - $\beta 8$) of the SN2 domain (Figure 2c). The preference for digesting loops is common for the majority of proteases and, hence, is not surprising. In contrast, the single cleavage site in human TSN for caspase-3 is located in a long helix ($\alpha 2$) of the Tudor-SN5 part (Figure 2b). Although the ability of caspase-3 to cleave α helices has been previously demonstrated,⁶⁰ this is not consistent with the structural analysis of the caspase-3-inhibitor complex.⁶¹ In this complex, the inhibitor inserts

an extended conformation segment into the active site, suggesting that cleavage sites must either have this conformation or acquire it by unfolding. Interestingly, the caspase-3-cleaved helix $\alpha 2$ of human TSN contains six Asp residues (including two Asp residue of the DAVD motif), creating a highly negatively charged patch on the surface of the helix (Figure 2b). The abundance of Asp residues around the cleavage site might facilitate its recognition by caspase-3. Alternatively, the high density of an uncompensated negative charge might trigger a transient unfolding of the helix, which would facilitate not only the cleavage site recognition but also

its digestion by caspase-3. Cleavage would likely to promote the unfolding of the entire Tudor-SN5 part and subsequent protein aggregation. In contrast, cleavage of spruce TSN in the loop regions (Figure 2c) is likely to generate soluble domains. Therefore, mclI-Pa-mediated cleavage of spruce TSN at multiple sites may provide an alternative mechanism (to a cleavage at a single site within the $\alpha 2$ helix) to ensure efficient proteolytic inactivation of TSN.

As the list of annotated members of the caspase degradome continues to increase, it becomes evident that cleavage of many substrates is just a bystander event, which does not contribute to cell death development.⁵⁸ However, cleavage of human TSN by caspase-3 is important for the execution of apoptosis for the following reasons. The cleavage breaks up the hook-like structure formed by the Tudor and SN5 domains. An aromatic cage within the hook binds methyl groups of small nuclear ribonucleoproteins, anchoring TSN to the spliceosome and stimulating mRNA splicing.⁵⁹ Accordingly caspase-mediated cleavage of TSN abrogates its stimulatory function in splicing.³³ As TSN is supposed to bridge transcription and splicing by interacting with the components of both processes, the cleavage by effector caspases will also uncouple these processes.

It is more difficult to explain the molecular mechanism underlying pro-cell death role of metacaspase-mediated cleavage of TSN because the molecular role of TSN in plant biology remains poorly understood. Furthermore, plant TSN is a cytoplasmic protein,^{33,57} which does not support its involvement in transcription and splicing activation. Frei *et al.*⁵⁷ have recently demonstrated that TSN confers stress tolerance in *Arabidopsis* through selective stabilization of mRNAs-encoding secreted proteins. Notably, a significant proportion of these proteins are cysteine and serine protease inhibitors, which are known to suppress cell death in plants.^{62,63} Although the mechanism of TSN-dependent stabilization of specific mRNAs remains unknown, metacaspase cleavage of TSN molecule in at least four sites should result in deregulation of this mechanism. We propose a model, where intact TSN protein helps to protect cells from death by keeping expression of protease inhibitors at the level sufficient to suppress activation of a subset of cell-death proteases (Figure 2d, left). Contrary, metacaspase-mediated cleavage of TSN abrogates this function and promotes cell death through increased activation of cell-death proteases (including metacaspases; Figure 2d, right). Experimental verification of this model will provide a fruitful avenue for the future research.

Conclusions

It has been approximately 10 years since metacaspases were discovered. During this time, the pendulum has shifted from naive hopes that metacaspases are responsible for cell death events and associated caspase-like activities in plants and other non-metazoans to well-grounded view that metacaspases are a distinct group of enzymes that can regulate (similar to caspases) various cell biological processes, including PCD. We expect that more mechanistic data will be obtained in the near future about metacaspases through integrated efforts of cell and structural biologists and

biochemists. This will enable to resolve a number of important issues and controversies, including (but not limited to) (i) molecular conformation of active metacaspase, and differences as well as similarities between type I and II metacaspases; (ii) whether activation of type I metacaspases occurs in multiprotein complexes similar to caspases-2, -8 and -9; (iii) whether the degradome of metacaspases is indeed much larger than that of caspases, considering a weak preference of metacaspases for specific residues located in P4-P2 and P1' positions in their substrates; (iv) is metacaspase-mediated proteolysis directly responsible for morphological changes that occur during PCD in non-metazoan organisms, which differs significantly from apoptosis in animals; (v) whether metacaspase functions differ in the organisms containing a single metacaspase (e.g. budding or fission yeast) and multiple metacaspases (e.g. *Arabidopsis*) and (vi) how precisely do metacaspases regulate downstream aspartate-specific proteases.

Anticipated discovery of the natural substrates of metacaspases using advanced techniques of protease degradomics^{64,65} will assess and further consolidate metacaspase specificity in a broader biological context that will allow a more precise and rational design of synthetic substrates and inhibitors. As metacaspases control vital cellular functions in protozoa, fungi and plants, development of approaches for artificial regulation of metacaspases can find important practical applications in the improvement of growth and disease resistance characteristics.

Conflict of Interest

The authors declare no conflict of interest.

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