Chapter 2

Programmed Cell Death-Related Proteases in Plants

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Additional information is available at the end of the chapter

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Abstract

From an ancient Greek term related to the "leavening of bread" (*en*, in; *zyme*, leaven), an enzyme can be defined as a substance showing the properties of a catalyst that is produced as a result of cellular activity. Every proteinaceous enzyme that performs hydrolysis of peptide bonds is appropriately termed "protease" (peptidase). All of them share aspects of catalytic strategy, but with some variation. As a result, the proteases are grouped into six different catalytic families: serine, threonine, cysteine, aspartic, glutamic and metallopeptidases (http://merops.sanger.ac.uk/). The larger families (cysteine, serine, aspartic and metallopeptidases) have a wide range of distribution on living organism groups, and are also present in the "controversial" viruses. As a well-represented family, the cysteine proteases play important roles in events such as signalling pathways, programmed cell death (PCD), nutrient mobilization, protein maturing, hormone synthesis and degradation. In the past two decades, an increased interest was driven to the study of the programmed cell death (PCD), mainly after the discovery of caspase-related proteins and caspase-like activities in organisms not metazoan. Caspases are cysteine proteases that cleave their substrate after aspartate residues and are part of signalling cascades of the apoptotic PCD process (also in inflammatory process), unique of metazoan. The caspase-related proteins are named paracaspases and metacaspases. Paracaspases are found on metazoan and *Dictyostelium*, whereas the metacaspases are present on plants, fungi and groups of protozoan. On plants, PCD has features that are distinct from that of animals and is an important pathway on developmental events, defensive and stress response (biotic and abiotic). All these events have their own particularities, but the participation of proteases seems to be universal with those responsible for caspase-like activities and metacaspases having an increasing number of reports that put them as important for plant PCD. In this chapter, we tackle important aspects of the proteases, in special that involved in plant PDC, as well as their specific regulators. Aspects of function, catalytic mechanisms and interaction with ligands will be on focus.

Keywords: plants, programmed cell death, metacaspases

1. Introduction

Every proteinaceous enzyme that performs hydrolysis of peptide bonds is correctly designed as protease (peptidase) [1, 2]. This term was first used by Vines [3] based on direct and indirect evidences from studies with algae, some fungi and Phanerogams. Long before this work, the word "proteolytic" was applied by Roberts [4] to describe the digestive process on human stomach, and in the first years of the twentieth century, the digestive proteins trypsin and pepsin as well as other autocatalytic enzymes were known as proteases, being pepsin credited to do "proteolysis" since 1877 [5]. In 1928, Grassmann and Dyckerhoff [6] established important definitions concerning the nature of the catalytic activities of proteases. In resume, the peptide cleavage pattern by proteases can be internal, for endopeptidases; on the N-terminal portion, for aminopeptidases; or C-terminal, for carboxypeptidases [2].

Despite this difference, all proteases share the same catalytic strategy, as they polarize the carbonyl group on the peptide bond of the substrate by the stabilization of the oxygen atom on an oxyanionic hole, what makes the carbon atom more vulnerable to the attack of an activated nucleophile. The nature of the nucleophile presents some variation and is determinant to the employed mechanism for enzymatic catalysis. As a result of these variations, the proteases are grouped into six different catalytic families: serine, threonine, cysteine, aspartic, glutamic and metallopeptidases (http://merops.sanger.ac.uk/). The larger families (cysteine, serine, aspartic and metallopeptidases) have a wide range of distribution on living organisms, and are also present in the "controversial" group of viruses [7].

About their function, these enzymes are well known for the promotion of protein degradation on amino acid unities. Besides this function, they regulate the destination of other proteins based on their cleavage specificity and participate on important cellular pathways, being key regulators in different response processes to environmental factors and developmental signals [2].

One of these cellular pathways is the event of programmed cell death (PCD) that, since the 2000s, is being studied with increased interest on non-metazoan organisms, in particular on plant models. On metazoan, one of the main constituent of the pathway are cysteine proteases known as caspases. They are not found in plants, but an increasing number of reports shows strong evidences that caspase-related proteases that belong to the metacaspase group, as well as proteases with caspase-like activities are eminent on plant PCD [8].

2. Programmed cell death in plants

Programmed cell death (PCD) is a genetically controlled physiological innate mechanism, which involves the selective death of individual cells, tissues or entire organs. It is a process different from necrosis as it occurs passively in response to environmental perturbations [9]. Together with the chromatin remodelling machinery, the cell cycle regulation mechanisms, the nuclear envelope and the cytoskeleton, this process is one of the major eukaryotic innovative aspects, which allowed the development of more complex organisms [10].

Molecular evidences have pointed out that the PCD machinery has evolved since very early stages of the evolutive history, and that this evolution has been processed through expansion and innovation of protein recruitment domains, as well as through the derivation of effector domains and horizontal gene transference events [11].

The best-understood models in PCD are metazoan organisms and in concern to the cell morphology and involved organelles, two main categories are known: apoptosis and autophagy [12–14].

Apoptosis is defined by three main morphological characteristics: nuclear DNA fragmentation, apoptotic bodies' formation and degradation of the apoptotic bodies on the lysosome of a phagocytic cell [12–14].

Autophagy is the main system of degradation and recycling in eukaryotic cells, contributing to the clearing of cellular compounds and cytosolic portions. This process can occur in two forms: through the cytosolic sequestration by the vacuole or through the sequestration of large portions of cytosol by a structure called autophagosome [15–17].

On situations where a cellular set is under a more intense stress, so that the cells are not able to activate the apoptotic PCD pathway, cell death occurs through necrosis, characterized by a protoplasmic swelling due to the loss of the osmoregulation control capability, and consequent water and ions migration to the cell [18].

In plants, PCD is observed under diverse circumstances through the entire life cycle of many species, as well as in response to biotic and abiotic stimuli, what allows wide biochemical and developmental plasticity [19], as, for example:

- on the degeneration of cells from tissues with transitory functions, such as cotyledons, suspensor, certain leaves, petals [20] and secretory tissues [21];
- on the elimination of excessive produced cells, as in the case of some unisexual flowers that initially produce male and female organs, and must eliminate one of these groups to become functional [22].

3. Programmed cell death-related proteases

Concerning to PCD, the main group of proteases performing important roles is the subfamily C14 from the CD clan of cysteine proteases. Their representatives include the metacaspases, paracaspases and caspases.

The first discovered and the most known in terms of structure and function are the caspases. The early reports of caspases and related proteins are those with the genes *cd-3* and *cd-4*, as well as their encoded products from *Caenorhabditis elegans* and the enzyme caspase-1 from mammals [23–27].

To date, caspases have been proven to be "in the heart" of a pathway that mediates the highly ordered process of apoptotic cell suicide [28], and, indeed, they are the convergence point of biochemical pathways on cellular substrates which lead to the activation of a "protease cascade" (the caspase cascade).

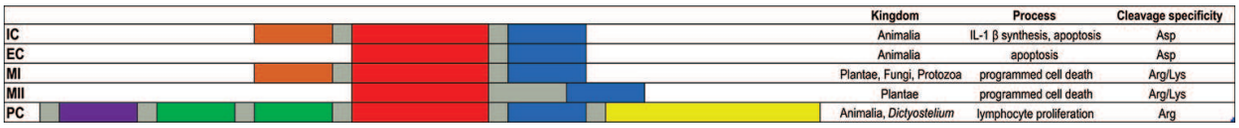

Figure 1. Representation of caspase, metacaspase and paracaspase major domains. IC: initiator caspase; EC: executor caspase; MI: type I metacaspase; MII: type II metacaspase; PC: paracaspase. Red: p20 domain; blue: p10 domain; orange: prodomain/recruitment domain; purple: death domain; green: immunoglobulin-like domain; yellow: paracaspase C-terminal region. Based on Vercammen et al. [8].

The importance of this cascade can be seen when disturbance of its regulation occurs on the cells, what causes immunodeficiency, carcinogenesis and other troubles related to aberrant PCD [29–31].

The cascade overview reveals two functional groups of caspases, concerning their position on the sequence of proteolytic events (**Figure 1**). The first caspases to be activated are the initiators which become active by the "induced proximity mechanism", often triggered by recruitment of adaptor protein complexes, such as recruitment domain of membrane receptors or by a huge protein complex called apoptosome. This induced proximity leads to the oligomerization of caspase molecules which form a heterodimer that becomes able to trigger the activation of the effector caspases by cleavage of a pro-domain, since these enzymes are synthetized as zymogens. After this cleavage, the effectors themselves also stay organized as heterodimer, and cleave the apoptotic substrates, leading to a typical cellular morphology [8].

Despite the positional and functional differences, all caspases belong to a group named ICE family (interleukin-1β-converting enzyme/caspase 1 family), since caspase 1 was the first characterized member.

About the phylogeny, these molecules are unique and well distributed among the Animalia Kingdom. Their presence ranges from vertebrate organisms such as *Homo sapiens* and *Danio rerio* to invertebrates such *C. elegans* and *Drosophila melanogaster* [32]. Also, their involvement with cell death processes is also well documented.

Alongside caspases, on clan CD, there are caspase relatives called paracaspases which are found in metazoans and in slime molds [4, 32] (**Figure 1**). In humans, paracaspase/MALT1 is associated to the lymphocyte activation by the NF-kB pathway [33, 34]. In *Dictyostelium*, the paracaspase gene disruption does not affect the developmental cell death process, what leads to the suggestion that the enzyme is not required for the phenomenon [35]. A function for human paracaspase MALT 1 on caspase-8 modulation is reinforced by Kawadler et al. [36], whose data show that MALT1 activates caspase-8 during TCR signalling but reduces the cleavage of caspase-3, avoiding apoptosis and inducing cell proliferation.

About its structural architecture, the human MALT 1 monomer has an apparent molecular mass of 41 kDa and the dimer, about 84 kDa and the overall predicted structure shows an N-terminal death domain, two immunoglobulin domains, a paracaspase domain and another immunoglobulin-like domain. The paracaspase domain is folded in a similar way to that of caspases and exhibits the ability to bind on substrate. Also, the enzyme, contrary to caspases, seems not to require cleavage of loop 4 to become active [37, 38].

The other member of CD clan of the cysteine proteases are the metacaspases (**Figure 1**). They are found in Fungi, Protozoa, Chromista and Plantae [8], and were first described in 2000 [32], on a study performing structural and sequence analysis, which revealed a great diversity of protease genes related to caspases in these Phyla. They were credited as strong candidates to perform central roles on PCD [11].

Based on their structural architecture, they were divided into two categories: type I and type II metacaspases. The type I subclass metacaspases have an N-terminal extension, a prodomain with a proline-rich repeat motif that is absent on the type II metacaspases, which, instead, present a 200 amino acid C-terminal extension. Also, many plant type I metacaspases have a zinc finger motif that is similar to that of the plant hypersensitive response/cell death protein lsd-1 [39].

Recently, a third group, named as type III metacaspases, has been proposed [40]. These proteins are found on many phytoplankton organisms and are not grouped with the other metacaspase groups, probably by a p10 domain rearrangement on which the motif SGCXDXQTSADV is located on the N-terminus rather than on the C-terminus, as usual for plant metacaspases. The studied organisms also revealed the presence of metacaspase-like proteases which possess only the p10 domain. These proteases are found on bacteria and may represent an evolutionary connection.

4. Metacaspases

4.1. Plant metacaspases

After the first finding of caspase-like activities on plants [41], as well as on other non-metazoan organisms, an extensive search for the enzymes responsible for these activities was performed [32]. For the model plant, *Arabidopsis thaliana*, for example, nine metacaspases encoded by its genome were found: 3, of type I and 6, of type II metacaspases [42]. The list is being extended, among other examples are: soybean (*Glycine max*) with 16 metacaspases (10, type I and 6, type II); wheat (*Zea mays*) with 12 metacaspases (8, type I and 4, type II) and *Oryza sativa* sp japonica with eight metacaspases (4, type I and 4, type II) [43].

The first observations using cell extracts suggested that metacaspases could be responsible for the caspase-like activities found in plants [44]. Nevertheless, recorded reports show that plant metacaspases are unable to cleave caspase synthetic substrates [45–47]. The caspases cleave their substrates after aspartate residues and metacaspases cleave after Asp or Lys residues at P1 position [43]. So, appears that the metacaspases are not directly responsible for the caspase-like activities found in plants.

Although metacaspases do not have caspase-like activities, many works suggest that they have a role in PCD [48]. The inhibition of a type II metacaspase (McIIPa) suppressed PCD in suspensor cells from an embryonic culture from *Picea abies*. During *P. abies* embryogenesis, it was shown that McIIPa is transported from cytosol to nuclei, where its presence was correlated to DNA fragmentation detection. Also, through experiments with a cell free system in the presence of a mutant form of this protein on its active site, the nuclear alterations were not detected [44]. These data reinforce that McIIPa is directly involved on a pathway which

generates nuclear degradation, an event present on most eukaryotic PCD programmes. In this pathway, the protease can play a role on the cleavage of nuclear proteins, similar to what happens to the metazoan effector caspases [46].

The heterologous expression of the *A. thaliana* metacaspases MCP1b and MCP2b on a *Saccharomyces cerevisiae* strain, disrupted for its metacaspase YCA1 gene, was able to trigger an apoptosis-like phenotype under hydrogen peroxide treatment, and this effect was seen to be abolished on the presence of the pancaspase inhibitor based on the sequence VAD. These findings reinforce a metacaspase role on PCD, as well as the requirement of proteins with caspase-like activity [42].

The involvement of metacaspases on PCD is also suggested by works with plants under pathogen attack. Some examples are given by the detection of gene expression for MCA1 on *A. thaliana* leaves infected by *Pseudomonas syringae* [49], and by the increasing levels of a type II metacaspase on tomato leaves under *Botrytis cynerea* infection, with the detection of cell death phenotype [50].

It cannot be excluded, though, that metacaspases could not be directly involved on PCD regulation, but indirectly involved on signalling cascades that leads to PCD [48].

In face of this, the role of metacaspases are still under discussion, as well as their classification, since there are evidences which favour, and others contrary, to their groupment together with caspases [51, 52]. A cascade mechanism comparable to that of caspases was proposed for vegetable systems concerning cysteine proteases on senescent leaves and seeds on maturation. Bozhkov et al. [46] reinforced the idea that the execution of PCD in plants is controlled by two groups of enzymes with separated cellular localization. One of them is accumulated on lytic compartments and vacuoles, and the other has cytoplasmic-nuclear localization, as in the case of MCIIPa.

4.2. Yeast metacaspases

The first report of a metacaspase on yeast was made by Madeo et al. [53], where the overexpression of the protein codified by theYor197w gene stimulated PCD associated-caspase-like activity on *S. cerevisiae* cells, under H_2O_2 stress. The authors proposed the name Yeast Caspase-1 (YCA1) for the protein. Also, a *S. cerevisiae* mutant strain with an inactive gene encoding a deubiquitinating enzyme (UBP10) showed an apoptosis-related phenotype which appears to be related to the YCA1 presence, in a manner similar to that observed when the cells were submitted to external stimuli, such as treatment with H_2O_2. The overexpression of YCA1 in cells lacking UBP10 resulted on a decrease of about 53% in viability [54].

A study with frataxin-deficient yeast cells (Δ*yfh 1*), sensible to pro-oxidant chemicals, showed that YCA1 is induced under H_2O_2 stress. When performance of metacaspase-deficient yeasts (Δ*yca1*) was evaluated under oxidative stressing condition, a resistance to cell death, reflected by higher glutathione concentrations than the wild-type, was detected, probably caused by the absence of the metacaspase [55].

By using an original approach of combining the techniques of a digestome analysis (an *in vitro* assay aimed to search putative specific substrates of proteases), cleavage of recombinant GAPDH by metacaspase and evaluation of protein levels *in vivo*, wild-type and YCA1 overexpressing yeast cells upon H_2O_2-induced apoptosis were evaluated. Under these oxidative conditions, the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was shown, by mass

spectrometric analysis, to be a specific target of metacaspase. This result suggests a link between programmed cell death and metabolism [56]. Besides the potential role in MCP, this metacaspase seems to have involvement on cell cycle regulation. Deficient *yca1* yeast revealed an increased level of proteins related to the vacuolar catabolism, including six peptidases, as well as chaperones involved on stress response and protein aggregates elimination. YCA1 was also copurified with chaperones that respond to protein aggregation and with a cell-cycle control protein; thus, a role on clearance of protein aggregates during mitosis and aging was proposed [57]. This potential on protein clearance is dependent on the presence of Hsp40 and Hsp104 chaperones, and is reduced by deletion of the proteasome regulator Rnp4, which reduces the proteasome levels. When overproduced, YCA1 extended the life span of the cells by 46–56% [58].

Metacaspase studies are also being performed with other fungi, as in the case of *Candida albicans* metacaspase 1 (CaMCA1). Mutant strains for this gene showed a lack of PCD under oxidative stress, as well as a decrease of energy metabolism intensity followed by accumulation of trehalose and increase on expression of genes related to trehalose biosynthesis, what is a well-known protective mechanism against oxidative stresses [59]. Other example is the study of two metacaspases from *Aspergillus fumigates*, whose deficiency was associated with loss of membrane phospholipid asymmetry and an increased growth under conditions of endoplasmic reticulum stress [60].

4.3. Metacaspases of protozoa

Among the first reports concerning metacaspases on protists was the work of Szallies et al. [61]. According to this, *Trypanosoma brucei* encodes five putative metacaspases. The expression of the *T. brucei* metacaspase MCA4 in *S. cerevisiae* caused a PCD-related phenotype, and the protein was found to be located on nuclei, alongside the yeast metacaspase MCA1. When overexpressed, MCA4 induced PCD.

Two metacaspases (LdMC1 and LdMC2) of *Leishmania donovani* were also characterized and displayed high homology among each other. The proteins were seen to be capable of cleaving trypsin substrates and were inhibited by classical trypsin inhibitors such as leupeptin and antipain. Despite not being able to cleave caspase substrates, when their genes were overexpressed in the parasite cells, a higher sensibility to PCD was observed [62]. The metacaspases MCA2, MCA3 and MCA 5 were detected *in vivo* in their full molecular form and seem not to suffer processing to become active in the parasite cells. They were found to be associated with recycling endosomes that contain recycling receptors and PCD-independent functions were proposed for this association with endosomes [63].

The capability to induce PCD was also investigated for the *Leishmania major* single metacaspase (LmjMCA), whose heterologous expression in a *S. cerevisiae* defective strain was successful on the induction of PCD by oxidative stress. The protease originated from the metacaspase gene revealed an arginine-specific activity and through approaches of site-directed mutagenesis, it was possible to determine that its activity is dependent of H147 and C202 [64]. This metacaspase was suggested to be essential for the parasite, since the tentative to obtain a null mutant was unsuccessful. The protein was detected in variable levels in different compartments at different stages of the cell cycle, in association with the dividing kinetoplast and

the nuclear spindle, what may suggests a role on DNA segregation. Also, it was seen that the protein was associated with cytoskeleton filaments. A role on differentiation and proliferation has been additionally proposed [65].

The activity of a *Plasmodium falciparum* metacaspase 1 (PfMCA-1) increased under high concentrations of calcium and induced PCD under stress conditions [66]. The authors have also presented evidences of the ability of PfMCA1 of triggering a downstream enzyme that is sensible to the pancaspase inhibitor z-VAD-fmk.

5. The caspase fold of metacaspases

Caspases, metacaspases and paracaspases have a conserved pattern of tridimensional organization and are then considered as structural homologues. The degree of this conservation is variable, but the overall structure is related to a conformation named caspase fold that is characterized by a core formed by a contiguous six-stranded β-sheet (β1–β4, β7 and β8) and helices α1–α5 region, present in every caspase structure. Also, the presence of three well-ordered loops (L1, L2 and L4) is well characterized [67].

As it was early discussed, metacaspases are divided into two groups based on the presence of a pro domain or a linker region. Type I metacaspases possess an N-terminal prodomain with length of about 80–120 amino acids [32], with two CXXC-type LSD1-like zinc finger structures as well as a proline/glutamine rich region [45, 68]. The type II metacaspases do not have prodomains but, otherwise, contain a large loop (linker region) between the p10 and p20 domains which ranges about 90–150 amino acids [32, 69].

The p10 and p20 domains are present in all of these proteins. For p10, there is a SGCXDXQTSADV consensus sequence, as well as other conserved short sequences [40]. The p20 domain contains the conserved catalytic dyad histidine/cysteine as a remarkable feature, where the two amino acids are distant from each other in about 29–47 amino acids. There are also other conserved regions/amino acids that together give about 80% of consensus on the entire sequence [32]. A noticeable signature is the motif DSCHSG in the surroundings of the catalytic Cys, which is highly conserved among all type II plant metacaspases [70].

Differently of metacaspases, all caspases contain a conserved QACXG (where X can be R, Q, or G) pentapeptide active-site motif. The catalytic residues histidine 237 and cysteine 285, and those involved in forming the P1 carboxylate binding pocket on caspase 1 (Arg-179, Gln-283, Arg-341 and Ser-347), are also conserved in all other caspases, except for the conservative substitution of the threonine for the serine 347 in caspase 8. This explains the requirement for an aspartate in the substrate P1 position. The residues that form the P2–P4 binding pocket are not well conserved, suggesting that they may determine the substrate specificities of the different caspases [71]. The metacaspases do not have these features and present cleavage specificity for lysine/arginine on the P1 position on the substrate, so their binding residues seem to be of opposite chemical nature from those of caspases.

The type II metacaspases present autoprocessing sites, whose cleavage seems to be necessary for their full activation: the residues Lys 260 and Arg 214, on the wheat type II metacaspase

[70]; Lys 269, from MCIIPa [46]; and the Arg 214, from AtMC9 [45]. Caspases, otherwise, have different cleavage site, always after Asp residues. Caspase 1 is cleaved after Asp-103, Asp-119, Asp-297 and Asp-316 [71]; caspase 7, after Asp-23, Asp-198 [72]; and caspase 3, after Asp-9/Asp-28 and Asp-175 [73].

Actually, there are only two metacaspases with elucidated structural organization. MCA2, from *T. brucei*, the first metacaspase with elucidated structure, is topologically more related to other metacaspases, such as YCA1, than to caspases, presenting the same number and organization of β sheets and α helix, although the enzyme showed 65% of secondary elements similarly to caspase 7. Also, the β-sheet region of MCA2 is sized two strands longer than caspases. As for YCA1, this characteristic prevents dimerization. Autocatalytic processing occurs only *in vitro* and is not required for enzyme activity, which was also shown to be dependent of calcium as in other metacaspases [74].

The crystal structure from the yeast metacaspase Yca1 shows the general patterns of the caspase fold, with the well-ordered loops, being L1 and L4 in opposing sides of the substrate interaction site, and the conserved caspase core. Concerning β conformations, yeast YCA1 presents two β-strands (β5 and β6) which are absent in caspases (caspase 3 and caspase 9), and these are located in a way that blocks dimerization. As a result, YCA1 cannot form dimer, as caspases do. The catalytic dyad consists on the residues Cys276 and His220 which are well conserved among other proteins with caspase fold. Also, the identity between the YCA1 and the caspases 3 and 9 is lower than 12%, and the sequence divergence greatly affected the root-mean-square deviation (RMSD) analysis. The superimposition of YCA1 and caspase 3 showed a higher structural variation than the superimposition of caspase 3 and 9. On *in vitro* assays, the presence of Ca^{2+}, and not of other divalent cations, was required to an enhanced capability of auto processing. Under this condition, two peptide fragments were formed revealing that the processing occurred after Arg72 and Lys86. When compared with MCA2, YCA1 shows 24.9% of amino acid sequence identity and a very similar structure through RMSD analysis [75].

6. Metacaspase activators and regulators

Despite the growing knowledge about the structure and function of CD clan members of cysteine proteases, the molecules involved on the control of their activities are still to be more unravelled. As discussed before, one of the main activation mechanisms of the caspases is its cleavage on specific sites, promoted by other caspases. This processing results on conformational changes and dimerization that enhance the substrate cleavage activity of the target caspase.

For this to happen, it is necessary the assemblage of huge protein complexes, which function as activation platforms. Examples of these are the Fas death-induced signalling complex (DISC), whose association is required for the caspase 8 (pro-caspase 8) self-activation and the apoptosome, which binds to caspase 9 (pro-caspase 9) prior to its activation [11, 28, 31]. It is not clear, if in the case of metacaspases, the auto processing and dimerization are always required for their activation as well as the formation of protein complexes [8].

Until now, biochemical studies have demonstrated that only type II metacaspases undergo autocatalytic activation, similar to the phenomenon observed for caspases. Contrary to that,

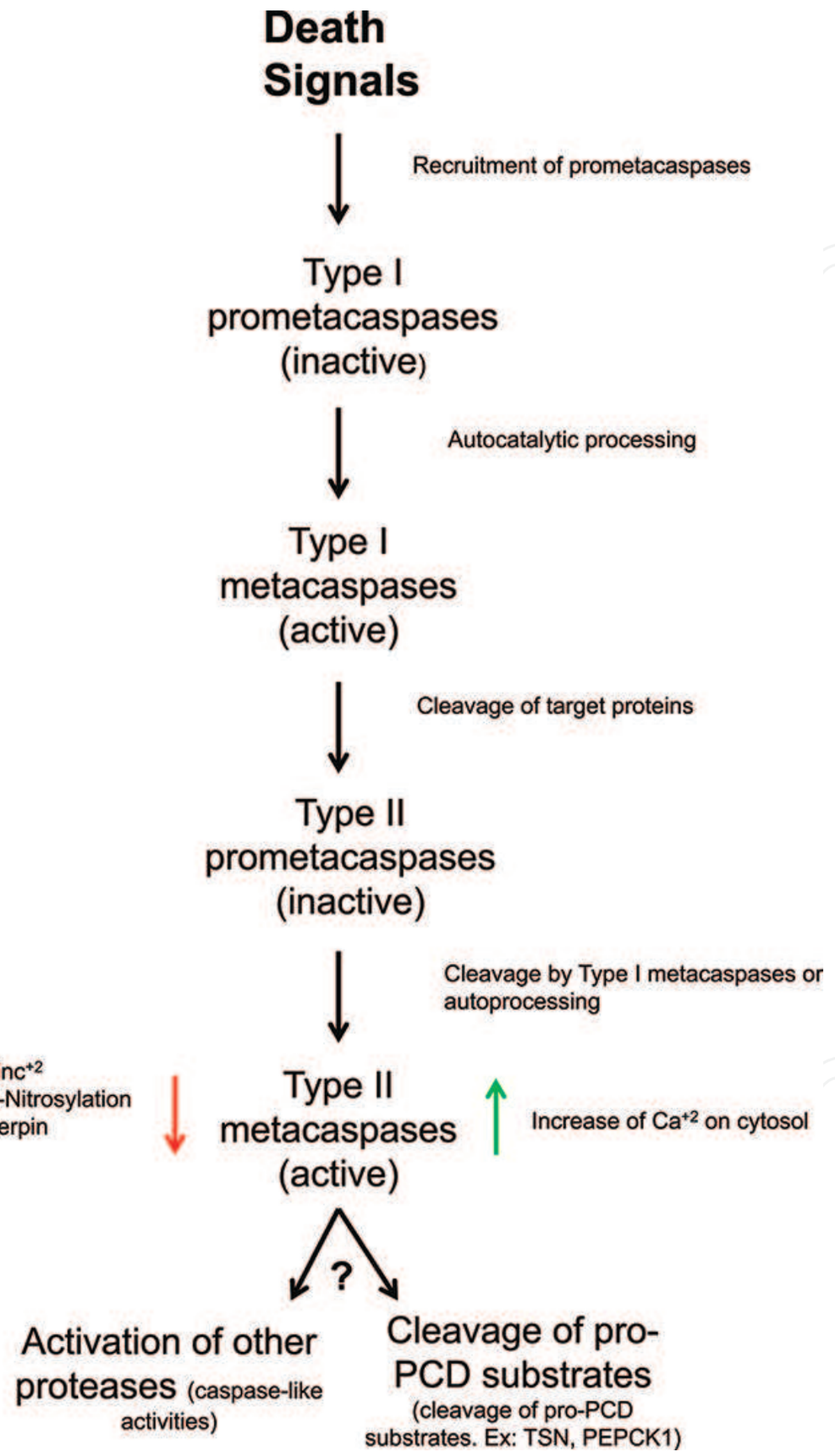

Figure 2. Proposed cascade model of plant proteolytic events triggered by a death inductor signal. The type II metacaspase inhibitors (red arrow) and stimulators (green arrow) are displayed, as well as possible substrates.

no proteolytic activation was observed for type I metacaspases. So a scenery, where type I prometacaspases become active by the action of death signals coupled to the activation of type II prometacaspases, which by their turn become active and able to cleave other proteases and trigger the degradation of cellular components, was proposed as a probable signal transduction pathway during PCD proteolysis in plants [69] (**Figure 2**).

Alongside with the proteolysis processing requirements, there are reports which show calcium dependence for type II metacaspases. By *in vitro* experiments, a type II recombinant metacaspase from *A. thaliana* exhibited Ca^{2+} dependence (10 mM) for its activation, on a pattern indicative of auto-processing [76]. A similar Ca^{2+} dependence was detected on *in vitro* assays with the *T. brucei* MCA2, whose activity peaks on the presence of 10 mM of Ca^{2+}, although the enzyme does not require autoprocessing [77]. This characteristic was shown to be valid for both *L. major* and *P. falciparum* metacaspases [66].

One candidate to be a metacaspase regulator is a serine protease inhibitor called Serpin. Serpin1 from *A. thaliana* was shown to have a potent cleavage activity towards the reactive centre loop of metacaspase 9, besides the ability of covalent binding to the target protein on *in vitro* assays. This study has also demonstrated that both proteins were localized on extracellular space, suggesting they could interact under *in vivo* conditions as well [78].

Other control mechanism proposed is the S-nitrosylation, that is used as a regulation strategy of certain proteins under basal NO levels. The S-nitrosylation of the catalytic cysteine (Cys-147) on *A. thaliana* metacaspase 9 (AtMC9) on its mature processed form does not affect its activity. This happens because other cysteine (Cys-29) residue can act as alternative nucleophile. Despite this, the enzyme can be kept inactive through S-nitrosylation, and otherwise, become active only under conditions of disturbance on cellular redox balance [79].

The effect of zinc on metacaspase activity was also investigated. The supplementation of plant embryos with extra zinc suppressed the terminal differentiation and death of the suspensors, delaying the embryo maturing, and also reducing the intensity of metacaspase activity between 96 and 168 h of development, which is the period when the suspensor death occurs [80]. These data, alongside the work of Bozhkov et al. [46], suggest that zinc may be part of a mechanism of posttranslational regulation of metacaspases, still to be further examined.

7. Metacaspase targets

Little is known about the metacaspase natural substrates, to the present date. This lack of information makes hard to construct a PCD pathway as was done for caspases. Despite this, the efforts aiming at the elucidation of this question are growing.

The first biological substrate discovered for a metacaspase is the Tudor Staphylococcal Nuclease (TSN), a protein involved on gene expression regulation, highly conserved phylogenetically. The cleavage of this protein prevents its function and is important for the execution of apoptosis; also, the protein is known to be a part of the human caspase 3 degradome. TSN was shown to be cleaved by the metacaspase McIIPa (type II metacaspase of *P. abies*) on an *in*

vitro assay. *In vivo,* the McIIPa activity was shown to be simultaneous to the decrease of TSN activity, what was deduced from TUNEL positive embryonic cells [81].

Another substrate found for a metacaspase was the already mentioned GAPDH. This protein was detected as a digest product of an YCA1 metacaspase-enriched extract from *S. cerevisiae* cells subjected to H_2O_2 treatment. As recombinant YCA1 was shown to cleave GAPDH *in vitro*, the *in vivo* evaluation of the GAPDH performed during a comparison between the wild-type and a mutant yeast strain, disrupted for YCA1, both under PCD triggering condition, showed a reduction of the enzyme levels on the wild-type (resistant to PCD). The GAPDH is also a caspase substrate, but the cleavage again happens on different sites of those targeted by the metacaspases. Yet, this is another evidence for the existence of conserved molecular members of a PCD pathway in metazoan and in metacaspase-bearing organisms [56].

Recently, as a remarkable effort, a proteome-wide-level study of *A. thaliana* seedlings, focusing on the identification of physiologic substrates of metacaspase 9, has been performed employing a digestome analysis strategy. Important features of the target proteins were prospected, and it was possible to map the frequencies of the amino acids sitting at the neighbourhood of the Arg or Lys P1-specific cleavage sites. Along with other interesting features, the enzyme has shown a strong tendency to prefer acid residues, as Asp and Glu at the P1′ position. Among the identified substrates, was phosphoenolpyruvate carboxykinase 1 (PEPCK1), a gluconeogenesis enzyme. This protein was shown to be cleaved *in vivo* in such a manner that its activity was enhanced, and thus, the glucose *de novo* synthesis pathway may be stimulated during PCD [82].

8. Molecular modelling of the metacaspase 4 from *Glycine max* (type II metacaspase)

For the comprehension of the structural organization of a type II metacaspase, the delimitation of the p20 and p10 domains of the metacaspase 4 from *G. max* was performed by our group, as well as the analysis of its catalytic amino acids residues and the motifs conservation with other metacaspases and caspases, through protein alignment. Also, the tridimensional structure of the protein was predicted. Metacaspase and caspase sequences of organisms from different taxa (**Table 1**) were aligned using the software Clustal X [83] (http://www.clustal.org/). The sequences were obtained from the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/) data bank and given a treatment for removal of prodomains and loops, for adjustment to the alignment. In this process, the works of Vercammen et al. [8] and of Uren et al. [32] were used as a guide to the delimitation of the domains and catalytic residues.

The p20 and p10 domains of the *G. max* metacaspase were confronted to the Protein Data Bank (http://www.wwpdb.org/) for the search of templates for molecular prediction employing the software Swiss Model [84] (http://swissmodel.expasy.org/). The visualization, the analysis, the validation and the improving of the protein structures were performed with the assistance of the software NOC [85] (http://noch.sourceforge.net).

Protein	Specie	Identification code (gi)
Caspase8	*Homo sapiens*	12862693
Caspase8	*Mus musculus*	29436722
Caspase8	*Gallus gallus*	16555407
Caspase8	*Xenopus laevis*	148228484
Caspase8	*Danio rerio*	46397548
CED3/NEDD2-like	*Drosophila melanogaster*	220901640
CED3	*Caenorhabdits elegans*	11967321
Metacaspase4	*Glycine max*	356556698
Type II metacaspase	*Nicotiana tabacum*	195963550
Metacaspase8	*Arabidopsis thaliana*	32482822
Metacaspase9	*Arabidopsis thaliana*	332191328
Type II metacaspase	*Picea abies*	328887884
Type II metacaspase	*Medicago truncatula*	357451305
Type II metacaspase	*Triticum aestivum*	267850617
Latexprotein	*Hevea brasiliensis*	4235430
Type I metacaspase	*Saccharomyces cerevisiae*	151945290
Type I metacaspase	*Schizosaccharomyces pombe*	19076003

Table 1. List of proteins utilized on the analysis of sequence alignment.

9. Results

9.1. Protein alignment

The initial alignment of caspases and metacaspases sequences with the whole protein sequences (**Figure 3**) divided them into two groups: sequences with larger pro-domains and shorter loops; and sequences with smaller pro-domains and larger loops. Using the position of the catalytic dyad His-Cys as guide and after the removal of pro-domains and loops, the core of p20 and p10 domains became evident. The number of amino acid residues counted 235 residues, being 146 from p20 and 85 from p10 domain. It was also possible to underline the approximate segment borders for the domains of the *G. max* metacaspase, and it was seen that, besides the conserved catalytic dyad position, their adjacent residues (on primary sequence) shared the same chemical nature, encompassing all the protein sequences, despite the phylogenetic distance. The same was seen for the residues close to these regions, considering the p20 segment. In relation to p10, its catalytic residues presented conserved position, despite the chemical divergence (Asp for metacaspases and Arg for caspases). This segment also revealed conservation along its extension, such as the Ser residues close to the catalytic site, amino acids with shorter lateral groups and polar residues. Concerning the prediction

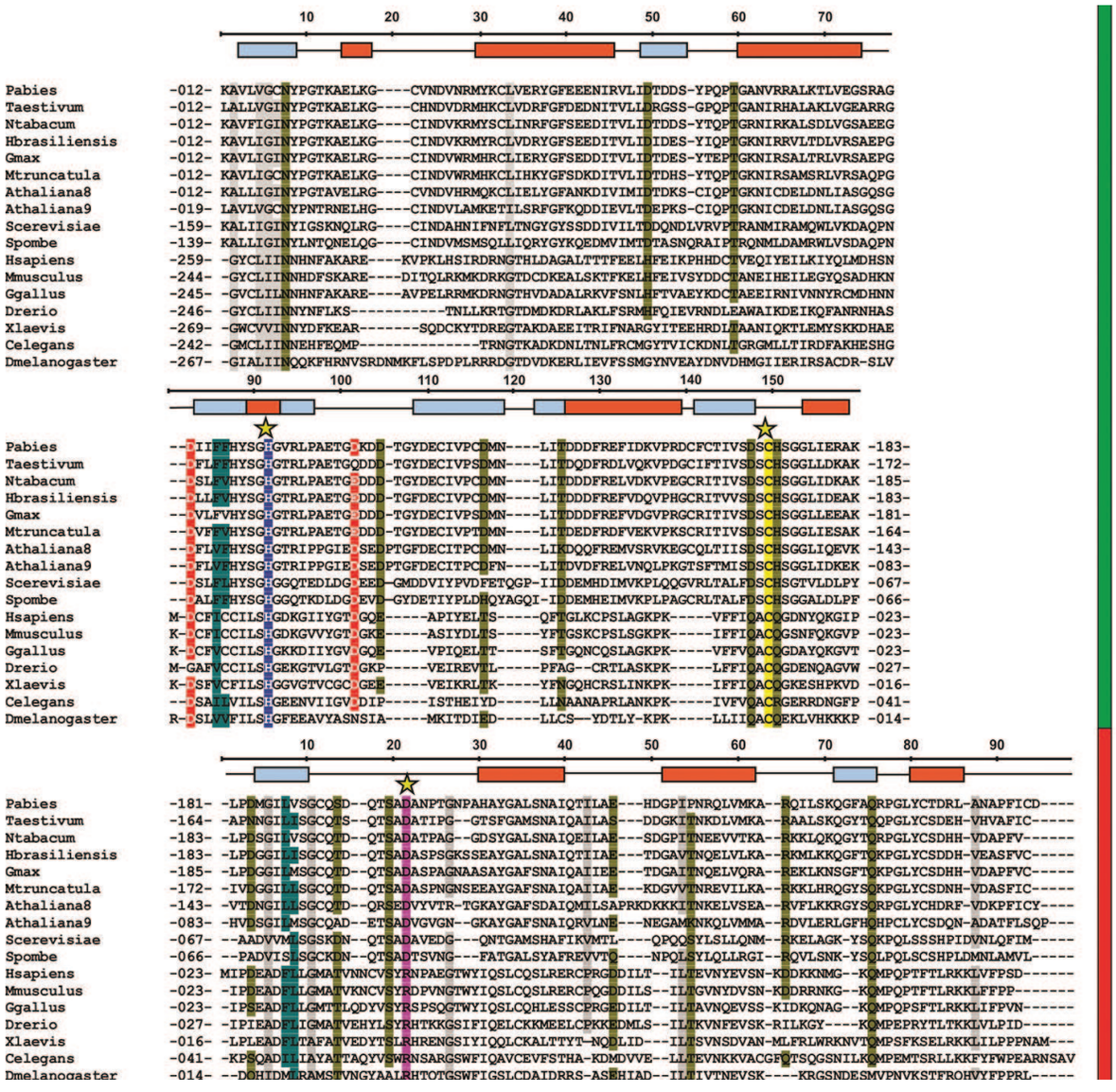

Figure 3. Sequence alignment of caspases and metacaspases, displaying the corresponding secondary structure. Blue: catalytic histidine; yellow: catalytic cysteine; brown: polar residues; grey: aliphatic residues; pink: contact aspartic acid and arginine residues; green: hydrophobic residues. Blue box: β conformation; red box: α-helix/3_{10}helix. Star: catalytic residues.

of secondary structure organization, it was possible to determine that about 33% of the sequence can form α-helix/3-10 helix and that about 23% can take part of β-sheet conformation (**Figure 3**).

9.2. Molecular structure prediction of *G. max* metacaspase 4

The search for a protein that could be applied as a template using the individual subunits from *G. max* metacaspase, resulted in only one choice with a significant sequence similarity rate for the p20 subunit: the chain A of a protein complex from *Geobacter sulfureducens* (PDB-3BIJ). The identity and similarity rates between the template (from the 60th to the

148th residue) and p20 were of 35 and 55%, respectively. As the analysis of tertiary structure of the subunits and the total protein sequences were compatible, even with the high primary structure difference, 3BIJ was used as a template for the construction of a structural model for metacaspase 4. Only one α-helix from 3BIJ was removed for adjustment to the target protein sequence.

Concerning the established structural model (**Figure 4**), it was possible to note that the amino acids residues from the catalytic dyad (His/Cys) of *G. max* metacaspase were closer arranged, spatially. The contact Asp residue also kept this position, suggesting that they are, in fact, intimately associated to the enzymatic catalysis. The model also presented a tridimensional structure close to that of caspases and related proteins, with a core of β-conformations originated from both p20 and p10 being encircled by α-helixes, also originated from both domains. It totalized two β-conformations on its central position which are originated from p10, while p20 contributed with tree of these secondary structures. Other two β-sheets occupied the model extremity. With relation to the α-helixes and 3–10 helixes, there are eight of those that encircled the β-conformation core. Three of those are originated from p20; other two are from p10. The remaining helixes are disposed on the model extremities.

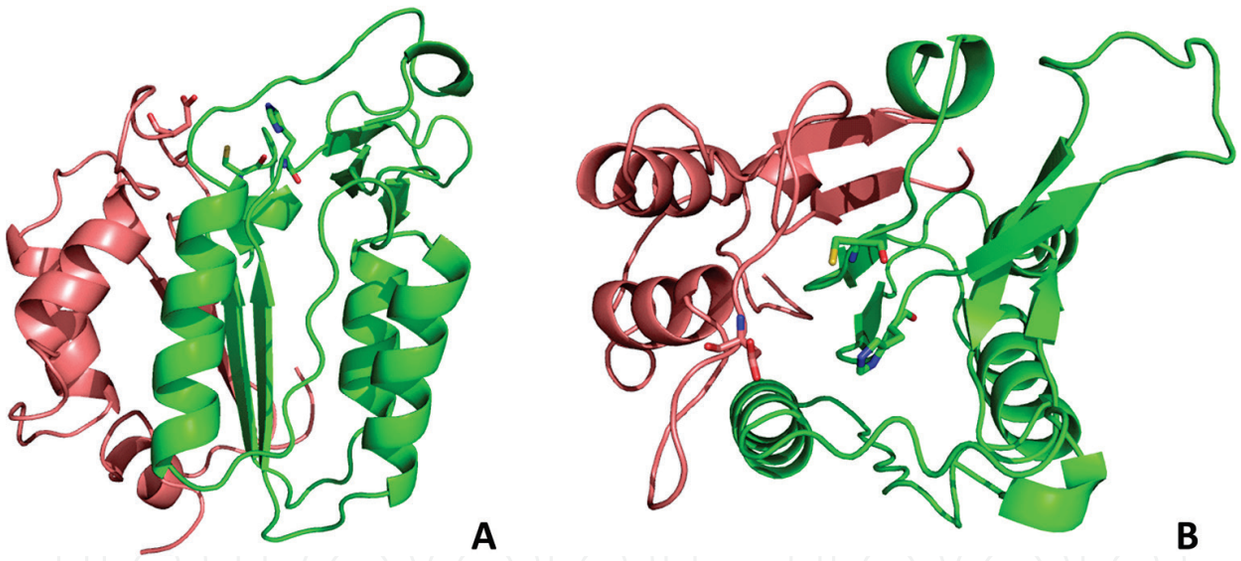

Figure 4. Structural model generated for metacaspase 4 of*Glycine max*. (A) Lateral and (B) top view from catalytic site. The p20 and p10 domains are colored in green and red respectively. The lateral chains from the catalytic amino acids are in evidence.

10. Discussion

The sequence comparison of metacaspases and caspases domains p20 and p10 clearly shows differences on the amino acid composition and disposition. Nevertheless, the segments, once aligned, displayed conserved positions of catalytic residues and of other amino acid residues with conserved physical-chemical properties, what is important to the arrangement on a

similar secondary structure. Among the p20 secondary structure, six peptide sequences participate on α-helixes and 3–10 helixes, and seven composes β-conformations; for p10, three sequences form helix structures and two originate β-conformations. Together, these structures are organized in a similar way to that observed for the protein from the CD clan of C14 family of the cysteine proteases, which includes caspases and metacaspases [86]. The constructed *G. max* metacaspase model shows that these sequences are organized in form to present a core of β conformations encircled by five helixes, with the amino acid residues which compose the active site localized in one of the enzyme central axis poles, out of the β-sheets and α-helixes region.

The used template here was the chain A of the protein not functionally uncharacterized 3BIJ protein of *G. sulfureduccens*. This is the same protein used by Dudkiewicz and Piszczek [87], for the prediction of a model for *Triticum aestivum* type II metacaspase. Interestingly, 3BIJ was seen to be a better template for the considered metacaspase than the *Homo sapiens* caspase 7, whose similarity with soybean metacaspase was also high.

Curiously, a number of recent reports have demonstrated a difference of cleavage specificity among caspases and metacaspases. The recombinant metacaspase McIIPa, from *P. abies,* was shown to be efficient on the cleavage of the peptide sequence EGR and GRR, but not of VEID and YVAD, which are processed by caspases [46]. In 2008, He et al. [88] demonstrated that the recombinant metacaspase 8 from *Arabidopsis thaliana* was efficient on the cleavage of the sequence GRR, being unable to process DEVD, VEID, IETD and YVAD. This difference on enzymatic activity nature of metacaspases and caspases are generating an open discussion on literature. The denomination "caspase" itself gives clues to these discussions concerning to the particularities presented by caspases and metacaspases. Carmona-Gutierrez et al. [51] suggested that metacaspases and caspases present characteristics that fulfil the homology criteria, as they participate of a common program, share substrates and by the fact that metacaspase genes are present in all organisms, except superior animal taxa. In this scenario, the caspase genes could be derived from metacaspases.

In response, Enoksson and Salvesen [52] defended that yeasts and plants would employ PCD programmes other than apoptosis, what would be an innovation when compared to animals. Also they argue that, even if metacaspases and caspases share the tridimensional structure, the cleavage specificity displayed by them could show that they are derived from a common ancestor, which was neither caspase nor metacaspase.

This scenery is reinforced by data from the work of Koonin and Aravind [11], which showed that metacaspases have similarities with α-proteobacteria homologues, the group of endosymbiotic mitochondria ancestors, being the metacaspases from prokaryotic origin. Also, it was demonstrated that bacterial homologues of caspase-related proteins showed a greater diversity of phyletic distribution, domain architecture and sequence than their eukaryotic counterparts, suggesting that events of gene transference from prokaryote to eukaryotes could be an explanation for the distribution of caspase-related genes, what could have been assured by multiple bacterial gene infusions [87].

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