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FUNCTIONAL ANALYSIS OF METACASPASES IN *ARABIDOPSIS THALIANA*

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EXAMINATION COMMITTEE

For the annotation of the *Arabidopsis* metacaspases, nomenclature was followed as in the first publication of *Arabidopsis* metacaspases (Vercammen et al., 2004), were they were annotated as **Atmc**.

Reference:

Vercammen, D., van de Cotte, B., De Jaeger, G., Eeckhout, D., Casteels, P., Vandepoele, K., Vandenberghe, I., Van Beeumen, J., Inze, 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.

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Chapter 1 - Introduction

Metacaspases, distant homologues of mammalian caspases, are a distinct family of cysteine proteases in plants.

1.1 Abstract

Cysteine proteases (CP) are proteases which need a catalytic cysteine/histidine dyad for their activity. They are involved in many aspects of plant development and are also known to play a role in developmental and defense-related programmed cell death, senescence, seed development, cell cycle and nodulation and nitrogen fixation. The activity of cysteine proteases is regulated by inhibitors and processing of their less active precursors. Within the CPs different families possess different substrate specificities. However, little (natural) substrates are characterized hitherto. The *Arabidopsis* genome contains at least 149 (putative) CPs which can be classified into four different clans (clan CA, CD, CE, CF) according to their evolutionary conservation. The expression profiles of all *Arabidopsis* CPs were analyzed in microarray databases relative to plant development, plant organs and in response to stress responses.

Recently, in plants, caspase-like activities have been detected and have been associated with cell death in many cases. However, despite the initial assumption that the metacaspases would be prime candidates for performing these caspase-like activities, other proteases have been characterized that were proposed to fulfill this role, such as the legumains and saspases. Indeed, the metacaspases were identified to have an arginine / lysine-specificity in contrast to their mammalian counterparts which possess an aspartate-specificity. In analogy to the mammalian caspases, the metacaspases undergo selfcatalytical processing, which was shown to be required for their activity (at least for the Type II). Likewise to the key regulatory role that caspases play in apoptosis, there are little arguments which suggest that metacaspases would also play an important role in plant programmed cell death.

1.2 General characteristics of cysteine proteases

Proteolysis is essential in many aspects of plant growth and development. It is a complex process which involves many enzymes and various proteolytic pathways in different cellular compartments. Proteases, peptidases, or peptide hydrolases are enzymes able to hydrolyze peptide bonds. According to their catalytic site they can be classified into four major **classes**: serine proteases, aspartic proteases, metalloproteases and cysteine proteases (Barrett, 1994). The cysteine proteases are grouped into different **clans** (CA, CD, CE, CF, CH, CJ, CK, CX, PA(C), PB(C) and PC(C)) based on their evolutionary conservation (Rawlings and Barrett, 1993). Crucial to the activity for all cysteine proteases (except for those in the clan PB(C) is the presence of a **catalytic cysteine-histidine (Cys / His) dyad**. Between the different clans the order of these catalytic residues is different and for some clans there are additional highly conserved amino acids which are important for activity. The Cys / His catalytic dyad is known to be sufficient for clans CD, CK and CJ; the other cysteine peptidases possess a third highly conserved amino acid within a catalytic **triad** which differs between the clans (Mottram et al., 2003)). In plants, cysteine proteases have a molecular mass between 21-30 kDa and most of them show the highest hydrolytic activity at an acidic pH (pH 4 - 6.5). Cysteine proteases play an **essential role** in plant proteolysis, their contribution is plant and organ dependent but stands for up to 30% of total proteolytic activity in mature non-senescing organs (Grudkowska and Zagdanska, 2004). However, internal and external stimuli strongly influence the activities of these proteases, and in some cases they are even responsible for up to 90% of the total proteolytic activity (Wisniewski and Zagdanska, 2001).

1.3 Classification of cysteine proteases

Based on their structural and evolutionary relationship cysteine peptidases are grouped into different clans and families (Rawlings and Barrett, 1993). A clan consist of a group of evolutionary related families. Despite the large differences in amino acid sequences, the families in such a clan are ultimately related, as seen by the conservation of their protein folds. Cysteine proteases have come from different evolutionary origins, within each clan the proteases possess distinctive structures and properties. The terminology for the families and clans is guarded by the the MEROPS database (http://merops.sanger.ac.uk/) (Rawlings and Barrett, 2000). Each identifier starts with a letter indicating the catalytic type: for the cysteine peptidases this is the letter C, for aspartic proteases, metalloproteases and serine proteases the letters A, M and S respectively .Proteases of clans starting with the letter “P” can have different catalytic residues. The identifier for a clan is completed with a letter following the catalytic class (e.g. clan CA), each family is characterized by a number (e.g. C14). These numbers were often assigned before the clan was even characterized and are as such unrelated to the clan to which the family belongs to. Currently, in plants at least six clans of cysteine prepidases are identified: clan CA, clan CD, clan CE and clan CF, PC(C) and PB(C) ((van der Hoorn and Jones, 2004); MEROPS database). The different clans are distinghuished by their order and identity of their catalytic residues (besides the essential cysteine residue: presence of the histidine residue, presence of an additional amino acid) (Table 1).

1.3.1 Overview of the different clans and families

In general, **clan CA** is characterized by the functionally important asparagine and aspartatic acid which occur in a defined order (Table 1). The clan consists of a large number of cysteine proteases which comprises the best characterized proteins of the cysteine proteases, namely the papain-like enzymes (C1 family). These proteases

are called papain-like enzymes as the protein fold of the peptidase unit resembles that of papain. This family represents the largest and most widespread family amongst plant endopeptidases (MEROPS). Papain-like enzymes are known to play a crucial role in plant pathogen defense (reviewed in (van der Hoorn and Jones, 2004)), mobilization of storage proteins in seeds and senescing tissues ((Schmid et al., 1999), (Gietl and Schmid, 2001)) and in plant cell death which is highly connected to plant development (reviewed in (Beers et al., 2000)). Plant papain-like enzymes are characterized by a broad *in vitro* substrate specificity with a preference for substrates with a bulky non-polar side chain at the P2 position. Furthermore, they are inhibited by cysteine protease inhibitors such as E-64 and cystatins (see 1.4). Another feature of these enzymes is the necessity of a glutamin residue which precedes the cysteine / histidine residues. In addition, they are synthesized as preproproteins or zymogens (40-50 kDa) which are processed to mature, active peptidases (22-35 kDa) (see 1.4). (Phyto)calpains (C2 family) are calcium-dependent proteases which micro- or millimolar concentrations of calcium for their activity (reviewed in (Margis and Margis-Pinheiro, 2003)). They possess a highly conserved structure in the catalytic domain, however an overall diversity outside of the catalytic domain (Ono et al., 1998). The protein fold of the peptidase unit of the calpains resembles that of the papain-like enzymes. The first calpain homolog in plants was identified through characterization of the *dek1* mutant, which showed a defective aleurone cell development in the endosperm (Lid et al., 2002). Virus-induced gene silencing of *Nicotiana benthamiana* calpain showed that the phytocalpain is responsible for the regulation of cell division and differentiation (Ahn et al., 2004). To date, few reports on the identification of phytocalpains have been published in plants and little is known about the functional implications of these calpains. The deubiquitinating enzymes can be divided into two groups based on their amino acid sequence and substrate specificity: the Ubiquitin (Ub) C-terminal hydrolases (UCHs, C12 family) and Ubiquitin-specific proteases (UBPs, C19 family) (for review: (Wilkinson, 1997)). UCHs are relatively small polypeptides which have the preference to cleave Ubiquitin from small adducts, such as peptides or amino acids. In addition, they act on a catalytic triad cysteine / histidine / aspartatic

Clan	catalytic residues
clan CA	Cys, His, Asn/Asp
clan CD	His, Cys
clan CE	His, Glu/Asp, Cys
clan CF	Glu, Cys, His
clan CH	Cys, Thr, His
clan CJ	Cys, His
clan CK	Cys, His
clan CX	unassigned
clan PA(C)	His, Asp, Cys
clan PB(C)	Cys
clan PC(C)	Cys, His, Glu

Table 1: Clans of Cysteine peptidases and the essential residues in catalysis. The order of the catalytic residues is as represented in the text. Cys = Cysteine, His = Histidine, Asn = Asparagine, Asp = Aspartic acid, Glu = Glumatic acid, Thr = Threonine (as derived from the MEROPS database).

acid (Johnston et al., 1997). In plants, there is a lack of functional data from these UCH enzymes. UBPs are larger polypeptides which have a more complicated structure than the ubiquitinyl hydrolases of the related UCH family. They preferentially cleave Ub linked to larger proteins by either peptide or isopeptide bond. The enzymes are characterized by the conservation of two catalytic motifs: the cysteine and histidine box. Outside these boxes, the amino acid sequences diverge which possibly reflects the specific targets and, concomitant with that, different cellular functions (Wilkinson, 1997). Large UBP families have been identified in different organisms. Despite their potential importance little is know about UBPs in plants: few knockout mutants have been characterized in plants. Insertion mutants of AtUBP1 and AtUBP2 has suggested a role for these proteases in abnormal protein turnover (Yan et al., 2000), and an insertion mutant of AtUBP14 a function in embryonic development (Doelling et al., 2001). The C65 family consists of novel identified deubiquitinating enzymes: the OTU (ovarian tumour gene)-like enzymes. This family was first discovered in mammalian eukaryotes (Makarova et al., 2000). In plants, nothing is known about their function.

For the activity of enzymes belonging to the **clan CD** the presence of the catalytic dyad, in the defined order histidine / cysteine in sequence, is sufficient (Table 1). Clan CD consists of the legumain family (C13 family), also called the vacuolar processing enzymes (VPEs). Most members of the family are endopeptidases with a restricted specificity for asparaginyl bonds and are therefore called asparginyl endopeptidases. There are four members in the VPE gene family: α, β, χ and δ. They can be categorized in two subfamilies: the seed type (β and δ) and the vegetative type (α and χ) VPEs. Seed-type specific VPEs are responsible for processing and assembly of seed storage proteins.(Gruis et al., 2002) and have also been associated with developmental cell death during embryogenesis (Nakaune et al., 2005). The VPE members expressed in the vegetative organs are found to be upregulated in response to various types of cell death and stress conditions (Kinoshita et al., 1999). Indeed, the vegetative-types are involved in defense-related hypersensitve cell death of plants (Rojo et al., 2004). Furthermore, both types of VPEs were demonstrated to exhibit caspase-1 like activity ((Nakaune et al., 2005); (Hatsugai et al., 2004); (Rojo et al., 2004)). VPEs are synthesized as inactive precursors, preprolegumains (see § 1.4.) and are only active at an acidic pH (Muntz and Shutov, 2002). The C14 family, or metacaspase family are of special interest and are reviewed in § 1.9. On the C50 family (separase) no studies have been led in plants. Arabidopsis contains only one hypothetical protein which belongs to that family.

Clan CE consists of a large family of *Ulp proteins (C48 family)*. This clan needs the presence of the catalytic triad histidine / glutamic acid – aspartatic acid / cysteine for its activity (Table 1). The C48 family contains SUMO (small ubiquitin-like modifier) deconjugating enzymes and are known to be involved in the processing of SUMO precursors and in the deconjugation of SUMO proteins from cellular targets. Ulp1 was first discovered in yeast, and found to specifically remove SUMO from proteins and required for cell cycle progression (Li and Hochstrasser, 1999). Hitherto, nothing is known about the function of these Ulp proteins in plants, also the reason for this multitude of Ulp-like enzymes in plants is unclear.

The catalytic triad glutamic acid / cysteine / histidine is required for the activity of the enzymes belonging to the **clan CF**. Clan CF consists of the *C15 family, pyrrolidone-carboxylate peptidases*, which are responsible for the release of N-terminal pyroglutamate residues from proteins (Singleton et al., 1999). The exact function of these proteins in eukaryotes remains elusive.

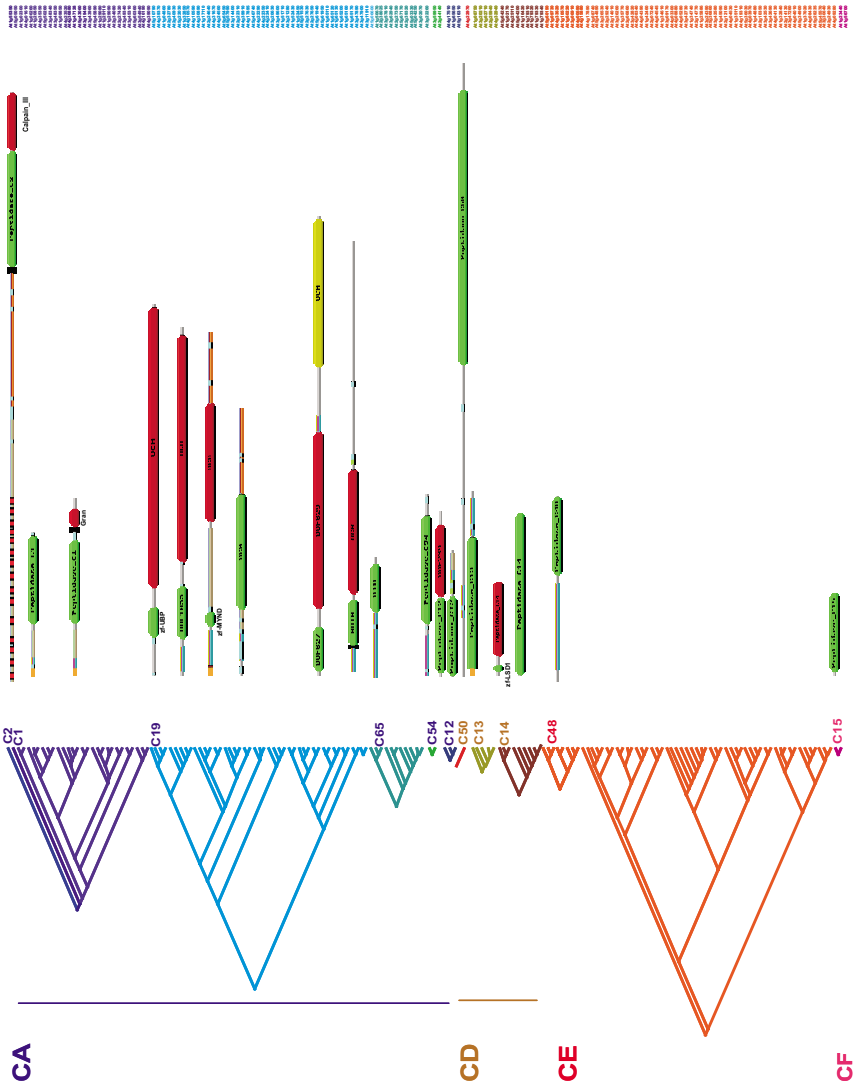
1.3.2 Arabidopsis thaliana cysteine proteases

The *Arabidopsis thaliana* genome contains at least 149 cysteine proteases. For the identification of all cysteine proteases of *Arabidopsis*, the MEROPS classification of *Arabidopsis* cysteine proteases was used and the domain characteristic of each clan and family was identified. Next, the Pfam database was examined for the presence of the respective domain amongst all *Arabidopsis* proteins, followed by retrieval of the full length sequences. Subsequently, the cysteine proteases present in the Arabidopsis genome were identified via their AGI code by blasting, using the TIGR annotation of 2006 (http://www.tigr.org/). A simple phylogenetic tree was constructed using a CLUSTALW alignment of all *Arabidopsis* cysteine proteases and is represented in Figure 1 (analysis performed by Dr. B. Belenghi). A complete description and annotation of the 149 *Arabidopsis* cysteine proteases is presented in Appendix 1. The cysteine peptidases can be divided in four major clans: clan CA, CD, CE and CF. The families which can be distinghuished within the different clans are characterized by the presence of specific catalytic and also non-catalytic domain(s) which reflects the evolutionary conservation amongst the different proteases. Clan CA comprises the families of papain-like and cathepsin-like enzymes (C1), the calpain-like proteases (C2), Ubiquitin (Ub) C-terminal hydrolases (UCHs, C12 family) and deubiquitinases (UBPs, C19 family), autophagy genes (C54) and OTU-like enzymes (C65). The C2 family of *Arabidopsis* consists of only one member and is characterized by a C2 peptidase domain and a calpain-specific domain (calpain large subunit, domain III). All members of the papain- / cathepsin-like family bear the C1 peptidase domain, a subgroup contains

also an additional domain, a Granulin domain. The deubiquitinating enzymes split into two families: the C12 family consists of the 20 to 30 kDa polypeptides, the C19 family of the 100 to 200 kDa polypeptides. Figure 1 shows that all members of the C19 family show a UCH (Ubiquitin C-terminal hydrolase) domain. Different subfamilies can be distinghuised by the presence of a Zn-finger (Zf-UBP and Zf-MYND), an N-terminal region with unknown function (DUF627 and DUF629) or a MATH domain. The C12 family is characterized by a C12 peptidase domain and for some proteases a DUF239 domain of unknown function. The metacaspase family (C14) are divided into two subfamilies, one containing an LSD1-like zinc finger domain. The C15 family, C48, C13, C50 and C54 are characterized by a peptidase domain, specific for its family. It has to be remarked that we have restricted our analysis to the most important and major clans of the cysteine peptidases. As such, members of the PC(C) and PB(C) clan were not taken into account, this is also due to the high number of unassigned peptidases.

Figure 1 (*other side*): Classification and phylogenetic tree of all 149 Cysteine proteases of *Arabidopsis thaliana* (B. Belenghi, unpublished results). The completion of the *Arabidopsis* genome sequence gave the opportunity to identify and classify all the cysteine proteases of this plant species (as described in the text). Four major clans can be distinghuished in the *Arabidopsis* genome, which can be further subdivided into families. These families are characterized by the presence of specific catalytic and non-catalytic domain(s) which reflects the evolutionary conservation amongst the different proteases.

Calpain_III = calpain large subunit, domain III: domain with unknown function; Gran = Granulin (Yamada et al., 2001); UCH = ubiquitin C-terminal hydrolase; Zf-UBP = Zinc finger in ubiquitin hydrolases (Hershko and Ciechanover, 1998); Zf-MYND = Myeloid, Nervy, and DEAF-1 (Gross and McGinnis, 1996); DUF1055 / DUF627 / DUF 629 / DUF239: domains with an unknown function; MATH = Meprin And TRAF-Homology (MATH) domain (Sunnerhagen et al., 2002); Zf-LSD1 = LSD1-like zinc finger (Dietrich et al., 1997).


C2
C1
C19
C65
C54
C12
C50
C13
C14
C48
C15
CA
CD
CE
CF

1.4 Regulation of proteolytic enzyme activity / Regulation of cysteine proteases.

Proteolytic enzyme activity is regulated in a highly strict manner as proteolysis at a wrong time and location may be lethal. Important regulating factors are inhibition of the activity by cysteine protease inhibitors and synthesis of the proteases as less active precursor molecules in order to prevent inappropriate proteolysis. Most cysteine proteases are activated by limited intra- or intermolecular proteolysis cleaving off an inhibitory peptide. These regulatory proenzyme peptides are thought to have a four-fold function: inhibition of the activity of the proenzyme (Wiederanders, 2003), ensuring the correct folding of the mature protein (Tao et al., 1994), stabilization of the protein in neutral and alkaline environments (Mach et al., 1994) and targeting of the enzyme. The latter was demonstrated for the plant thiol protease aleurain where the prodomain contained an N-terminal vacuolar targeting domain NPIR (Holwerda et al., 1992).

The **processing of protease precursors** results in an activation (or maturation) of the enzymes and includes one or more limited proteolytic cleavages within the polypeptide backbone as well as at the N- and C-termini, respectively (reviewed in (Wiederanders, 2003)(Mach et al., 1994)). Processing of a papain-like enzyme and a VPE is represented in Figure 2. The processing can be catalyzed by the molecule itself (intramolecular) or by a second molecule (intermolecular). Some proteases can autoprocess *in vitro*, while others need the assistance of another active protease. Protease precursor activation *in vivo* is generally very complex involving other factors such as the pH but also other proteases, their endogenous inhibitors and clusters of negative charged surfaces (Mason and Massey, 1992) which can influence the maturation process. For example, for processing of the vacuolar papain-like enzyme SH-EP, an asparaginyl endopeptidase of *Vigna mungo* seeds (VmPE-1) would be required (Okamoto et al., 1999a); (Okamoto and Minamikawa, 1999). In addition, upon release of the propeptides of the enzymes these propeptides can show affinity for the active enzyme and form as such additional inhibitors (Wiederanders, 2003). However,

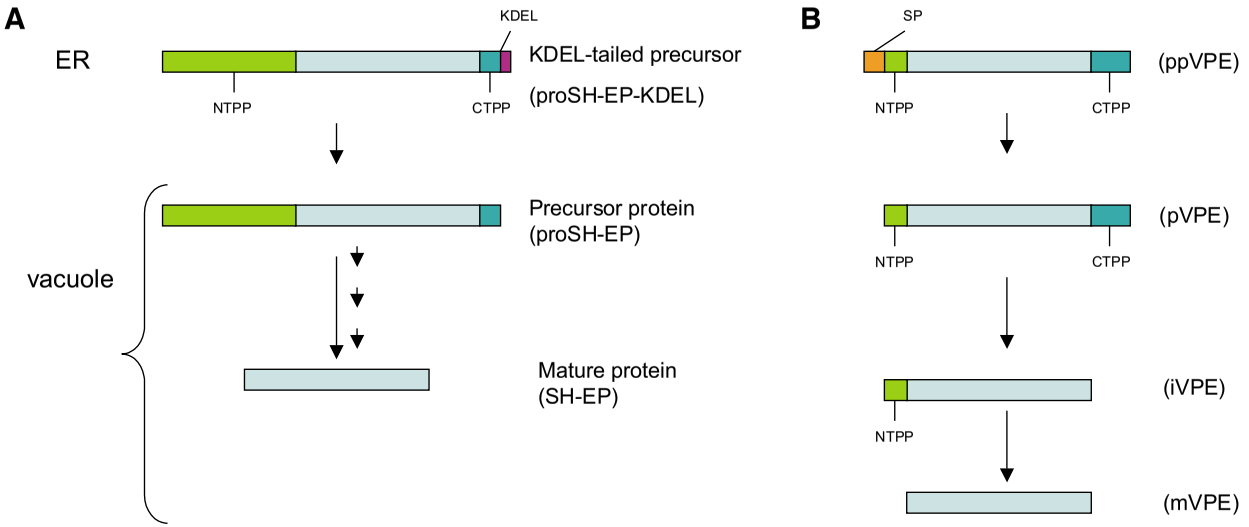


Figure 2: Processing of cysteine proteases results in the formation of an active, mature enzyme. Representation of processing of papain-like enzymes (SH-EP protease from *Vigna mungo* seeds) (A), and of legumains (B). **(A)** SH-EP possesses both a large N-terminal propeptide (NTPP) and a C-terminal propeptide (CTPP). A C-terminal KDEL ER retention signal peptide is located at the C-terminus. Cleavage of this KDEL signal, either while the protein is still in the ER or immediately after its exit from the ER, results in translocation to the vacuole. At that stage, the enzyme is still in an inactive form (Okamoto et al., 1999b). Upon translocation, the acidic pH leads to (auto)processing of the precursor protein into the fully active mature enzyme, which occurs through different intermediates. It was indicated that a VPE would be involved in processing of SH-EP (Okamoto et al., 1999a). **(B)** Release of the signal peptide of the preproVPE on the ER results in the inactive proform (pVPE) and translocation to the vacuole. Subsequent self-catalytic cleavage of the CTPP yields an intermediate form of the VPE (iVPE) which is an active form of the enzyme. Further release of the NTPP results in mature VPE, however, is not essential to activate the enzyme (Kuroyanagi et al., 2002).

most split products may be rapidly degraded upon their liberation in the acidic environments where they arise and as such the (*in vivo*) action of these peptides is not very likely (Maubach et al., 1997).(Rowan et al., 1992) The fact that proteases are synthesized as inactive (or less active) proenzymes prevents accidental or inappropriate proteolysis. The mode of propeptide inhibition has been clarified. It was demonstrated that an antiparallel orientation of the prodomain towards its peptidyl substrate covers the prodomain from cleavage while access of the substrate to the active site is hindered (Groves et al., 1998).

For plant **papain**-like CPs the processing from propeptides to active peptidases occurs in a multi-step manner in post-Golgi, lytic compartment(s) such as the vacuole which possess the required acidic pH and reducing conditions for activation ((Holwerda et al., 1990); (Okamoto et al., 1999b)) (Figure 2A). These enzymes are not expected to be active in the lumen of the ER where these activation conditions are not favourable. As such, the inactive proproteins are accumulated in the ER and only activated upon secretion or sequestration to acidic compartments of the cell. Several plant papain-like enzymes possess putative ER retention signals at their C-termini such as KDEL residues (Guerrero et al., 1998), which is thought to be responsible for accumulation of these inactive proenzymes. Posttranslational removal of the C-terminal KDEL signal probably results in secretion of the proteins to other compartments for activation and occurs prior to maturation / processing of the proenzyme (Okamoto et al., 1999b). However, it has to be remarked that removal of the KDEL signal is not always needed for secretion (Jones and Herman, 1993). Most papain-like enzymes contain an N-terminal prodomain which is cleaved off to produce an active enzyme. However, some plant papain homologues, such as actidinin, possess a carboxyterminal prodomain in addition. The C-terminal extension is necessary for proper autocatalytic cleavage of the proprotein (Paul et al., 1995).

Plant pro**legumains** are activated by self-catalytic removal of a short N-terminal and a much longer C-terminal propeptide which flank the mature enzyme (Figure 2B). Under acidic conditions, the 56 kDa precursor protein (pVPE) was self catalytically converted to an intermediate form (iVPE) and then to the 40-kDa mature form (mVPE).

It was shown that sequential removal of the C-terminal propeptide and N-terminal propeptide produced mVPE. As both iVPE and mVPE exhibited the activity, while pVPE exhibited no activity it could be implied that the removal of the C-terminal propeptide is essential for activating the enzyme. Further removal of the N-terminal propeptide from iVPE is not required to activate the enzyme (Kuroyanagi et al., 2002).

Furthermore, it was demonstrated that for the **metacaspases**, at least for the type II metacaspases, cysteine-dependent processing is required for their activity. Hereby processing includes cleaving off a short N-terminal prodomain and concomitant cleavage and separation of the large and small subunits. Processing of the type II metacaspases is in complete analogy with that of the mammalian caspases (see § 1.9.3).

Regulation of CP enzymatic activity by **cysteine protease inhibitors (CPI)** forms another important aspect of regulation. Cysteine protease inhibitors inactivate proteases by trapping them in a reversible or irreversible tight complex (Barrett et al., 1998). The cystatin superfamily of tight and reversibly binding inhibitors of the papain-like cysteine proteinases (clan CA, family C1) have been divided into three animal families, namely the stefins, the cystatins and the kinenogens, and one plant cystatin family, the so-called phytocystatins (Abrahamson, 1994). The primary sequences of phytocystains have a high degree of homology with the members of the cystatin family, but they resemble stefins by the absence of disulfide bonds and cysteine residues (Laing and McManus, 2002), as such the phytocystatins are classified as a new cystatin family. **Plant cystatins** are characterized in several monocots and dicots, including rice (Abe et al., 1987), maize (Abe et al., 1992), soybean (Botella et al., 1996), Chinese cabbage (Lim et al., 1996), chestnut (Pernas et al., 1998), potato (Waldron et al., 1993), *Arabidopsis* (Belenghi et al., 2003) and tomato (Bolter, 1993). It has been shown that members of the cystatin superfamily interact directly with the active site cleft of papain at three regions of the mature cystatin. These are an N-terminal region with a conserved glycine residue, a central loop containing the highly conserved Q-X-V-X-G motif and a carboxy-terminal region with a conserved tryptophan residue (Corr-Menguy et al., 2002).

The plant cystatins are further characterized by a consensus sequence (LVI)-(AGT)-(RKE)-(FY)-(AS)-(VI)-X-(EDQV)-(HYFQ)-N within a predicted amino-terminal alpha-helix. Plant cystatins are thought to have several possible functions besides regulating the activity of endogenous cysteine proteinases during different physiological processes such as seed maturation and germination ((Arai et al., 2002); (Corr-Menguy et al., 2002)), programmed cell death (Solomon et al., 1999) and acting as plant defensive proteins to biotic and abiotic stresses in a variety of plants. As a response to wounding and methyl jasmonate expression of cystatins genes was induced in soybean (Botella et al., 1996); in barley the expression of cystatin-encoding genes was increased in vegetative tissues in response to anaerobiosis, dark and cold shock (Gaddour et al., 2001). Overexpression of *Arabidopsis* cystatin blocks cell death activated by either avirulent pathogens or by oxidative and nitrosative stress in both *Arabidopsis* cultured cells or tobacco plants (Belenghi et al., 2003). In addition, cystatins seemed to confer resistance against plant-parasitic nematodes when expressed in transgenic plants ((Cowgill et al., 2002); (Urwin et al., 1997)).Transgenic tobacco plants expressing CPI genes have an increased resistance against different potyviruses and potentially also against other viruses whose replication involves cysteine proteinase activities (Gutierrez-Campos et al., 1999). Transgenic rice plants expressing a phytocystatin showed potent inhibitory activity against proteinases that occur in the gut of the insect pests (Irie et al., 1996), a CPI from pearl millet seeds exhibits antifungal activity (Joshi et al., 1998), a plant defensive cystatin (soyacystatin) targets cathepsin L-like digestive cysteine proteinases in the larval midgut of western corn rootworm, and by attenuating digestive proteolysis growth and development of western corn rootworm is substantially inhibited (Koiwa et al., 2000). Moreover, the cystatin from chestnut seeds is active against digestive proteinases from the insect *Tribolium castaneum* and the mite *Dermatophagoides farinae*, two important agricultural pests (Pernas et al., 1998). Transgenic *Arabidopsis* plants expressing modified oryzacystatin show resistance to the field slug, by inhibiting the major proteinase present in the digestive gland of the slug and as such suppressing the growth rate of the slug (Walker et al., 1999).

1.5 Substrates of cysteine proteases

In plants, little is known about the substrates of CPs, the least of the natural substrates. As described in §1.4. autocleavage leads to activation of cysteine proteases. This means that the proform of the cysteine proteases can also be considered as a substrate of the enzymes, however they will not be taken into account in this section.

Legumains (VPEs; C13) cleave peptide bonds with asparagine (Asn) or aspartatic acid (Asp) (the latter less efficiently) at the P1 position (Becker et al., 1995). The actual amino acid upon which proteolytic cleavage of the polypeptide sequence occurs, is termed the P1 position. The three amino acids more upstream in the sequence are hence the P2, P3 and P4 positions. These positions are important for substrate specificity. *In vitro* processing assays with purified VPE and seed protein precursors showed that VPE is capable of processing several seed proteins, including the 2S (pro)albumins, 11S (pro)globulins (or legumins) and a (pro)51 kDa membrane associated protein of the castor bean ((Hara-Nishimura et al., 1991); (Hara-Nishimura et al., 1993)). *In vivo* processing of the precursors of the storage proteins, 12S globulins and 2S albumins was shown in *Arabidopsis*. *Arabidopsis* mutants that accumulate detectable amounts of the precursors of those protein precursors had a defect in the βVPE, indicating that βVPE is involved in the processing of storage proteins. In 11S seed storage (pro)globulins the α- and β-chains of the 11S globulin subunits which are linked by a disulfide bond are cleaved at an Asn-Gly site (Muntz and Shutov, 2002). It was shown that mutants of 11S proglobulin that lack Asn at the specific processing site are not processed into the α- and β-chains neither *in vitro* nor *in vivo* (Jung et al., 1998). Mature 2S albumins are also composed of two subunits which are linked by disulfide bonds. During the maturation the pro2S albumins are cleaved at no less than four sites, however there are also other proteases which complement in this processing. Initial processing of the storage proteins by the VPEs promotes cleavage by other proteases such as aspartic proteinases (Shimada et al., 2003). Cleavage of synthetic substrates by the legumains has also been reported. One of the best synthetic substrates is Z-Ala-Ala-Asn-NHMec (MEROPS).In addition, VPE was shown to exhibit

a caspase-1 like activity detected by cleavage of a synthetic fluorogenic substrate Ac-xVal-Ala-Asp-AMC ((Hatsugai et al., 2004); (Rojo et al., 2004)).

For the **metacaspases**, an Arginine/Lysine (Arg/Lys) -specificity of the recombinant purified proteases was shown also by cleavage of synthetic fluorogenic substrates with Arg/Lys at the P1 position ((Vercammen et al., 2004); (Watanabe and Lam, 2005)).

Although **papain-like enzymes** bear a broad range *in vitro* specificity towards their substrates, they preferentially cleave peptide bonds with Arg in the P1 position (Fischer et al., 2000) and Phe at the P2 position ((Menard and Storer, 1998); (Okamoto et al., 1999a)). *In vitro* REP1, a rice papain-like CP digested both the acidic and basic subunits of glutelin, the major seed storage protein in rice (Kato and Minamikawa, 1996). In addition, i*n vitro* digestion of seed globulin of dry vetch seeds by papain-like cysteine proteases pointed seed storage proteins as substrates of these enzymes (Fischer et al., 2000).

Deubiquitinating enzymes catalyze the hydrolysis of bonds involving the C-terminal Gly of ubiquitin (Ub). UCHs cleave α-amino linked (linear) polyubiquitin chains and UBPs cleave branched polyubiquitin chains linked internally via ε-amino isopeptide bonds or via α-amino peptide bonds (Vierstra, 2003). α-amino peptide bonds are bonds to the the N-terminus of another ubiquitin molecule, isopeptide bonds to the sidechain of Lys48 in another ubiquitin molecule or to the sidechain of a Lys residue in another protein. Recombinant *Arabidopsis* AtUBP2 protein was assayed against a variety of substrates both *in vitro* and *in vivo* ((Callis et al., 1995); (Callis et al., 1989); (Varshavsky, 1997); (van Nocker and Vierstra, 1993)). AtUBP2 seemed to have broad specificity, capable of cleaving (the C-terminal Glycine of) ubiquitin attached to a variety of substrates via peptide (α-amino) and/or isopeptide (ε-amino) bonds. AtUBP3, AtUBP4 and AtUBP5 were also tested against a variety of substrates such as Hexa-ubiquitin and Tetra-ubiquitin, and they all seemed to exhibit preference for Ub-Ub linkages (Rao-Naik et al., 2000). Recombinant AtUBP14 protein of *Arabidopsis* was assayed *in vitro* with Lys48-linked multiubiquitin chains and *in vivo* with several substrates containing ubiquitin linked via an α-aminopeptide bond and this enzyme seemed to have a specificity for free C-terminal glycine (Doelling et al., 2001).

Table 2 summarizes the specificity of the CPs against both natural and synthetic substrates. Characterizing substrates of proteases is very useful in helping to elucidate the function of the enzymes. Indeed, when a substrate is known to belong to a certain pathway, the proteases can be linked to the same pathway and hereby it can be assumed they play an important role in it. Therefore the hunt for substrates is of big importance and should receive further attention.

	synthetic substrates	**natural substrates**
legumains	Z-Ala-Ala-Asn-AMC Ac-xVal-Ala-Asp-AMC	seed proteins: oa (pro)albumins (pro)globulins
metacaspases	Z-Phe-Arg-AMC Boc-Gly-Arg-Arg-AMC Boc-Gly-Lys-Arg-AMC	/
papain-like enzymes	Z-Phe-Arg-AMC	seed proteins: (pro)glutelin (pro)globulin
deubiquitinating enzymes	/	polyubiquitin proteins (see text for details)

Table 2: Substrate specificity for the plant cysteine proteases legumains, metacaspases, papain-like enzymes and deubiquitinating enzymes. Specificity against both synthetic and natural substrates are summarized. AMC = amido-4-methylcoumarin, Z= benzyloxycarbonyl, Ac = acetyl, Boc = t-butyloxycarbonyl.

1.6 Caspase-like proteolytic (CLP) activity in plants

In animal systems, caspases are a family of aspartatic acid-specific cysteine proteases that play a central role in signalling and executing apoptosis, (Cryns and Yuan, 1998). Some of the morphological and biochemical features associated with apoptosis such as compaction and shrinkage of the cytoplasm, nucleus, and DNA and nuclear fragmentation are similar in animal cells undergoing apoptosis and dying plant cells (Wang et al., 1996). These observations suggested a similar apoptotic machinery in animal and plant cells. In animal cells these typical hallmarks of apoptosis are ascribed to caspase-mediated processing of specific target molecules. In plants, no close caspase homologues have been identified in genomes, however recently caspase-like or aspartatic acid-specific activity have been associated with many plant cell death processes (reviewed in Rotari et al., 2005; (Woltering, 2004)). Evidence for the existence of **caspase-like proteases (CLPs)** and their putative involvement in plant PCD is largely based on both the activity of the CLPs (CLP activity) and the inhibitory effects on cell death of caspase-specific inhibitors *in vitro* or *in vivo* (Rotari et al., 2005).

1.6.1 Studies based on synthetic substrates and inhibitors

Various caspase-like activities have been detected in plants (summarized in Table 3). As this observed aspartate-specific proteolytic activity was not due to the action of “real” caspases, however from (mostly unidentified) caspase-like proteases, this activity was called caspase-like activity. Protease activities are denominated after the four amino acid substrate cleaved, using the single letter codes. Using **synthetic fluorogenic substrates** to caspase-1 (YVADase activity) and caspase-3 (DEVDase) proteinase activity with properties similar to animal caspases was detected for the first time in plant cells (reviewed in (Woltering et al., 2002)). Later on, additional caspase substrates have been used in recent studies and therefore several novel activities had

been discovered. This is the case for VEIDase and TATDase. For almost all observed caspase-like activities it was suggested that they were **implicated in plant PCD** (Table 3) and in some cases the activities correlated with the level of PCD (Woltering et al., 2002). Furthermore, CLP activities have been associated with all kinds of PCD (Table 3). An involvement of CLP proteinases in **hypersensitive response (HR)-related cell death** could be suggested by several reports. Caspase-1 like activity was detected in extracts from tobacco mosaic virus (TMV)-infected tobacco leaves and caspase-specific peptide inhibitors could abolish bacteria-induced plant PCD, which suggested an involvement of CLP proteinases in HR-related PCD (del Pozo and Lam, 1998). However, no activity was detected using caspase-3 fluorogenic substrate (Ac-DEVD-AMC) (Lam and del Pozo, 2000). Victorin-induced PCD in *Avena sativa* is associated with DEVDase activity and proteolysis of Rubisco. This activity was shown to be prevented by caspase-3 specific inhibitors, however not by E64 or leupeptin, general cysteine protease inhibitors which indicates that, in this study, the proteases involved in CLP activities are not cysteine proteases (Coffeen and Wolpert, 2004) (also see §1.6.3). Caspase-1 like activity was detected in TMV (Hatsugai et al., 2004)- and *Pseudomas* (Rojo et al., 2004) -infected tobacco leaves. Inhibition of the CLP activity resulted in reduction of lesion formation (Hatsugai et al., 2004). A TATDase proteolytic activity was detected during the N gene-mediated hypersensitive response in tobacco plants infected with TMV. A specific inhibitor biotinyl-TATD-CHO was able to counteract TMV-triggered HR *in vivo* (Chichkova et al., 2004). **Abiotic stress induced PCD** was also associated with the involvement of CLPs. Ultraviolet-C light overexposure induces programmed cell death in *Arabidopsis*, and is associated with increased YVADase and DEVDase activity. These activities possibly mediate the observed DNA fragmentation. In addition, caspase-1 and caspase-3 inhibitors and the pan-caspase inhibitor p35 were able to suppress DNA fragmentation and cell death, however, broad-range cysteine protease inhibitors did not seem to affect CLP activity (Danon et al., 2004). The latter could imply that the proteases responsible for the described CLP activities are not cysteine proteases. Caspase-1 and caspase-3 like protease activity were activated in isopentenyladenosine (iPA)-induced apoptosis of BY-2 cells. Cell

	Experimental system	CLP activity	effect of caspase-specific inhibitors	Reference
CLP activity associated with cell death				
defense-related cell death	TMV-infected tobacco leaves	YVADase	Ac-YVAD-CMK: abolish cell death	del Pozo and Lam, 1998
		no DEVDase	Ac-DEVD-CHO: abolish cell death	
	victorin-induced PCD in *Avena sativa*	DEVDase	Ac-VAD-CMK: reduced Rubisco proteolysis	Coffeen and Wolpert, 2004
	TMV-infected tobacco plants	TATDase	biotin-TATD-CHO: inhibits necrotic lesion formation	Chichkova et al., 2004
			Ac-YVAD-CHO: inhibition of DNA fragmentation and degradation of PARP	
	TMV-infected tobacco leaves	YVADase	Ac-YVAD-CHO: inhibition of lesion formation	Hatsugai et al., 2004
	Pseudomonas syringae -infected tobacco leaves	YVADase		Rojo et al., 2004
abiotic-stress related PCD	UV-C induced PCD in *Arabidopsis*	YVADase	Ac-YVAD-CHO: inhibition of DNA fragmentation, suppression of PCD	Danon et al., 2004
		DEVDase	Ac-DEVD-CHO: inhibition of DNA fragmentation, suppression of PCD	
	IPA-induced PCD in BY2 suspension cells	YVADase	Z-VAD-fmk, Z-YVAD-FMK: reduce PCD	Mlejnek and Procházka, 2002
		DEVDase		
	heat-shock induced PCD in tobacco suspension cells	DEVDase / PARP		Tian et al., 2000
	chemical-induced PCD in tomato suspension cells	/	Ac-YVAD-CMK: reduced cell death	De Jong et al., 2000
	menandione-induced PCD in tobacco protoplasts	/	Ac-DEVD-CHO: inhibition of DNA fragmentation and degradation of PARP	Sun et al., 1999
developmental PCD	germination of white spruce seeds	DEVDase	Ac-DEVD-CHO: reduction of PCD	He and Kermode, 2003
	self-incompatibility (SI)-triggered PCD in papaver pollen	DEVDase	Ac-DEVD-CHO: inhibits DNA fragmentation, pollen tube growth	Thomas and Franklin-Tong, 2004
	embryogenesis-related PCD in norway spruce	VEIDase	Z-VAD-FMK, Z-VEID-FMK: reduce PCD	Bozhkov et al., 2004
	PCD in seondary shoots of *Pisum sativum*	DEVDase	Ac-DEVD-CHO: suppession of PCD	Belenghi et al., 2004
		YVADase		
other	addition of cytochrome c in carrot suspension extracts	/	Ac-YVAD-CHO: inhibition of apoptotic-like changes	Zhao et al., 1999
			Ac-DEVD-CHO: inhibition of apoptotic-like changes	
CLP activity associated with other / not identified	addition of mitochondria to barley embryonic cells	YVADase	block CLP-activity	Korthout et al., 2000
		DEVDase	block CLP-activity	

Table 3: Involvement of caspase-like proteases in plant PCD, based on cleavage of synthetic caspase-specific substrates and/or inhibition by caspase-specific inhibitors. Amino acids are indicated by their one-letter code: Y = Tyrosine, V = Valine, A = Alanine, D = Aspartaic Acid, E = Glutamic acid, T = Threonine, I = Isoleucine. PARP = Poly (ADP-ribose) polymerase. CHO = aldehyde, CMK = chloromethyl ketone, FMK = fluoromethyl ketone, AMC = amido-4-methylcoumarin, Ac = acetyl, Z= benzyloxycarbonyl.

death in tobacco BY-2 cells was markedly reduced by a caspase-1 inhibitor (Mlejnek and Prochazka, 2002). Heat shock-induced apoptosis in tobacco suspension cells was found to be associated with activation of caspase-3 like proteases (Tian et al., 2000). The suggestion of an implication of caspase-like proteases in chemical- (De Jong et al., 2000) and menandione-induced (Sun et al., 1999) PCD is based on the inhibition of the programmed cell death process by caspase-1 and caspase-3 specific inhibitors, respectively. CLPs also seemed to be involved in **developmental PCD**. DEVDase activities were detected three days after germination of white spruce seeds. In addition, upon treatment of germinated seeds with a caspase-3 inhibitor, both the CLP activities and the death of megagametophyte cells were delayed. This suggests that CLP activity is involved in PCD of white spruce megagametophyte cells (He and Kermode, 2003). Programmed cell death in papaver pollen, triggered by self-incompatibility (SI) in order to reject incompatible “self” pollen, is linked to DEVDase activity. This activity is associated with inhibition of SI-triggered inhibition of pollen tube growth and DNA fragmentation and caspase-3 specific inhibitors could attenuate this fragmentation and growth inhibition. (Thomas and Franklin-Tong, 2004). Norway spruce development is associated with the activation of (a) protease(s) with the preferential cleavage of caspase-6 specific substrate (VEIDase activity). This activity is maximal at the early stages of embryo development while it is nearly detectable in mature embryos. The increased CLP activity during this process suggests that it is required for embryo shape remodelling and it was found to be involved in the terminal differentiation and death of the embryo suspensor (Bozhkov et al., 2004). Caspase-3 like activity was found to be induced in senescing secondary shoots, which could be suppressed by a caspase-3 specific inhibitor (Belenghi et al., 2003). Addition of cytochrome c in cytosolic extracts from carrot suspension cells was able to induce apoptotic like changes including chromatin condensation, formation of apoptotic bodies and DNA fragmentation in purified mouse liver. This could be blocked by caspase-1 and caspase-3 inhibitors (Zhao et al., 1999). YVAD and DEVD-ase activity was detected in cytosolic extracts from barley embryonic suspension cells, however an implication in cell death was not demonstrated. The activities could be blocked by the specific caspase-3 inhibitor and not by cysteine protease inhibitors (Korthout et al., 2000).

1.6.2 Studies based on a (endogenous) plant substrate and physiological macromolecular caspase inhibitors

The involvement of CLP in plant PCD was not only based on the cleavage of synthetic caspase substrates or the inhibition by synthetic caspase-specific inhibitors. Proteolytic activity in plant cells undergoing PCD has also been studied using poly ADP Ribose polymerase (PARP) as a substrate and macromolecular caspase inhibitors. In both mammals and plants two different types of PARP exist and both types are presumably involved in DNA repair. Cleavage of **endogenous (plant) PARP** occurs during during menadione-induced PCD in tobacco protoplasts and this was inhibited by caspase-1 and caspase-3 inhibitors (Sun et al., 1999). During heat shock-induced apoptosis in tobacco suspension cells a caspase-3-like protease was activated associated with the cleavage of endogenous PARP (Tian et al., 2000).

Additional evidence for an intimate link between caspase-like activities and the completion of PCD have been obtained by the use of viral or cellular **macromolecular caspase inhibitors** which are thought to interact directly with the protease, such as the inhibitors of apoptosis (IAPs) and in particular p35. Baculovirus **p35** is a pan caspase inhibitor and forms a complex with the caspase (mechanism-based inactivation). p35 is shown to be effective at blocking cell death in plants in several instances. *Agrobacterium tumefaciens*-induced PCD in maize can be suppressed by ectopic expression of p35 (Hansen, 2000). Likewise, tobacco plants expressing p35 were partially inhibited in HR cell death, whereas different mutant versions with loss of the caspase cleavage site lost their inhibitory activity when expressed in tobacco (Lam and del Pozo, 2000). Transgenic tomato plants bearing the p35 gene were also protected against AAL-toxin-induced death and pathogen infection (Lincoln et al., 2002). Ultraviolet-C induced cell death could also be inhibited by p35 (Danon et al., 2004). **IAP proteins**, conserved between numerous organisms, interact with caspases in such a way that substrate access is blocked without directly docking into the substrate pockets on the enzyme surface (Salvesen and Duckett, 2002). It has been reported that *Agrobacterium*

tumefaciens-induced PCD in maize cells with typical features of apoptosis can be suppressed by ectopic expression of the baculovirus IAP (Hansen, 2000). Conclusively, these studies suggest that both IAP and p35 inhibit plant proteases that are expected to have substrate- and site-specificity equivalent to animal caspases.

1.6.3 Which proteases are responsible for CLP activity in plants?

Based on the structural homology and evolutionary relationship to caspases, **metacaspases** were the first candidates to encode proteases with caspase-like activities. However; to date there are also elements to argue the opposite.

Several reports showed a **metacaspase-dependent CLP activity**. Upon overexpression of yeast caspase YCA1, hydrogenperoxide-induced cell death was correlated with caspase-like protease activity (IETDase activity) and could be blocked by a general caspase inhibitor (Z-VAD-fmk) (Madeo et al., 2002). Upon overproduction of *Trypanosoma cruzi* metacaspase 3 (TcMCA3) cell death was induced, which was paralleled by an increase in peptidase activity against a typical substrate (z-YVAD-AFC) (Kosec et al., 2005). In plants, during embryo spruce development VEIDase activity was associated with the accumulation of mcII-Pa in the embryonic tissues that are committed to death (Suarez et al., 2004). Overexpression of *Arabidopsis thaliana* metacaspases in yeast provoked cell death which could be prevented by addition of a caspase inhibitor (Watanabe and Lam, 2005).

However, in none of the cases, this caspase-like activity was shown to originate directly from the metacaspases. In addition, bacterial and yeast extracts containing recombinant *Arabidopsis* metacaspases exhibited **Arg/Lys-specific endopeptidase activities** but could not cleave caspase-specific substrates (Watanabe and Lam, 2005). This Arg/Lys-specificity of the *Arabidopsis* metacaspases was previously shown by Vercammen et al. (2004). These metacaspases displayed no specificity towards the classical caspase substrates, only against P1-Arg/Lys substrates (Vercammen et al., 2004). Bozhkov *et al*. (2005) demonstrated that mcII-Pa silenced lines showed both a

reduction in VEIDase and EGRase activity, the latter being a P1-Arginine substrate. Moreover, recombinant mcII-Pa does not cleave classical caspase substrates, including the VEID sequence-containing substrate however it does cleave P1-Arg/Lys substrates (Bozhkov et al., 2005).

All these findings suggest that **metacaspases are not directly responsible** for reported caspase-like activities in plants, yeast, and *Trypanosoma*. However, **activation of other upstream caspase-like protease(s)** triggered through either direct or indirect proteolysis by a metacaspase could mediate execution of yeast apoptosis or plant PCD.

Recently, it has been shown that **VPEs are responsible for caspase-like activity in plants**, at least for HR-related cell death (reviewed in (Lam, 2005)). Despite the fact that VPE is structurally unrelated to caspases it was shown to exhibit caspase-1 like activity and to be sensitive to a caspase-1 specific inhibitor (Hatsugai et al., 2004). In addition, infection with an avirulent strain of Pseudomonas syringae results in an increase of caspase-1 activity, and this increase is partially suppressed in VPEg mutants (Rojo et al., 2004). As such, VPE has been suggested to act like a plant caspase that regulates hypersensitive cell death in defense. Whether or not these VPEs are activated by upstream metacaspases remains to be elucidated.

In addition, subtilisin-like proteases from oat have also been identified to play an important role as caspase-like proteases in the execution of plant cell death. Subtilisin-like proteases are serine proteases that possess strong caspase-3 like activities (Table 3). They are involved in the proteolysis of Rubisco during victorin-induced plant cell death and are very sensitive to numerous caspase-specific inhibitors (Coffeen and Wolpert, 2004). Therefore, subtilisin-like proteases were denominated **saspases**. In contrast to VPEs which are cysteine proteases, saspases are not caspase-like proteases in as strict sense because they are serine proteases.

1.7 Plant cysteine proteases and their multifunctional role

In general, CPs play a crucial role in metabolic degradation of peptides and proteins playing a housekeeping function to remove abnormal, misfolded proteins ((Schaller, 2004); (Grzonka et al., 2001)). **Mammalian** CPs are known to be involved in many processes such as food digestion, complement activation, blood coagulation or immune system function. They are implicated in the development and progression of many diseases that involve abnormal protein turnover ((Henskens et al., 1996); (Dickinson, 2002)). **Plant** cysteine proteases are widely distributed and play essential roles in various aspects of plant growth, development and environmental responses. They are implicated in seed maturation and seed germination by the accumulation of storage proteins in seeds and storage protein mobilization. They also play a role in developmental and defense-related programmed cell death (PCD), and senescence (Grudkowska and Zagdanska, 2004). In addition, they are also involved in cell cycle and mediate plant-microbe interactions (Sheokand and Brewin, 2003). Proteolysis in plants provides a mechanism of protein turnover and reutilization of nitrogen for cellular homeostasis and growth.

Microarray datasets relative to plant organs, growth stages and stress responses were analyzed for their expression of the *Arabidopsis* CPs. As little functional data are available for many of the CPs, this analysis can be useful to assign a (novel) function to the proteases or confirm an already described function in literature (see §1.8). The putative roles of the best characterized plant CPs in plant PCD, seed maturation/ germination and other processes are summarized in Table 4. A clear multifunctional role for the CPs in plant development can be demonstrated.

Cysteine Protease	Programmed cell death			seed maturation and germination	others (nodulation, cell cycle)
	developmental	defense (abiotic/*biotic*)	senescence		
papain-like enzymes	inhibitor[1] activity[2] expression[2,3]	inhibitor[8,9,10] activity[8, 10, 11,12] expression[12,13,14,15] mutation analysis[14]	expression[19,20,21,22,23]	activity[25,26] expression in germinating seeds[27]	expression[34]
legumains (VPEs)	expression[4] knock-out mutant[4]	knock-down mutant[16]	expression[24]	activity[28,29,30,31] predominant seed expression for β- and δVPEs[28,32] knock-out mutants[32,33]	
metacaspases	knock-down mutant[5] expression[5,6] activity[6]	expression[17,18]			
(phyto)calpains	overexpression/knock-down[7]				silencing[35]

References

1 Woffenden et al., 1998
2 Ye and Varner, 1998
3 Funk et al., 2002
4 Nakaune et al., 2005
5 Suarez et al., 2004
6 Bozhkov et al., 2004
7 Lid et al., 2002
8 Solomon et al., 1999
9 *Belenghi et al., 1999*
10 *Konno et al., 2004*
11 Hayashi et al., 2001
12 *Pechan et al., 2000*
13 Koizumi et al., 1993
14 *Kruger et al., 2002*
15 *Avrova et al., 1999*
16 *Hatsugai et al., 2004*
17 *Hoeberichts et al., 2003*
18 *Watanabe and Lam, 2005*
19 Valpuesta et al., 1995
20 Nadeau et al., 1996
21 Cercos et al., 1999
22 Schmid et al., 1999 purif and prod
23 Lohman et al., 1994
24 Kinoshita et al., 1999
25 Kato and Minamikawa, 1996
26 Mitsuhashi et al., 1986
27 Okamoto and Minamikawa, 1999
28 Hara-Nishimura et al., 1991
29 Shimada et al., 1994
30 Hiraiwa et al., 1997
31 Yamada et al., 1999
32 Gruis et al., 2002
33 Shimada et al., 2003
34 Goetting-Minesky and Mullin, 1994
35 Ahn et al., 2004

Table 4: Multifunctional role of plant papains, VPEs, metacaspases and phytocalpains. On which study (inhibitor studies, mutant analyses, activity or expression) the assignment of the function was made, is indicated in the table.

1.7.1 Cysteine proteases in seed maturation, seed germination and seedling growth

Plants acuumulate protein reserves (or storage proteins) during **seed maturation**, also called **storage protein deposition**. In dicotyledonous seeds such as *Arabidopsis* seeds, these protein reserves consist predominantly of two-types of seed storage proteins: legumin-type globulins (Sjodahl et al., 1991) and napin-type albumins (van der Klei et al., 1993). During the middle and late maturation stages the storage proteins are deposited in dedicated membrane-bounded compartments called protein storage vacuoles (PSVs) where they act as nitrogen sink (Muntz et al., 2001). After the storage proteins has entered the PSV they are prone to Asn-specific cleavage or processing by the seed-type specifc legumains or VPEs. The seed-specific legumains are the βVPEs and δVPEs as seen by their predominant expression in seeds ((Hara-Nishimura et al., 1991); (Gruis et al., 2002)). Their role in seed storage protein processing was demonstrated genetically in an *Arabidopsis* transposon-insertion line. Seeds of a ˜VPE knock out mutant contain increased levels of propolypeptide forms of legumin-type globulins (Gruis et al., 2002). In addition, screening T-DNA tagged *Arabidopsis* lines for defects in seed storage protein maturation isolated six βVPEs mutants (Shimada et al., 2003). In addition, VPEs have found to be responsible for conversion *in vitro* of several seed proprotein precursors (of both legumain- and napin-type) into the mature forms ((Hara-Nishimura et al., 1991); (Shimada et al., 1994); (Hiraiwa et al., 1997); (Yamada et al., 1999)). Processing of the storage proteins at conserved Asn residues is required for the assembly into multimeric structures (Figure 3). Upon processing of the precursor polypeptides, e.g. proglobulins into globulins, by the VPEs and their subsequent transformation into multimeric structures, the globulins are finally deposited in the PSV of storage tissue cells of maturing seeds, where they remain protected from unlimited proteolysis by VPEs and as such remain undegraded during their storage in the PSVs. Protection of the storage proteins during their deposition is linked with their conformational stabilization, as Asn-residues potentially susceptible to proteolytic

cleavage by VPEs are located in structurally ordered regions where they are protected from uncontrolled proteolysis. As such, seed storage proteins stably accumulate in PSVs where they coexist with active VPEs.

Upon the transition to **seed germination** and subsequent seedling growth, the seed storage proteins are prone to degradation, which contributes to **protein mobilization**. This results in reutilization of the nitrogen source. Both **VPEs** and **papain-like** enzymes are assumed to work cooperatively in protein degradation and N-mobilization during seed germination (see Figure 3) (reviewed in (Muntz et al., 2001)). Upon germination, a loss in conformational stabilisation results in the complete degradation of seed storage proteins. Legumains contribute to the activation of a major

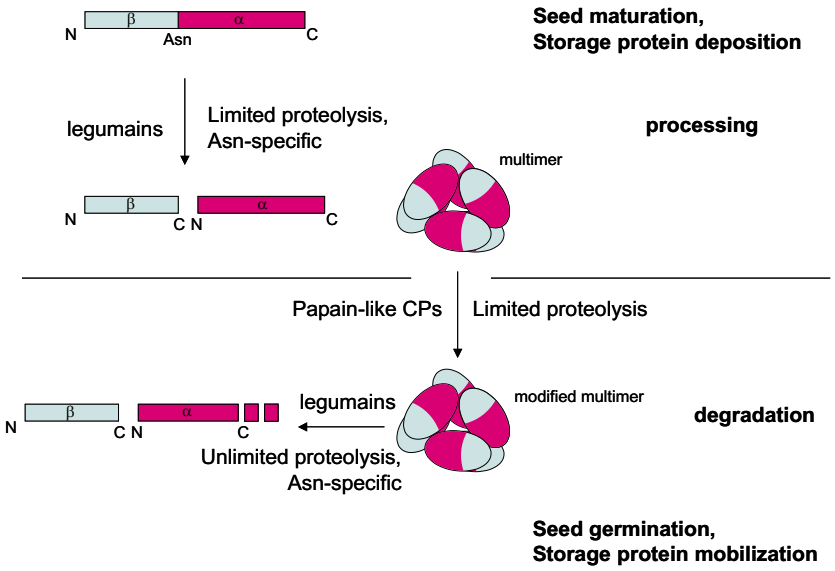


Figure 3: Overview of protein processing and degradation. As example, 11S globulin deposition during seed maturation and 11S globulin mobilization during seed germination. During seed maturation, processing of the 11S proglobulin into globulin results in separation of the α- and β-chains of globulin via Asn-specific cleavage by the legumains. This cleavage is accompanied by formation of multimeric structures which protects the storage proteins from further uncontrolled proteolytic attack. Upon seed germination, limited proteolysis by papain-like CPs results in conformational change, which allows further Asn-specific degradation by the legumains.

storage protein degrading protein with broad specificity, a papain-like proteinase ((Okamoto and Minamikawa, 1999); (Okamoto et al., 1999a)).

Limited proteolysis by papain-like CPs results in conformational changes which overcomes the conformational protection. Evidence for the involvement of papain-like CPs in seed germination was founded by the fact that endopeptidase activity increased in day-9 germinated seeds of rice, reaching a maximum on day 18. One of the proteases responsible for this activity was isolated and identified as papain-like CPs called REP-1. REP-1 was able to digest rice glutelin, the major seed storage protein of rice ((Kato and Minamikawa, 1996); (Kato et al., 1999)). In *Vigna mungo* a papain-like enzyme was found to be expressed in germinating seeds (Okamoto and Minamikawa, 1999)(Okamoto et al., 1999a) and responsible for the degradation of seed storage protein (Mitsuhashi et al., 1986).

Loss of conformational protection by papain-like mediated cleavage thus permists further Asn-specific unlimited proteolysis or degradation by legumains (Shutov et al., 1982). As such, control of protein conformation forms a mean of natural regulation for the function of legumains in protein processing and protein breakdown. Both the processing and degrading activities of legumains are crucial for the function of vacuoles in plant cells (Muntz and Shutov, 2002).

1.7.2 Cysteine proteases in programmed cell death (PCD), plant defense and senescence

Programmed cell death occurs as part of normal plant development as well as in response to biotic and abiotic stress stimuli (defense-related cell death) and during senescence. While in animals, cysteine proteases have emerged as key enzymes in the regulation of animal PCD, evidence demonstrating an important role for cysteine proteases in all types of plant PCD has been slowly accumulating. The involvement of papain-like cysteine proteases, legumains, metacaspases and (phyto)calpains in developmental cell death, defense-related cell death and senescence is reviewed (also see Table 4).

1.7.2.1 Developmental programmed cell death

The terminal process of xylogenesis, autolysis, is essential for the formation of a tubular system for conduction of water and solutes throughout the whole plant (Ye, 2002). In *Zinnia elegans* cell suspensions, cysteine proteases play a prominent role during tracheary element (TE) differentiation (xylogenesis) (Beers et al., 2000).

Activity gel assays (Ye and Varner, 1996) and pharmacological studies using both a broad spectrum cysteine protease inhibitor (E-64) (Minami and Fukuda, 1995) and peptide-aldehyde inhibitors of **papain-like enzymes** (Woffenden et al., 1998) indicated the involvement of papains in this process. Promotor::GUS constructs of both papain-like enzymes XCP1 and XCP2 specifically showed XCP promoter activtiy in TEs and vacuolar localisation of XCP1 (Funk et al., 2002). CPs are necessary for autolysis during this TE differentiation process, as they provide a complete removal of intracellular contents of differentiated TE elements, which is necessary for the formation of this water-conducting tissue.

δVPE is specifically expressed in two cell layers of the inner integument of the seed coat at an early stage of seed development. δVPE deficiency delays targeted cell death and nuclear degradation in the cell layers of the inner integument (Nakaune et al., 2005). As such, δVPE is a key player in the developmental PCD of these limited cell layers which results in the formation of a seed coat.

Downregulation of a **type II metacaspase** (mcII-Pa) suppresses PCD in the suspensor cells of an embryogenic culture of *Picea abies* (Norway spruce) ((Suarez et al., 2004); (Bozhkov et al., 2005)). As such, mcII-Pa is thought to be responsible for plant embryogenesis (also see §1.9.2). It was suggested that the metacaspase might exhibit its function by cleaving nuclear structural proteins, which results in disassembly of the nucleus and subsequent facilitated DNA fragmentation.

The only plant member of the **calpain** superfamily (C12), DEK1, is required for aleurone cell development in the endosperm of maize grains (Lid et al., 2002). Mutation in the Arabidopisis thaliana *DEK1* calpain gene perturbs embryo development displaying

irregular mitotic divisions in the embryo and suspensor, while overexpression affects overall organ development of trichomes, leaves and flowers (Lid et al., 2005). DEK1 plays a role in maintaining the aleurone cell fate, probably by acting as a cysteine protease on a -not yet identified- substrate in aleurone cell signalling. In addition, DEK1 is also involved in restricting the aleurone cell fate to only one layer, the surface layer of the endosperm. It was suggested that this is mediated trough interaction of the DEK1 protein with molecules on the endosperm surface which results in activation of its CP activity.

1.7.2.2 Defense-related cell death

Increases in cysteine protease activities have been associated with both environmental (abiotic) and biotic stress conditions. A function for the CPs in response to environmental abiotic and biotic factors has been proposed in rebuilding cellular proteins. Indeed, degradation of damaged or denatured proteins under stress is closely related with the production of new proteins, like cold and heat shock proteins, dehydration-induced proteins and pathogenesis-related proteins, since the released amino acids are reused for the synthesis of new proteins (Schaffer and Fischer, 1990). In case of biotic stress responses, CPs have many different roles in the defense response: pathogen perception, signalling and execution (reviewed in (van der Hoorn and Jones, 2004)).

The induction of CPs and their activity is often associated with **environmental stress** conditions. In wheat leaves, CPs are induced in response to water deficit and proteolytic activity increased significantly upon drought (Zagdanska and Wisniewski, 1996). Drought and low temperature stress could induce expression of a CP gene in *Brassica napus* (Stroeher et al., 2004), nitrogen starvation regulates the expression of a CP in *Oryza sativa* (Ho et al., 2000), glucose starvation induced expression of a CP in maize root tips (Chevalier et al., 1995) and wounding induced a CP in tobacco (Linthorst *et al.*, 1993).

More in specific, **papain-like enzymes** were involved in salt stress and drought stress. Salt stress induced the fusion of vesicles that originate from the ER, called ER bodies, with each other and with the vacuole. These ER bodies are found back in the epidermal cells of *Arabidopsis* seedlings and contain a papain-type enzyme RD21A. Upon fusion with the vacuole, this enzyme might become processed and subsequently active (Hayashi et al., 2001). Therefore, the ER bodies are considered as protease-storing systems which assist in cell death in the epidermal cells. A strong induction of the papain-type enzymes RD19 and RD21 mRNAs was observed under drought stress in *Arabidopsis thaliana* (Koizumi et al., 1993).

Activity of a **Calcium-dependent cysteine proteinase** has been associated with anoxia-induced root tip death in maize (Subbaiah et al., 2000). The exact identity of the calcium dependent protease has not been characterized yet but the protease would be functionally similar to the calpain-type of CPs. The protease was suggested to play a role in the initiation of the cell death process, probably by acting as a molecular switch.

In general, it is important to identify the molecular targets of the CPs and events downstream of the CP-induction to better understand the role of the CPs in the different stress responses.

Concerning **biotic stress responses**, an important role for many **papain-like enzymes** has been established in pathogen defense. In soybean cells, ectopic expression of a papain-type inhibitor cystatin upon *Pseudomonas*-induced PCD led to the abolishment of induced CP activity and suppression of cell death. Similar expression of serine protease inhibitors was ineffective (Solomon et al., 1999). In *Arabidopsis thaliana* avirulent pathogen-induced cell death could be blocked by a cystatin from *Arabidopsis* (Belenghi et al., 2003). In addition, papain-like enzymes are also involved in plant defense against certain insects. A papain enzyme in latex of Papaya trees is a crucial factor in the defense against lepidopteran larvae and polyphagous pests (Konno et al., 2004). Maize genotypes resistant to the insect *Lepidoptera* showed an accumulation of Mir1, a unique 33-kD papain-like cysteine proteinase, upon larval

feeding. A strong induction of the CP was observed at the site of larval feeding immediately after the feeding. Mir1 may have a directl toxic effect on the larvae, or its proteolytic activity may lead to the release of other toxic compounds or essential signalling intermediates (Pechan et al., 2000). In tomato, a papain-like cysteine protease, RCR3, is specifically required for the function of the disease resistance gene Cf-2, that mediates recognition of the Avr2 avirulence gene of the fungal pathogen *C.fulvum*. It is proposed that RCR3 may cleave Avr2, Cf-2 or other plant proteins in order to activate the defense response (Kruger et al., 2002). The expression of a RD21-like protease of potato is also induced upon pathogen infection with *Phytophtora infestans* (Avrova et al., 1999).

Recently, it was shown that the **VPEs** are involved in defense-related cell death in plants. Virus-induced hypersensitive cell death is mediated by a tobacco VPE. VPE deficiency was able to prevent virus-induced HR in tobacco plants (Hatsugai et al., 2004). In addition, the levels of the VPEg protein in *Arabidopsis* plants were increased upon pathogen infection and VPEγ mutants were more susceptible to pathogen infection. The function of the VPE in cell death would be a dismantling of the cell by degrading cellular components, similar to the animal caspases. Alternatively, the protease might also be involved in the initiation phase of the cell death process by regulating the accumulation of proteins that control the intiation (Rojo et al., 2004).

Transcripts of a tomato **metacaspase** gene (C14) were found to be upregulated upon infection with *Botrytis cinerea* (Hoeberichts et al., 2003). Interestingly, all Type I *Arabidopsis* metacaspases contain a LSD1-like zinc-finger domain in their aminoterminal prodomain. LSD-1 (lesions simulating disease resistance-1) is a negative regulator of the HR (Dietrich et al., 1997). However, no functional data are available that hint for a role of plant metacaspases in HR.

1.7.2.3 Senescence

Senescence is a highly regulated process which occurs as a final stage of plant development, or can be prematurely induced by stress conditions. The main purpose of senescence is the active turnover and recycling of cellular material for use in other organs. The final death stage of senescence is actively delayed untill all nutrients (nitrogen, phosphorus, metal ions and minerals) have been remobilized (Hortensteiner and Feller, 2002). Leaf senescence is characterized by yellowing of the leaves due to chlorophyll degradation and subsequent loss of photosynthetic activity (Yoshida, 2003).

Programmed cell death, more specifically senescence, can be associated with the specific expression of several proteins, in particular **cysteine proteases**. Senescence in leaves, flowers and ovaries is associated with the induction of cysteine proteases ((Griffiths et al., 1997); (Hensel et al., 1993); (Buchanan-Wollaston and Ainsworth, 1997); (Chen et al., 2002); (Li et al., 2000); (Granell et al., 1992); (Guerrero et al., 1998); (Nadeau et al., 1996); (Wagstaff et al., 2002)). Genes encoding CPs are amongst the most abundantly expressed genes in leaf senescence (Guo and Gan, 2005). It is assumed that a general function for the CPs during senescence is the degradation of cellular constituents prior to their mobilization to other tissues, this role is similar to the role that the CPs perform during seed germination.

Senescence seemed to be associated with the accumulation and release of **KDEL-tailed papain-type cysteine proteases** from ricinosomes, called after the first discovery of this ER-derived organelle in castor bean or *Ricinus communis*. The precursors of these cysteine peptidases (proCys-EP) are targeted and retained in the ER due to their KDEL ER retention signal. Upon release from the ricinosomes in the cytoplasm, the precursor enzymes are processed and hence activated. This ultimately leads to the breakdown of cytosolic constituents during the final stages of cell death. Later on it became clear that ricinosomes are not restricted to castor bean but may be a more general feature of senescing tissues where they function as “suicide bombs”, having an important role in degradation of cellular proteins for use in other organs (Schmid et al., 2001).

KDEL-tailed CPs were found to be upregulated in senescing daylily flowers (Valpuesta et al., 1995), or to be specifically expressed in late stages of ovule development in the outer integument of *Phalaenopsis* orchid (Nadeau et al., 1996). Late stages of fruit maturation were associated with the expression and increased endopeptidase activity of a similar CP (Tanaka et al., 1993). A CP which shows some similarity to papain-like enzymes is induced during ovary senescence in *Pisum sativum* (Cercos et al., 1999). A KDEL-containing papain-like CP (Cys-EP) is accumulated during senescence in the endosperm of castor bean seeds (*Ricinus communis*) which undergo programmed cell death during germination (Schmid et al., 1999).

VPEs are also implicated in the senescence process as indicated by the upregulation of α- and γ-type VPEs in vegetative organs during senescence of *Arabidopsis* (Kinoshita et al., 1999).

Furthermore, leaf senescence is associated with the preferential expression of a specific set of 'senescence-associated genes' (**SAGs**). The upregulation of SAGs during leaf senescence is associated with decline in photosynthesis, degradation of macromolecules, mobilization of nutrients, and ultimate cell death (Guo and Gan, 2005). Some of the SAGs genes are cysteine proteases and there is one found in the genome of Arabidopsis which belongs to the C1 family, SAG12. SAG12 is one of the senescence-associated genes which shows the highest level of induction during senescence. The SAG12mRNA appears to be expressed only during senescence, as there was no detectable mRNA in non senescent leaves. The levels of SAG12 mRNA increase throughout progression of senescence and reach maximum levels at the stage of leaf senescence where there is more than 75% loss of chlorophyll (Lohman et al., 1994). This high induction of SAG12 during senecence was also detected in the microarray data of (Swidzinski et al., 2002).

1.7.3 Cysteine proteases in cell cycle

CPs play a role in other processes as well, such as the cell cycle process. DEK1, a **phytocalpain**, is implicated in the regulation of the balance between cell proliferation and differentiation (Ahn et al., 2004). Virus-induced gene silencing (VIGS) of a *Nicotiana benthamiana* calpain inhibits the development of the major organs, leaves, stems and flowers. This developmental abnormality was accompanied with hyperproliferating cell masses in the epidermal layers of leaves and stems and primarily caused by uncontrolled cell proliferation and delayed or inhibited cell differentiation. In the abnormal organs of the NbDEK VIGS plants, protein levels of D-type cyclins and proliferating cell nuclear antigen (PCNA) were strongly increased, and transcription of E2F (E2 promoter binding factor), E2F-regulated genes, retinoblastoma (Rb), and KNOTTED1 (KN1)-type homeobox genes was enhanced. These results suggest that phytocalpain is a key regulator of cell proliferation and differentiation during plant organogenesis. It is suggested to exhibit its function by controlling the CycD/Rb pathway by regulation of the activity of target proteins in this pathway, probably by degradation (Ahn et al., 2004).

Proteases of the C12 and C19 family consist of ubiquitin carboxyl-terminal hydrolase family (UCHs) and ubiquitin (Ub) -specific proteases (UBPs). Both deubiquinating families are important in the Ub/26S proteasome pathway, which is a major route for selectively degrading cytoplasmic and nuclear proteins in eukaryotes (reviewed in (Vierstra, 2003)). In this pathway, chains of ubiquitins become attached to short-lived proteins, signalling recognition and breakdown of the modified protein by the 26S proteasome. During or following target degradation, the attached multi-ubiquitin chains are released and subsequently disassembled by ubiquitin-specific proteases to regenerate free ubiquitin monomers for re-use. The **Ub/26S proteasome pathway** plays a pervasive role in plant biology being involved in numerous biological processes (Vierstra, 2003) due to the diversity of the substrate proteins. In many cases, the target protein subjected to degradation has a role in signalling or cell cycle control (Estelle, 2001). However, a more precise role specifically for deubiquitinating enzymes in cell cycle has not been established yet.

1.7.4 Cysteine proteases in plant growth and development

As already described in §1.7.2.1 many examples have been given in which plant cysteine proteases are important for developmental cell death, and as such in the general development of the plant. However, cysteine proteases are also important in plant development in a non-cell death related manner. For example, a T-DNA insertion mutation in the gene that encodes **AtUBP14 (C19 family)** causes embryo arrest at the globular stage. The arrested seeds display considerably increased levels of multi-ubiquitin chains, which indicate a defect in ubiquitin recycling. Hereby, an essential role for the ubiquitin/26S proteasome pathway in general and for AtUBP14 in particular during early plant development was demonstrated (Doelling et al., 2001).

1.7.5 Cysteine proteases in nodulation and nitrogen fixation

Many studies have reported on the specific expression of CPs in root nodules. Papain-like CPs were found to be expressed in *Alnus glutinosa* root nodules (Goetting-Minesky and Mullin, 1994). In addition, a cysteine protease was detected in pea nodules, in senescent infected tissue at the base of the nodule (Kardailsky and Brewin, 1996). A nodule-specific expressed CP was detected in Chinese milk vetch (Naito et al., 2000) and soybean (Panter et al., 2000). Nodule-expression was also assessed for another CP from pea, Pscyp15a (Vincent et al., 2000). In addition, cyp15a also showed proteolytic activity in nodule extracts (Vincent and Brewin, 2000). Increased CP activity was also detected during nodule senscence of pea and could be inhibited by E64, a general CP inhibitor (Groten et al., 2006). In most cases the exact physiological role of the CPs in root nodulation is not clear yet, however CPs have been suggested to be involved in several important processes in development and function of legume root nodules. Namely, in defence response to root invasion by microorganisms; protein turnover associated with the formation of new tissue; cellular homeostasis and housekeeping; adaptation to physiological stresses and regulation of nodule senescence (Sheokand and Brewin, 2003).

The multifunctional role of the best characterized plant cysteine proteases (papain-like enzymes, VPEs, metacaspases and phytocalpains) as described in literature is summarized in Table 4.

1.8 In silico analysis of expression profiles of all *Arabidopsis* cysteine proteases

Expression profiles of transcripts of all CPs of *Arabidopsis* (for an overview see Appendix 1) were verified, using the Meta-analyzer tool of Genevestigator (for more information see https://www.genevestigator.ethz.ch/at). This tool is used for studying the expression profiles of gene families from a given list relative to plant organs, growth stages or different stress responses. For growth stages and plant organs, absolute expression values are derived from Genevestigator. As such they were divided by the mean signal intensity value of that gene in the series of experiments, to become ratio values. Subsequently, a coefficient of variation (CV) was used to select for genes which are expressed differentially. The CV was calculated as the ratio of the standard deviation on all values of a gene and the mean signal intensity value of that gene. All genes with a CV e > 0.5 were retained and the expression data were visualized with Genesis software (Sturn et al., 2002). For stress response experiments, relative expression values are derived from Genevestigator which could be visualized directly with the Genesis software. Annotation was based on the Arabidopsis Information Resource (TAIR, Rhee et al., 2003; http://www.arabidopsis.org/), and the affy25k_array_elements-2006-01-06 version was used for ATH1 Affymetrix array element information. From the 149 *Arabidopsis* CPs, only 115 CPs had an array element name (also see Appendix 1) and could hence be taken into this analysis. Such an *in silico* analysis can be useful to predict a function to proteases with a hitherto unknown function, or confirm an (assumption of) an already described function in literature. Notably, it must be taken into account that differential gene expression cannot always be reflected as such in changes in protein levels, as there are some additional factors such as posttranscriptional and posttranslational modifications which also play a role in that aspect. Even more, an induction at the protein level also does not mean per se an induction of protein activity as the protein might have to undergo some modifications (e.g. processing) to be active. Nevertheless, studying gene profiles, and knowledge of which profiles cluster together within different data sets, is very informative and can be indicative for the predictions of functions.

1.8.1 Expression profiles of *Arabidopsis thaliana* cysteine proteases during plant development

Genome-wide expression profiles of all *Arabidopsis* cysteine proteases relative to plant developmental stage were generated with the meta-analyzer Genevestigator toolbox using *Arabidopsis* 22k array data sets. The data sets comprise chronological developmental stages such as seed germination, leaf production, rosette growth, inflorescence emergence, flower production and silique ripening (Schmid et al., 2005). The transcriptomic changes associated with plant growth stages of all the differentially expressed CPs were visualized with Genesis software and represented in Figure 4. Of the 116 CPs represented on the ATH1 Affymetrix microarrays only 24 CPs were differentially transcriptionally regulated during plant development. Genes which were upregulated during seed germination were predominantly members from the papain-like family and VPEs. Both families were already described for their prominent and well-established function during seed development (see Table 4). Silique senescence seems to be associated with the upregulation of the senescence-associated gene SAG12 during silique ripening; different families appear to be involved in silique shattering such as VPEs, a metacaspase, an OTU-like protease, an UBP protease and members of the papain-like family. Some members of the papain-like family and VPEs are possibly implicated in leaf development and flowering, as indicated by their downregulation during this process. Remarkably, from the largest family of the CP in *Arabidopsis*, namely the Ulps, there is only member which is differentially transcriptionally regulated. Moreover, also from the UBP family only one member shows differential transcriptional regulation. Of the Ulp and UBP families little is known about their function. For the Ulp family it is predicted that many of the genes are pseudogenes, and hence this result could be expected. However, it must be taken into account that there are many aspects of gene regulation, besides the transcriptional regulation, which can reflect on the function of a protein. Therefore, it does not implicate that when a gene is not differentially transcriptionally regulated in a process that the protein is not implicated in that process.

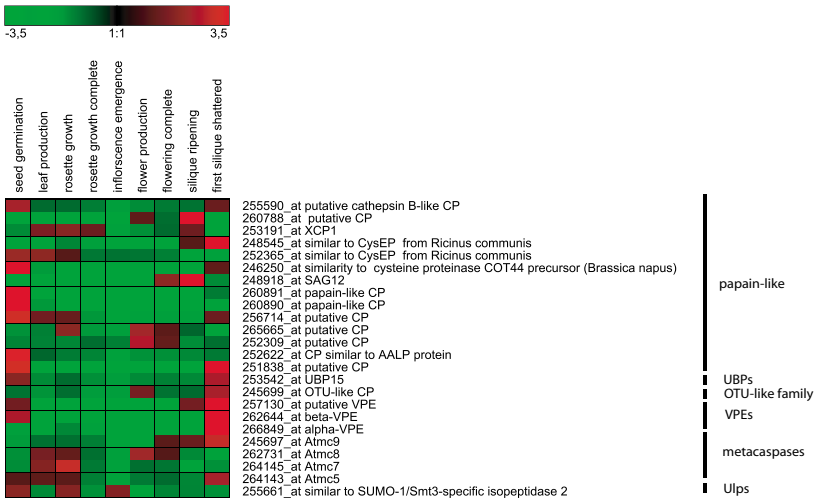


Figure 4: Expression profiles of differentially regulated (CV e > 0.5) *Arabidopsis* CP transcripts during plant development. Absolute expression values obtained from Genevestigator were divided by mean signal intensity values during development. The resulting values were visualized with Genesis software (Sturn et al., 2002). Red indicates upregulation, green downregulation. Intensity of the colors is proportional to the fold difference. Annotation was based on TAIR, version 2006-01-06 was used for ATH1 Affymetrix array element information. The figure also indicates the different families to which the CPs belong to.

1.8.2 Expression profiles of *Arabidopsis thaliana* cysteine proteases in plant organs

A specific expression of genes in certain plant organs could also be established by microarray analysis (microarrays developped by CAGE consortium) (Figure 5). Plant organ-specific expression can be very interesting as it is clearly indicative for the protein's function. Interestingly, the Type II metacaspases were clearly highly expressed in the roots. This root-related expression of the Type II metacaspases was already demonstrated for some metacaspases by promoter-GUS fusions (D. Vercammen and T. Beunens, unpublished results). The expression of the metacaspases in carpel, ovary and stigma is lower than the average expression of the proteins across all organs. A clear expression of the VPEs (also the vegetative types α and γ) can be assessed in

the seeds, as is also the case for part of the differentially expressed papain-like enzymes. This is concomitant with the fact that these genes are also highly upregulated during seed germination. Similar as for the metacaspases, an average lower expression in carpel, ovary and stigma is observed for almost all members of the VPEs and part of the papain-like enzymes. However, for the VPEs and papain-like enzymes this is largely associated with a lower expression in the leaves as well. Not surprisingly, the senescence-associated gene (SAG12) is highly expressed in senescent leaves. Two members of the only eight-membered OTU-like family and three members of the UBP family are highly expressed in stamen, and four members of the large Ulp family are highly expressed in pollen. Three other members of this Ulp family also show a higher expression in cell suspensions. No other protease family-specific expression patterns could be observed.

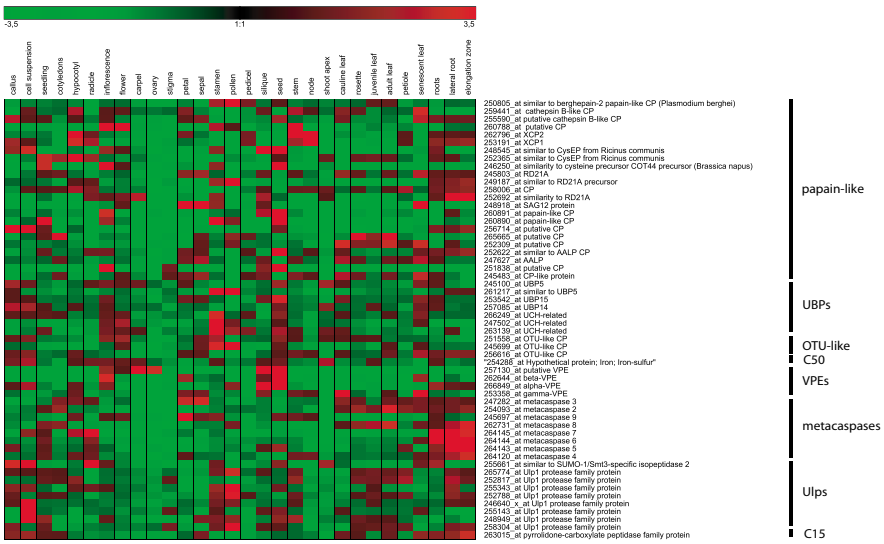
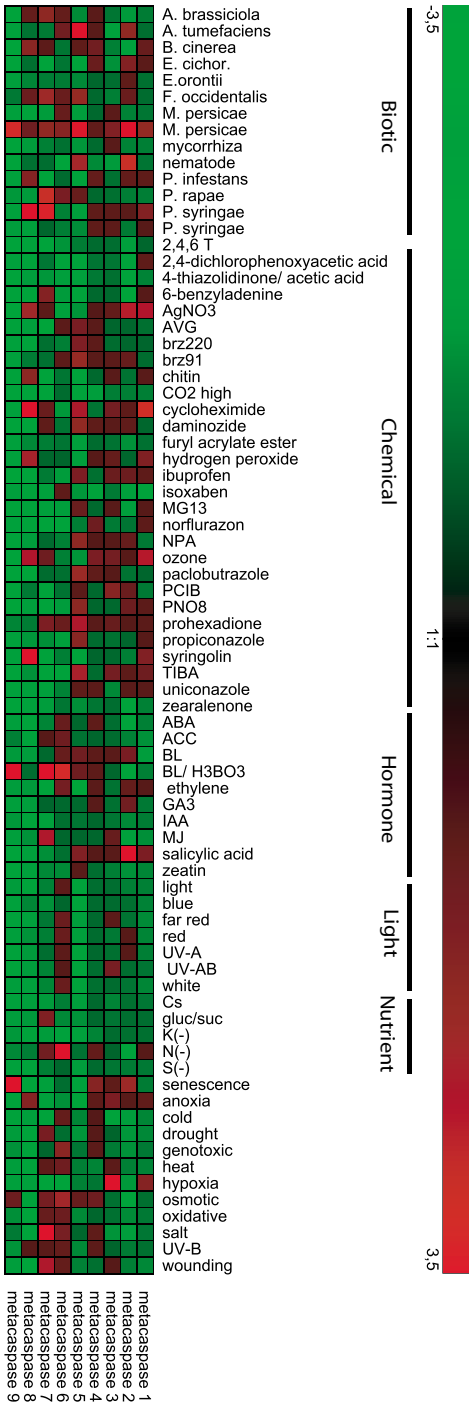


Figure 5: Expression profiles of differentially regulated (CV e > 0.5) *Arabidopsis* CP transcripts in different plant organs. Absolute expression values obtained from Genevestigator were divided by mean signal intensity values calculated over all plant organs. The resulting values were visualized with Genesis software (Sturn et al., 2002). Red indicates higher abundance of the transcripts of a particular gene in a particular organ, green a lower abundance. Intensity of the colors is proportional to the fold difference. Annotation was based on TAIR, version 2006-01-06 was used for ATH1 Affymetrix array element information. The figure also indicates the different families to which the CPs belong to.

1.8.3 Expression profiles of *Arabidopsis thaliana* cysteine proteases upon different abiotic and biotic stress stimuli

The response of the *Arabidopsis* CP transcripts on abiotic and biotic stress stimuli was found to be particularly scattered without obvious family-specific patterns. Therefore, only the response of the metacaspase transcripts on the different stress stimuli is represented in figure 6. In general, besides the peach-potato aphid *Myzus persicae*-induced upregulation of all metacaspases, there is no obvious pattern of regulation shared amongst the different metacaspases upon the various stress stimuli. The response of the metacaspases to the different stimuli is very diverse and in addition variable amongst the different metacaspases. Atmc9 shows downregulation upon almost all stress stimuli except to *Myzus persicae*, boric acid and during senescence. What can be remarked for the other metacaspases is that Atmc1 shows the highest induction upon application of the chemical stress cycloheximide, Atmc2 on the hormone salicylic acid and the aphid *M. persicae*, Atmc3 in response to hypoxic conditions. In addition, Atmc2 is also downregulated in response to benzyladenine (chemical stress) and boric acid (hormone stress). For Atmc4 no strong up- or downregulation on any of the tested stress stimuli could be revealed. Both a significant down- and upregulation of Atmc5 on different pathogens could be detected and a significant downregulation on few chemical stresses. Atmc6 was downregulated upon nematode infection and a chemical stress, and upregulated during nitrogen starvation and treatment with boric acid. Atmc7 was strongly downregulated in response to various stress stimuli, with the highest induction during salt stress and strongest downregulation upon ethylene treatment. Atmc8 was also differentially regulated upon various stress conditions, the strongest downregulation upon growth in high CO2 conditions and highest induction upon a chemical stress (syringolin).

In conclusion, some of the already described functions in literature were largely confirmed by the *in silico* analysis, such as a role for papain-like enzymes and VPEs in seed development. For the large Ulp1 family one can assume that many are indeed


A. brassiciola
A. tumefaciens
B. cinerea
E. cichor.
E.orontii
F. occidentalis
M. persicae
M. persicae
mycorrhiza
nematode
P. infestans
P. rapae
P. syringae
P. syringae
2,4,6 T
2,4-dichlorophenoxyacetic acid
4-thiazolidinone/ acetic acid
6-benzyladenine
AgNO3
AVG
brz220
brz91
chitin
CO2 high
cycloheximide
daminozide
furyl acrylate ester
hydrogen peroxide
ibuprofen
isoxaben
MG13
norflurazon
NPA
ozone
paclobutrazole
PCIB
PNO8
prohexadione
propiconazole
syringolin
TIBA
uniconazole
zearalenone
ABA
ACC
BL
BL/ H3BO3
ethylene
GA3
IAA
MJ
salicylic acid
zeatin
light
blue
far red
red
UV-A
UV-AB
white
Cs
gluc/suc
K(-)
N(-)
S(-)
senescence
anoxia
cold
drought
genotoxic
heat
hypoxia
osmotic
oxidative
salt
UV-B
wounding
Biotic
Chemical
Hormone
Light
Nutrient
-3,5
1:1
3,5
metacaspase 1
metacaspase 2
metacaspase 3
metacaspase 4
metacaspase 5
metacaspase 6
metacaspase 7
metacaspase 8
metacaspase 9

pseudogenes as indicated by their overall non-differential expression profile relative to plant development. Only for three members a high expression in cell suspension was assessed. For the OTU-like proteins a possible implication in reproductive plant development could be suggested based on a high expression in pollen for two of the eight members of the family, although this is very premature to make conclusive assumptions. The same pollen-specific expression could be detected for two UCH proteases, although based on the high number of members in this family (52) this is even more speculative.

Figure 6 (other side): Expression profiles of all *Arabidopsis* metacaspase transcripts upon various stress stimuli. Relative expression values (mutant to wild type or stress to control) obtained from Genevestigator were visualized with Genesis software (Sturn et al., 2002). Red indicates an upregulation compared to the control line or unstressed condition, green a downregulation. Intensity of the colors is proportional to the fold difference. Annotation was based on TAIR, version 2006-01-06 was used for ATH1 Affymetrix array element information.

1.9 Metacaspases, members of the C14 family

1.9.1 Metacaspases: general features

In mammalian systems, caspases are known to play key regulator functions in apoptosis. However, plant homologs were detected only recently when Uren and co-workers identified two families of distant caspase relatives in plants, fungi, protozoa, *Dyctiostelium,* and animals, by an iterative position-specific iterated homology search (PSI-BLAST). Paracaspases are restricted to the metazoa, and metacaspase genes are present in the genomes of **plants, fungi and protozoa** (Uren *et al*., 2000). The metacaspases are **members of the CD clan** of the cysteine proteases due to the presence of the catalytic dyad in the defined order histidine – cysteine in sequence. In *Arabidopsis*, Uren et al. identified eight metacaspases (2001). However, a homology search of the published sequences against a database of predicted *Arabidopsis* protein-encoding genes revealed one extra putative metacaspase gene, finally leading to a total of nine metacaspase genes in the genome of *Arabidopsis*, designated *Atmc1* to *Atmc9* (Vercammen *et al*., 2004). Genomic analysis of the *Arabidopsis* metacaspases revealed that the *Atmc8* gene is linked with genes *Atmc4*, *Atmc5*, *Atmc6* and *Atmc7* by an internal duplication event on chromosome I. Moreover, *Atmc4* to *Atmc7* genes are present as a tandem, suggesting that block duplication of the *Atmc8* gene was followed by a tandem duplication. Consequently, a high degree of similarity exists between the members of this clusters, with amino acid sequence similarities varying from 56% to 71% (Vercammen et al., 2004).

Metacaspases possess a similar **structure** to that of the mammalian caspases. Caspases consist of an N-terminal prodomain followed by a large (p20 subunit) and and a small (p10) subunit. Caspases with large prodomains are involved in the initiation process of apoptosis and are called initiator caspases. In the prodomains of these initiator caspases two related motifs have been found: the death effector domain (DED) and the caspase recruitment domain (CARD). They are important to promote

interactions of these initiator caspases with adaptor proteins and regulatory proteins. Caspases with short prodomains are involved in the execution phase of the cell death process and are called executioner or effector caspases. In some procaspases, the large and small subunits are separated by a small spacer which is excised during zymogen maturation. The p20 subunit possesses the histidine/cysteine catalytic dyad (Riedl and Shi, 2004). Three of the *Arabidopsis* metacaspase proteins (Atmc1, Atmc2 and Atmc3) belong to the **Type I metacaspases**. They possess an N-terminal extension ranging from about 80 to 120 amino acids in length (Uren et al., 2000) which is probably representing a prodomain, like in the mammalian upstream initiator caspases (Figure 7). These prodomains are rich in proline (Atmc1 and Atmc2) or glutamine (Atmc3), and all contain two putative CXXC-type zinc finger structures, similar to the lesion-simulating disease-1 (LSD1)-protein, a negative regulator of the hypersensitive response ((Uren et al., 2000); (Dietrich et al., 1997)). So far, zinc-finger proteins and proline-rich proteins have been implicated in cytoplasmic protein-protein interaction and.or binding to nuclear DNA. As such, it is very likely that this prodomain, in analogy to the mammalian initiator caspases, may be responsible for protein-protein interactions between metacaspases and/or other oligomerizing components of different signalling complexes, leading to subsequent metacaspase activation (also see below) ((Earnshaw et al., 1999); (Boatright and Salvesen, 2003)). Interestingly, the prodomains of paracaspases from humans, zebrafish and *C. elegans*, contain a death domain (DD) followed by either one or two immunoglobulin (Ig) domains. This DD is a homotypic interaction module, like DED and CARD of mammalian initiator caspases. The other metacaspases (AtMC4 to AtMC9) do not possess a large prodomain, although the occurrence of a short prodomain is possible. They were designated as the **Type II metacaspases** (Uren *et al.*, 2000). In both types, a conserved region of about 150 amino acids could correspond to the p20 subunit of caspases, while a conserved region at the C-terminius probably represents the p10 subunit. The putative p20 subunit contains the conserved catalytic Histidine/Cysteine dyad, as present in the mammalian caspases. Consequently, this feature combined with the structural domain homology, designate the metacaspases as distant caspase homologues. Between the putative

p20 and p10 subunit there is a variable region, which differs considerably between type I and type II metacaspases. This linker region is about 20 amino acids in type I metacaspases, while in the type II metacaspases the size varies between 90 (for Atmc9) and 150 (for Atmc4 to Atmc8) amino acids (Figure 7) (Vercammen *et al.*, 2004).

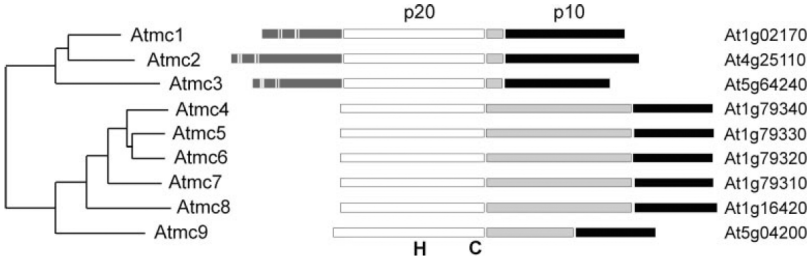


Figure 7: Phylogenetic tree of the metacaspases of *Arabidopsis thaliana* (from (Vercammen et al., 2004)). Three *Arabidopsis* metacaspase proteins (Atmc1, Atmc2 and Atmc3) belong to the type I metacaspases and possess an N-terminal extension ranging from about 80 to 120 amino acids in length (prodomain), whereas the Type II metacaspases (Atmc4 - Atmc9) lack such a prodomain.

1.9.2 Role for the meta- and paracaspases in programmed cell death (PCD)?

So far, little is known about the function of metacaspases, and the yeast metacaspase YCA1 remains the best studied. The question raises whether these metacaspases are the functional equivalents of caspases concerning their involvement in cell death. The mammalian caspases play a key regulator role in cell death, and they are involved in both the initiation and the execution phase of cell death (Earnshaw *et al.*, 1999). There are **some arguments that support a role for metacaspases in PCD**. First of all, the metacaspases possess a strong homology in their secondary structure to the mammalian caspases. Not only the conservation of the domain structure (p20, p10 subunit, and for the type I metacaspases a prodomain), also the presence of a conserved His/Cys catalytic dyad within the p20 subunit. This suggests

that there is an analogy concerning the structure of the proform and the mature oligomeric enzyme for both the caspases and the metacaspases. Secondly, perturbation of metacaspase levels in yeast, *Trypanosoma* and plants provides evidence for a putative role of the metacaspases in PCD. In yeast, Madeo et al. (2002) showed that hydrogenperoxide or ageing was able to induce accelerated apoptosis in yeast cells overproducing yeast caspase-1 (YCA1). A similar effect was obtained upon hydrogenperoxide- or ageing-induced cell death in yeast cells overexpressing *Arabidopsis* metacaspases. The cell-death-inducing activities of the metacaspases seemed to depend on their catalytic center cysteine residues (Watanabe and Lam, 2005). In addition, heterologous expression of *Trypanosoma brucei* metacaspase TbMCA4 in yeast cells resulted in growth inhibition, mitochondrial dysfunction and clonal death (Szallies et al., 2002). Furthermore, H_2O_2,-induced apoptosis seemed to be abrogated in a yeast strain lacking the YCA1 gene (ΔYCA1) (Khan et al., 2005). Yeast cells with a mutation in the *LSM4* gene showed increased mRNA stability and subsequent apoptosis. However, upon additional deletion of YCA1 apoptosis could be inhibited (Mazzoni et al., 2005). Similarly, UBP10Δ yeast cells lacking a deubiquitinating enzyme showed apoptosis, probably induced by an internal stimulus which activates the ectopic metacaspase. The additional deletion of YCA1 could abrogate apoptosis, while the additional expression of the YCA1 gene in UBP10Δ yeast cells resulted in an increase of apoptotic cells (Bettiga et al., 2004). Hyperosmotic stress-induced cell death could be suppressed in ΔYCA1 yeast cells. These cells also showed a reduced metacaspase activation (Silva et al., 2005). In *Trypanosoma cruzi*, overproduction of the TcMCA5 metacaspase resulted in an increased sensitivity towards fresh human serum (FHS)-induced PCD. Together with a change in subcellular localization of the metacaspase to the nucleus during FHS-induced PCD this suggested that the metacaspase might be involved in PCD of the parasite (Kosec et al., 2005). In plants, downregulation of a Type II metacaspase (mcII-Pa) suppresses PCD in the suspensor cells of an embryogenic culture of *Picea abies* and accumulation of mcII-Pa is detected in those embryonic tissues that are committed to death (Suarez et al., 2004). Bozhkov et al. (2005) demonstrated that the function of mcII-Pa during plant embryogenesis

relies on its Cysteine-dependent arginine-specific proteolytic activity. During plant embryogenesis, mcII-Pa is translocated to the nucleus in terminally differentiated cells which will undergo apoptosis, where it causes nuclear envelope disassembly and DNA fragmentation. Mutation of the catalytic cysteine abolishes the proteolytic activity of mcII-Pa and blocks nuclear degradation, which demonstrates that the metacaspase is as an executioner of PCD during embryo patterning. Furthermore, mRNA levels of LeMCA1, a tomato (*Lycopersicon esculentum*) Type II metacaspase, were increased upon infection of leaves with *Botrytis cinerea*, a fungal pathogen that induces cell death, and this increase seemed to be correlated with the formation of primary necrotic lesions (Hoeberichts et al., 2003).

In contrast to the metacaspases, there is currently no evidence for a possible involvement of the **paracaspases** in cell death. Inactivation of the only paracaspase gene of *Dictyostelium* did not result in altered cell death or alteration in development, which indicates that programmed cell death in development did not seem to require paracaspase (Roisin-Bouffay et al., 2004). A role for the human paracaspase, in mucosa-associated lymphoid tissue **(MALT) lymphoma** has been established. MALT lymphoma is a common type of lymphoma in extranodal sites and seems to be associated with chromosomal translocations. Fusion oncoproteins that arise from these chromosomal translocations often play critical roles in tumorigenesis. Both paracaspase (MALT1) and Bcl10 are involved in two recurrent chromosomal translocations in MALT lymphoma, t(1;14)(p22;q32) and t(11;18)(q21;q21) (Bertoni et al., 2002). The first translocation leads to overexpression of Bcl10, while the second translocation leads to production of a fusion protein, c-IAP2/MALT1, that contains the N-terminal portion of a inhibitor of apoptosis protein (c-IAP2) linked to the caspase-like domain of paracaspase. The consequence of overexpression of either Bcl10 or this chimeric protein is potent NF-kB activation, which is thought to contribute to increased B cell proliferation and survival promoting tumorigenesis. This ultimately resuls in MALT lymphoma (Ruefli-Brasse et al., 2003). The mechanism of the NF-kB activation by the chimeric protein is linked to its deregulated ubiquitin ligase activity (Zhou et al., 2005).

The fusion protein polyubiquitinates the regulatory subunit of the inhibitor of NF-kB (IkB), and hereby activates the NF-kB pathway. It was demonstrated that the caspase-domain is required for function of the fusion protein, since mutation of the conserved catalytic cysteine could attenuate NF-kB activation (Uren et al., 2000).

1.9.3 Metacaspases show Arg/Lys-specific activity and undergo (auto)processing

Mammalian caspases possess a strict **aspartate specificity (Stennicke and Salvesen, 1999).** Recently, in plants such aspartate specific proteolytic activities, so called caspase-like activities, associated with programmed cell death have been reported (also see §1.6). The metacaspases were prime candidates to be responsible for these activities. However, recent studies demonstrating **Arginine/Lysine specificity** of the **metacaspases** towards fluorogenic substrates suggested that the metacaspases are not directly responsible for the observed caspase-like activities ((Vercammen et al., 2004); (Watanabe and Lam, 2005)). Nevertheless, metacaspases might be responsible for the activation of more downstream proteases. Concerning the **paracaspases**, although they contain the highly conserved cysteine and histidine dyad required for catalysis by cysteine proteases, **no protease activity** whatsoever has been reported to date (Snipas et al., 2004).

Mammalian caspases undergo selfcatalytic **(aspartate-specific) processing**, which is dependent on the catalytic cysteine residue(Figure 8) (reviewed in (Stennicke and Salvesen, 1999)). Processing of the procaspases involves two cleavages in an ordered fashion whereby the first one results in removal of the prodomain and the second one in separation of the large and small subunit. Each active caspase is derived from processing and from two procaspase zymogens. The active form of animal caspases is a **heterotetrameric form** consisting of two large and two small subunits. Caspases are known to act in a **caspase cascade** (Figure 9A) (reviewed in (Czerski and Nunez, 2004)). Once the initiator caspases are (self-catalytically) activated upon

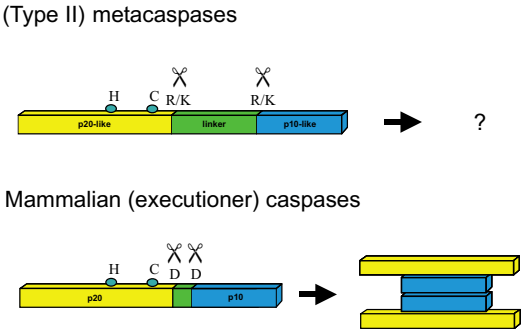


Figure 8: Activation of caspases and metacaspases . Processing of (Type II) metacaspases is Cysteine dependent and Arginine / Lysine-specific, whereas processing of mammalian caspases is Aspartate-specific. The active form of the mammalian caspases is a tetrameric form composed of two small subunits (p10) and two large subunits (p20). It was demonstrated that for Type II metacaspases processing is a prerequisite for activation, however if processed / active (Type II) metacaspases are also tetrameric proteins is hitherto unknown. For the Type I metacaspases possibly another mechanism of activation exists, whereby active dimerization is required and sufficient for their activation (and not cleavage), which is analogous to the mammalian executioner caspases.

an apoptotic stimulus, they can on their turn cleave and activate the effector caspases. The activated caspases will eventually cleave their substrates which results in cell death. Interestingly, it is known for mammalian caspases that **initiator caspases and executioner caspases possess different mechanisms of activation (Boatright and Salvesen, 2003)**. Whereas executioner caspases are activated by interdomain cleavage, initiator caspases are activated by dimerization. This is linked with the fact that initiator caspases exist as inactive monomers in the cytosol, while executioner caspases exist as inactive dimers (Boatright et al., 2003). The monomeric zymogens of the initiator caspases require dimerization to assume an active conformation and this activation is independent of cleavage ((Stennicke et al., 1999); (Boatright et al., 2003); (Donepudi et al., 2003)), whereas the dimeric zymogens of the executioner caspases are activated by limited proteolysis within their interdomain linker which is carried out by an initiator caspase or occasionally by other proteases under specific circumstances. Remarkably, cleavage of the initiator caspases does not lead to

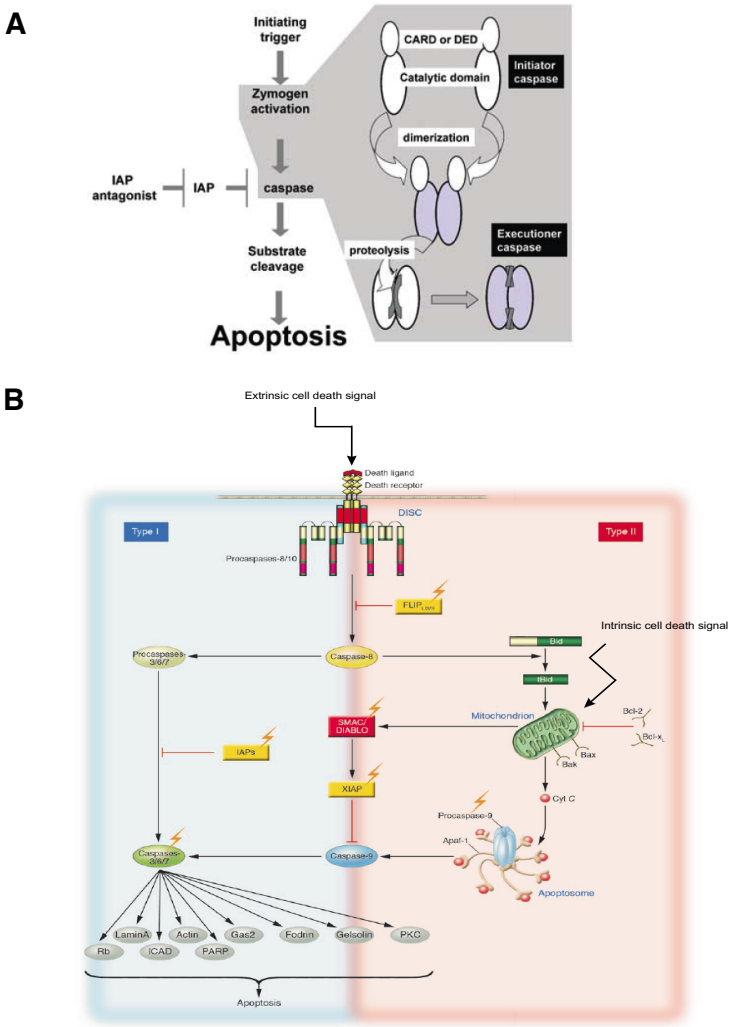


Figure 9: Activation of caspase cascade in mammalian systems. Activation of initiator caspases occurs upon an apoptotic stimulus via proximity-induced oligomerization either at the apoptosome (intrinsic death-inducing signal) or at the receptosome (extrinsic signal). Active initiator caspases at their turn cleave and hence activate the executioner caspases, which results in cleavage of a plethora of substrates leading to the morphological and biochemical changes associated with apoptotic cell death. (A) Simplified overview of the caspade activation leading to apoptosis (from (Salvesen and Abrams, 2004)). (B) Extrinsic and intrinsic signalling pathway leading to caspase activation and subsequent cell death (adapted from (Lavrik et al., 2005)).

activation, however it was suggested that cleavage may stabilize activity (Boatright et al., 2003). Nevertheless, the fundamental mechanism of zymogen latency is conserved for both types of caspases as activation of both types requires translocation of an activation loop. For the executioners, this translocation is blocked by steric hindrances imposed by the interdomain linker, for the initiators, dimerization must first occur to allow the activation loop to interact with the adjacent monomer (Boatright and Salvesen, 2003). The dimerization event occurs at multiprotein activating complexes, to which the zymogens are recruited by virtue of their N-terminal recruitment domain or prodomain. The result of the recruitment is an increase in local concentration of the caspases, creating a microenvironment in which the zymogens are oligomerized to yield fully active initiator caspases, this is called proximity-induced oligomerization (jnduced proximity). The activating complex involved depends on the origin of the death stimulus, which is classified as either being extrinsic (procaspase-8 and procaspase-10), triggered by cell surface receptors, or intrinsic, in response to environmental stimuli (procaspase-9) (Figure 9B). In the extrinsic pathway, formation of the cell death complex (death-inducing signalling complex or DISC) leads to caspase-8 activation. Two signalling pathways downstream from the receptor were established. In Type I cells, caspase-3 is directly cleaved by caspase-8, which results in active executioner caspases cleaving a plethora of substrates and hence leading to the morphological and biochemical changes associated with cell death. However, in Type II cells an additional amplification loop is required, involving tBid-mediated release, which induces apoptosome formation. In the intrinsic pathway, a mitochondria-mediated apoptosome is formed which leads to caspase-9 activation, followed by active executioner caspases and destruction of the cell (Figure 9B). As such, activation of the caspase cascade, namely activation of the initiator caspases by induced proximity and subsequent activation of the executioner caspases by active initiator caspases, is crucial to the execution of apoptosis.

Similarly to the caspases, **metacaspases also show cysteine-dependent autoprocessing**. However, this processing is arginine / lysine-specific (Figure 8). For

Type II metacaspases of *Arabidopsis* it has already been demonstrated that they undergo autocatalytic cysteine-dependent processing upon overexpression in bacteria (Vercammen et al., 2004). Likewise to mammalian caspase processing, this autoprocessing includes separation of the large and small subunits and concomitant removal of a short N-terminal prodomain. It has been shown for Atmc9 that autoprocessing is required for its activity (Vercammen et al., 2004). On the other hand, mere overexpression of **Type I** metacaspases is not sufficient to induce processing in bacteria (Vercammen et al., 2004), yeast (Watanabe and Lam, 2005) or plants (Chapter 2). However, processing of the Type I metacaspases in yeast occured after application of an apoptotic stimulus (Watanabe and Lam, 2005). Whether processing of the Type I metacaspases is required for their activity remains obscure, as it is possible that, analogous to the mammalian initiator caspases, **active dimerization** is sufficient and required for their activation. This active dimerization is probably not achieved by mere overexpression. Interestingly, the **human paracaspase did not undergo autoprocessing** when transfected in human cells, even when artificially oligomerized (Uren et al., 2000).

However, if the active form of the metacaspases is also a tetrameric form, or to what extent the activation mechanism of the metacaspases is similar to that of the mammalian caspases remains hitherto unclear. In addition, if a similar cascade also exists in plants is not known yet. These issues are of current interest to better understand the similarities between metacaspases and caspases, not only concerning their structures and mechanisms of activation, but derived from this knowledge also to unravel putative similar function(s) of the metacaspases.

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Appendix: The Cysteine proteases of *Arabidopsis* with their AGI code, description and Affymetrix ATH1 array element names. Annotation is based on the Arabidopsis Information Resource (TAIR) from 2006-01-06. If no array element was found for a particular gene this is indicated with "/".

	AGI code	Description	array element name
papain-like			
	At1g02300	cathepsin B-like cysteine protease	259441_at
	At1g06260	putative cysteine proteinase	260788_at
	At1g09850	cysteine protease, papain-like XBCP3	264687_at
	At1g20850	cysteine endopeptidase, papain-type XCP2	262796_at
	At1g29080	peptidase C1A papain family	260891_at
	At1g29090	peptidase C1A papain family	260890_at
	At1g29110	putative cysteine proteinase	260892_at
	At1g47128	cysteine proteinase RD21A	245803_at
	at2g27420	putative cysteine proteinase	265665_at
	at2g34080	putative cysteine proteinase	256714_at
	at3g19400	cysteine proteinase	258006_at
	At3g43960	cysteine proteinase similarity to cysteine proteinase RD21A	252692_at
	At3g45310	cysteine proteinase similar to AALP protein	252622_at
	At3g48350	cysteine proteinase similar to cysteine endopeptidase precursor [Ricinus communis]	252365_at
	At3g49340	putative cysteine proteinase	252309_at
	At3g54940	putative cysteine proteinase	251838_at
	at4g01610	putative cathepsin B-like cysteine	255590_at
	at4g16190	cysteine proteinase like protein	245483_at
	at4g23520	cysteine proteinase - like protein	254237_at
	at4g35350	papain-type cysteine endopeptidase xcp1	253191_at
	At4g36880	strong similarity to cysteine proteinase COT44 precursor [Brassica napus] (Rape)	246250_at
	At4g39090	cysteine proteinase RD19a	/
	At5g05050	peptidase C1A papain family protein weak similarity to berghepain-2 [Plasmodium berghei] GI:17978639	250805_at
	at5g43060	cysteine protease component of protease-inhibitor complex.similar to cysteine proteinase RD21A precursor (thiol protease) GI:435619	249187_at
	At5g45890	senescence-specific SAG12 protein	248918_at
	at5g50260	similar to cysteine endopeptidase precursor CysEP from [Ricinus communis]	248545_at
	at5g60360	cysteine proteinase aalp	247627_at
Calpain-like			
	At1g55350	calpain-type cysteine protease	259646_at
UCHs			
	At1g65650	ubiquitin carboxyl-terminal hydrolase family 1 protein similar to 26S proteasome regulatory complex subunit p37A	264639_at
	at4g17510	ubiquitin carboxyl-terminal hydrolase family 1 protein	245425_at
	at5g16310	ubiquitin carboxyl-terminal hydrolase family 1 protein	250126_at

UBPs			
	At1g04860	ubiquitin-specific protease 2 (UBP2)	261128_at
	At1g17110	ubiquitin-specific protease 15 (UBP15)	/
	At1g32850	ubiquitin-specific protease similar to ubiquitin-specific protease UBP5	261217_at
	At1g51710	ubiquitin-specific protease 6, putative (UBP6)	256179_at
	At1g52430	ubiquitin carboxyl-terminal hydrolase-related	/
	At1g65110	ubiquitin carboxyl-terminal hydrolase-related	263139_at
	At1g65120	ubiquitin carboxyl-terminal hydrolase-related	/
	At1g65130	ubiquitin carboxyl-terminal hydrolase-related	257453_at
	At1g65170	ubiquitin carboxyl-terminal hydrolase family protein	257454_at
	At2g22310	ubiquitin-specific protease 4 (UBP4)	263428_at
	at2g24640	ubiquitin-specific protease similar to ubiquitin-specific protease 15 (UBP15)	263798_at
	at2g27630	ubiquitin carboxyl-terminal hydrolase-related	266249_at
	at2g27650	ubiquitin carboxyl-terminal hydrolase-related	266248_at
	At2g32780	ubiquitin-specific protease 1, putative (UBP1)	267551_at
	at2g40930	ubiquitin-specific protease 5 (ubp5)	245100_at
	At3g11910	ubiquitin-specific protease ubiquitin-specific protease 12 (UBP12)	258780_at
	At3g14400	ubiquitin-specific protease 25 (UBP25)	258372_at
	At3g20630	ubiquitin-specific protease 14, putative (UBP14)	257085_at
	At3g21280	ubiquitin-specific protease 7, putative (UBP7)	258045_at
	At3g47890	ubiquitin carboxyl-terminal hydrolase-related	/
	At3g49600	ubiquitin-specific protease 26 (UBP26)	252275_at
	At4g10570	ubiquitin-specific protease similar to ubiquitin-specific protease UBP5	/
	at4g17890	human Rev interacting-like family protein	254709_at
	At4g17895	ubiquitin-specific protease 20, putative (UBP20)	254708_at
	at4g22290	ubiquitin carboxyl-terminal hydrolase family protein similar to pVHL-interacting deubiquitinating enzyme 1 type II	/
	at4g22410	ubiquitin carboxyl-terminal hydrolase family protein similar to U4/U6.U5 tri-snRNP-associated 65 kDa protein [Homo sapiens]	254308_at
	At4g24560	ubiquitin-specific protease 16, putative (UBP16)	254128_at
	At4g30890	ubiquitin-specific protease 24, putative (UBP24)	253552_at
	at4g31670	similar to ubiquitin-specific protease 15 (UBP15)	253542_at
	at4g39370	ubiquitin-specific protease 27, putative (UBP27)	252854_at
	at4g39910	ubiquitin-specific protease 3 (UBP3)	252832_at
	At5g06600	ubiquitin-specific protease 12 (UBP12)	250693_at
	At5g10790	ubiquitin-specific protease 22 (UBP22)	250405_at
	At5g22030	ubiquitin-specific protease 8, putative (UBP8)	245683_at
	At5g46740	ubiquitin-specific protease 21 (UBP21)	248841_at
	At5g57990	ubiquitin-specific protease 23, putative (UBP23)	247838_at
	At5g61940	ubiquitin carboxyl-terminal hydrolase-related	247502_at
	At5g65450	ubiquitin carboxyl-terminal hydrolase family protein	247184_at

	AGI code	Description	array element name
autophagy			
	At3g59950	autophagy 4b (APG4b) identical to autophagy 4b [Arabidopsis thaliana]	251439_at
	At2g44140	autophagy 4a (APG4a) identical to autophagy 4a [Arabidopsis thaliana]	/
OTU-like			
	at1g50670	OTU-like cysteine protease family protein	261854_at
	at2g27350	OTU-like cysteine protease family protein	265630_at
	at2g39320	OTU-like cysteine protease family protein	267020_at
	At3g02070	OTU-like cysteine protease family protein	258855_at
	At3g22260	OTU-like cysteine protease family protein	256616_at
	At3g57810	OTU-like cysteine protease family protein	251558_at
	At5g04250	OTU-like cysteine protease family protein	245699_at
	at3g62940	hypothetical protein	251149_at
VPE / legumains			
	At1g08750	GPI-anchor transamidase,	264810_at
	at1g62710	vacuolar processing enzyme, beta-isozyme precursor (beta-vpe).	262644_at
	at2g25940	vacuolar processing enzyme, alpha-isozyme precursor (alpha-vpe)	266849_at
	At3g20210	vacuolar processing enzyme, putative / asparaginyl endopeptidase,	257130_at
	at4g32940	vacuolar processing enzyme, gamma-isozyme precursor (gamma-vpe).	253358_at
metacaspases			
	At1g02170	metacaspase 1	264178_at
	At1g16420	metacaspase 8	262731_at
	At1g79310	metacaspase 7	264145_at
	At1g79320	metacaspase 6	264144_at
	At1g79330	metacaspase 5	264143_at
	at1g79340	metacaspase 4	264120_at
	At4g25110	metacaspase 2	254093_at
	at5g04200	metacaspase 9	245697_at
	at5g64240	metacaspase 3	247282_at
separases			
	at4g22970	Hypothetical protein; Iron; Iron-sulfur	254288_at

ULP			
	at1g09730	low similarity to SUMO-1-specific protease 1	264707_at
	At1g10570	Ulp1 protease family	263260_at
	At1g34610	Ulp1 protease family protein	/
	At1g34740	Ulp1 protease family protein	/
	At1g35770	Ulp1 protease family protein	/
	At1g37020	Ulp1 protease family protein	261287_at
	At1g42460	Ulp1 protease family protein	262265_at
	At1g60220	Ulp1 protease family protein	264268_at
	at2g02210	pseudogene, Ulp1 protease family	/
	at2g04980	pseudogene, Ulp1 protease family	/
	at2g05450	pseudogene, Ulp1 protease family	/
	at2g05560	pseudogene, Ulp1 protease family	/
	at2g07200	pseudogene, Ulp1 protease family	266430_at
	at2g07240	Ulp1 protease family protein	265774_at
	At2g10350	Ulp1 protease family protein	/
	at2g11480	pseudogene, Ulp1 protease family	/
	at2g12100	Ulp1 protease family protein	/
	at2g14010	hypothetical protein	/
	at2g14130	Ulp1 protease family protein	/
	at2g15140	pseudogene, Ulp1 protease family	/
	at2g15190	pseudogene, Ulp1 protease family	/
	At2g24930	Ulp1 protease family protein	263523_at
	at2g29240	Ulp1 protease family protein	/
	At3g06910	Ulp1 protease family protein similar to sentrin/SUMO-specific protease	258548_at
	At3g30640	Ulp1 protease family protein	258304_at
	At3g42530	Ulp1 protease family	252814_at
	At3g42580	Ulp1 protease family protein	252817_at
	At3g42690	Ulp1 protease family protein	252788_at
	At3g42730	Ulp1 protease family protein	252760_x_at
	At3g42820	hypothetical protein hypothetical proteins	252766_at
	At3g47260	Ulp1 protease family protein	/
	At3g47260	Ulp1 protease family protein	/
	At3g47260	Ulp1 protease family protein	/
	At3g47260	Ulp1 protease family protein	/
	At3g47260	Ulp1 protease family protein	/
	at4g00690	similar to SUMO-1/Sm43-specific isopeptidase 2	255661_at
	at4g04010	Ulp1 protease family protein	/
	at4g04130	Ulp1 protease family protein	/
	at4g04530	Ulp1 protease family protein	255343_at
	at4g05280	Ulp1 protease family protein	255273_at
	at4g07580	pseudogene, Ulp1 protease family	255208_at
	at4g07680	pseudogene, Ulp1 protease family	/
	at4g08340	Ulp1 protease family protein	255135_at

Chapter 2: Transgenic plants with elevated metacaspase levels: molecular analysis and metacaspase activity *in planta*

2.1. Abstract

Based on different forward genetic approaches where metacaspases are overproduced in different model organisms, an implication of metacaspases in the programmed cell death (PCD) process has been suggested. Hereby, cell death was often accompanied with caspase-like activity. However, it is doubted whether this caspase-like activity is caused directly by the metacaspases, as it has been shown that metacaspases are Arginine/Lysine-specific. To help decipher the role of metacaspases in plants, *Arabidiopsis* plants overproducing type I or type II metacaspases were generated. Expression analysis could provide more insight whether the metacaspase is present in its processed / active form and if this can be correlated with metacaspase activity in the transgenic plants. Mere overproduction of **type II** metacaspases in *Arabidopsis thaliana* was sufficient to induce clear autocatalytic processing of the metacaspase into its p20- and p10-like subunits. This processing was also translated in proteolytic metacaspase activity as shown by both fluorometric and in-gel assay of Atmc4 and Atmc9 in the corresponding transgenic lines. In contrast, overproduction of **type I** metacaspases in *Arabidopsis* did not induce detectable processing in the tested conditions, which was also the case upon bacterial overexpression (Vercammen et al., 2004). Possibly, Type I and Type II metacaspases use different mechanisms of activation, which might be reflected in the functional divergence between type I and type II metacaspases, as is also seen with animal initiator and executioner caspases.

2.2. Introduction

In mammalian systems, **caspases** are known to play key regulator functions in apoptotic cell death, in both the initiation as well as the execution process of cell death. Upon an apoptotic stimulus, initiator caspases are activated which in their turn are able to activate the downstream executioner caspases. Finally, these active executioner caspases cleave their substrates which contributes to the biochemical and morphological features associated with apoptotic cell death (reviewed in (Earnshaw et al., 1999). Lately, various **caspase-like activities** have been discovered in plants (see Chapter 1: §1.6). Moreover, many of these caspase-like activities have been associated with programmed cell death in plants (for overview see Chapter 1: Table 3). Although plants do not have close homologues of caspases, a phylogenetically distant family of cysteine proteases was recently discovered in plants, fungi and protozoa, named **metacaspases** (Uren et al., 2000). The question raised whether these metacaspases would be the functional equivalents of their mammalian counterparts concerning their involvement in cell death. Recent studies suggested that metacaspases are possibly involved in **programmed cell death**.

Perturbation of metacaspase levels in different model organisms provides arguments for a possible role of metacaspases in PCD. In yeast, overproduction and concomitant processing of the yeast metacaspase YCA1 as such had little effect. However, when combined with mild stresses, such as sublethal concentrations of H_2O_2 or cell culture aging, it resulted in apoptotic cell death (Madeo et al., 2002). Similarly, overexpression of *Arabidopsis* metacaspases in yeast significantly promoted cell death of both wild type and YCA1•strains (lacking *yca1* gene) during oxidative stress and early aging process (Watanabe and Lam, 2005). Heterologous expression of a metacaspase of *Trypanosoma brucei* in yeast inhibited growth and caused loss of respiratory competence and clogenicity (Szallies et al., 2002). Additional deletion of yeast metacaspase in yeast cells mutated in the *ubp10* gene, a deubiquitniating enzyme of *S. cerevisiae*, was able to suppress apoptotic cell death. However, YCA1

overexpression in the mutant cells was accompanied by an increase of apopototic cells (Bettiga et al., 2004). Yeast cells carrying a mutation in the *lsm4* gene, which is involved in mRNA decapping, led to increased mRNA stability and apoptosis in yeast. Additional deletion of YCA1 prevented mitochondrial fragmentation and rapid cell death during chronological aging of the culture (Mazzoni et al., 2005). Lack of YCA1 drastically reduced metacaspase activation and decreased cell death upon hyperosmotic stress (Silva et al., 2005). Overproducing *Trypanosoma cruzi* metacaspase 5 (TcMCA5) led to a higher susceptibility to fresh human serum-induced PCD and overexpression TcMCA3 seemed to have a lethal effect (Kosec et al., 2005). In plants, arguments are also accumulating for the involvement of metacaspases in PCD. RNA interference studies showed that a type II plant metacaspases from *P.abies* (mcII-Pa) was responsible for plant embryogenesis. An accumulation of mcII-Pa was found in the embryonic tissues that are committed to death. Silencing of mcII-Pa resulted in the absence of embryonic pattern formation (Suarez et al., 2004). Furthermore, one of the tomato genes is upregulated during *Botrytis cinerea*-induced plant cell death (Hoeberichts et al., 2003).

Currently, there is evidence which shows there is a **link between metacaspases and caspase-like activities**. Upon overexpression of yeast caspase YCA1, hydrogenperoxide-induced cell death was correlated with caspase-like protease activity (IETDase activity) and could be blocked by a general caspase inhibitor (Z-VAD-fmk) (Madeo et al., 2002). Upon overproduction of *Trypanosoma cruzi* metacaspase 3 (TcMCA3) cell death was induced, which was paralleled by an increase in peptidase activity against a typical substrate (z-YVAD-AFC) (Kosec et al., 2005). In plants, during embryo spruce development VEIDase activity was associated with the accumulation of mcII-Pa in the embryonic tissues that are committed to death (Suarez et al., 2004). Overexpression of *Arabidopsis thaliana* metacaspases in yeast provoked cell death which could be prevented by addition of a caspase inhibitor (Watanabe and Lam, 2005). However, in none of the cases, this caspase-like activity was proven to originate directly from the metacaspases. In addition, bacterial and yeast extracts containing recombinant

Arabidopsis metacaspases exhibited **Arg/Lys-specific endopeptidase activities** but could not cleave caspase-specific substrates (Watanabe and Lam, 2005). This Arg/Lys-specificity of the *Arabidopsis* metacaspases was previously shown by Vercammen et al. (2004). These metacaspases displayed no specificity towards the classical caspase substrates, only against P1-Arg/Lys substrates (Vercammen et al., 2004). Bozhkov *et al*. (2005) demonstrated that mcII-Pa silenced lines showed both a reduction in VEIDase and EGRase activity, the latter being a P1-Arginine substrate. Moreover, recombinant mcII-Pa does not cleave classical caspase substrates, including the VEID sequence-containing substrate however it does cleave P1-Arg/Lys substrates (Bozhkov et al., 2005). All these findings suggest that **metacaspases are not directly responsible** for reported caspase-like activities in plants, yeast, and *Trypanosoma*. However, **activation of other upstream caspase-like protease(s)** triggered through either direct or indirect proteolysis by a metacaspase could mediate execution of yeast apoptosis or plant PCD.

Recently, it has been shown that **VPEs are responsible for caspase-like activity in plants**, at least for HR-related cell death (reviewed in (Lam, 2005). Despite the fact that VPE is structurally unrelated to caspases it was shown to exhibit caspase-1 like activity and to be sensitive to a caspase-1 specific inhibitor (Hatsugai et al., 2004). In addition, infection with an avirulent strain of Pseudomonas syringae results in an increase of caspase-1 activity, and this increase is partially suppressed in VPEg mutants (Rojo et al., 2004). As such, VPE has been suggested to act like a plant caspase that regulates hypersensitive cell death in defense. Whether or not these VPEs are activated by upstream metacaspases remains to be elucidated.

In addition, subtilisin-like proteases from oat have also been identified to play an important role as caspase-like proteases in the execution of plant cell death. Subtilisin-like proteases are serine proteases that possess strong caspase-3 like activities (see Chapter 1, Table 3). They are involved in the proteolysis of Rubisco during victorin-induced plant cell death and are very sensitive to numerous caspase-specific inhibitors

(Coffeen and Wolpert, 2004). Therefore, subtilisin-like proteases were denominated **saspases**. In contrast to VPEs which are cysteine proteases, saspases are not caspase-like proteases in as strict sense because they are serine proteases.

As caspase-like activities were often linked to the metacaspases, however, recombinant metacaspases were shown to display Arg/Lys-specific-peptidase activities, it is assumed that these metacaspases are responsible for the activation of downstream proteases such as VPEs or saspases.

Nevertheless, studies with perturbed metacaspase expression levels in yeast, *Trypanosoma* and plants have shown that there is a conserved link between metacaspases and programmed cell death, the metacaspases might not act as bona fide homologues of animal caspases (cfr substrate specificity). However, the function of these plant caspases remains largely unknown. Perturbation of endogenous plant metacaspase levels in *Arabidopsis thaliana* plants can help to provide more insight in which biological processes the metacaspases are involved and point towards new insights into the functions of the metacaspases. In this work, *Arabidopsis thaliana* plants which overproduced both type I or type II metacaspases were obtained. A molecular analysis was performed to study whether these metacaspases are processed in plants, and whether processing is correlated with activity. Moreover, overexpression plants were tested for an enhanced/decreased susceptibility towards both biotic and abiotic stress stimuli (Chapter 3).

2.3. Results and discussion

2.3.1. Molecular analysis of transgenic *Arabidopsis thaliana* lines with higher metacaspase expression levels

2.3.1.1. Cloning of Arabidopsis thaliana metacaspases in plant overexpression vector pB7WG2

To assess the function of *Arabidopsis* metacaspases, transgenic *Arabidopsis* plants that overexpress wild type or catalytically inactive mutant versions of the metacaspases were produced.

Wild-type and mutant versions of the open reading frames (ORF) of eight annotated *Arabidopsis thaliana* metacaspases were cloned into the entry vector pDONR201 of the Gateway recombinatorial cloning system. All but one metacaspase, namely Atmc8, was cloned in the entry vector generating pDONR201-Atmc1 to pDONR201-Atmc9 and pDONR201-Atmc1CA to pDONR201-Atmc9CA, respectively (Vercammen et al., 2004). The ORF of *Atmc8* could not be amplified. Probably this gene is only induced under very specific conditions and could therefore not be amplified from tissues derived from plants grown under normal conditions. Alternatively, *Atmc8* is a pseudogene, as no expressed sequence tags corresponding to *Atmc8* could be found in public databases (Vercammen et al., 2004). In the mutant versions the presumable catalytic cysteine residue is mutated to alanine (C/A mutation). Subsequently the ORFs were cloned into the binary vector pB7WG2 via Gateway recombination. In the resulting vector the metacaspase ORF is under transcriptional control of the cauliflower mosaic virus 35S (CaMV 35S), and the BASTA ammonium resistance gene is present to allow for selection in planta (Karimi et al., 2002).

2.3.1.2. Generation of transgenic Arabidopsis lines overexpressing wild type and C/A mutant metacaspases

Constructs for overexpression of both wild type and mutant metacaspases were transferred to *Arabidopsis* via *Agrobacterium tumefaciens*-mediated floral dip transformation (Clough and Bent, 1998). The number of primary transformants for the eight metacaspases are summarized in the table below (Table 1). No transgenic lines were obtained for Atmc2CA-Atmc7CA. Transgenic lines were subjected to segregation analysis and expression levels of the transgene were checked by Western and/or Northern analysis. Subsequently, homozygous transgenic lines containing a single locus and displaying diverse expression levels were selected for seed upscale, further analysis and experiments. Segregation and expression data for all transgenic lines are displayed in annex I.

Atmc	# primary
pB7WG2-Atmc1	21
pB7WG2-Atmc1CA	43
pB7WG2-Atmc2	22
pB7WG2-Atmc3	13
pB7WG2-Atmc4	41
pB7WG2-Atmc5	51
pB7WG2-Atmc6	47
pB7WG2-Atmc7	27
pB7WG2-Atmc9	9
pB7WG2-Atmc9CA	10

Table 1: Number of primary transformants for every transgenic line. No transgenic lines for the mutant metacaspases Atmc2CA to Atmc7CA. It should be remarked that the number of transformation rounds varied amongst the different lines.

2.3.1.3. Expression analysis of transgenic Arabidopsis lines overexpressing wild type and C/A mutant metacaspases

a. Atmc1/Atmc1CA

As shown in Figure 1, western analysis on **leaf** (A) and **root** (B) material of selected lines of 35S::Atmc1 and 35S::Atmc1CA lines revealed differential expression of the transgene. Western blot on both leaf and root extracts, using a polyclonal rabbit antibody raised against recombinant Atmc1, displayed a band around 45 kDa. This band most probably represents the unprocessed zymogen or full length protein as it has approximately the size of the theoretical calculated molecular weight of the proform (40 kDa). Both 35S::Atmc1 and 35S::Atmc1CA lines showed clear overexpression of this full length protein when compared to the wild type line. In both the 35S::Atmc1 and 35S::Atmc1CA lines only the full length protein can be detected, which means that no processing of the Atmc1/Atmc1CA protein occurs in leaves or roots of these transgenic plants. Conclusively, mere overproduction of the full length protein does not lead to (detectable) processing of wild type Atmc1, nor Atmc1CA. However, for Atmc1CA which is a catalytic inactive mutant protein, this could be expected.

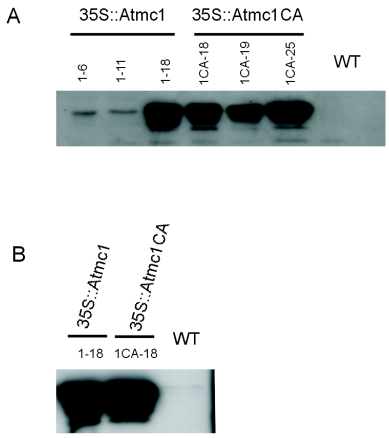

Figure 1: Overproduction of Atmc1 in leaves (A) and roots (B) of 35S::Atmc1 and 35S::Atmc1CA plants. Detection of *Arabidopsis* Atmc1 protein levels by western blot with an antibody against Atmc1 (PS729-Atmc1). For the complete western blot see Annex II.

b. Atmc2/Atmc2CA

Figure 2 shows expression of the transgenic protein for the selected 35S::Atmc2 lines. A Western blot was performed on extracts from **leaf** material, using a polyclonal antibody raised against recombinant Atmc2. A band around 60 kDa could be detected, and most probably represents the unprocessed zymogen or full length protein, which is higher than the theoretical molecular weight (45 kDa). The fact that this protein migrates higher can be due to posttranslational modifications *in vivo* of the protein. In the 35S::Atmc2 lines the transgenic Atmc2 protein is clearly overproduced when compared to the wild type lines, and also in this case no processed forms of the Atmc2 protein could be observed. For Atmc2, there were no CA lines with elevated levels of the transgenic mutant protein, as detected by Western blot (data not shown). Only levels similar to the endogenous wild type levels could be observed. Hitherto, it remains unclear why no line could be discovered which overproduces the mutant form of Atmc2. Possibly, there is a dominant negative effect caused by the mutant protein which masks the function of the wild type isoform, and thereby causes lethality. Consequently, only plants with levels comparable to the endogenous protein can survive, and hence be detected on a Western blot.

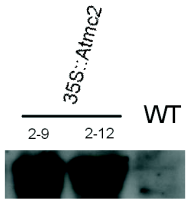

Figure 2: Overproduction of Atmc2 in leaves of 35S::Atmc2 plants. Detection of *Arabidopsis* Atmc2 protein levels by western blot with an antibody against Atmc2 (PS769-Atmc2). For the complete western blot see Annex II.

c. Atmc3/Atmc3CA

Expression of the transgenic Atmc3 protein in the 35S::Atmc3 and Atmc3CA lines was checked by Northern analysis on **leaf** extracts. Figure 3 shows the transcript levels of Atmc3 in the 35S::Atmc3 and 35S::Atmc3CA lines using a probe targeting the coding sequence of Atmc3. All tested 35S::Atmc3 lines showed a clear elevated expression of Atmc3 when compared to the wild type line. However, in none of the tested 35S::Atmc3CA lines the expression level was clearly higher than wild type. Whether this observation can also be linked to a dominant negative effect of the mutant protein causing lethality, remains to be studied. Lacking an appropriate antibody recognizing Atmc3, we do not know whether the protein levels can be correlated to the RNA levels, and whether the protein is processed or not.

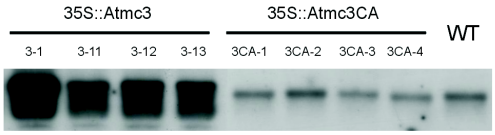

Figure 3: Overproduction of Atmc3 in leaves of 35S::Atmc3 plants. Detection of *Arabidopsis* Atmc3 transcript levels by northern blot using a probe generated against the coding sequence of Atmc3.

Taken together, these results suggest that mere overproduction of type I metacaspases is not sufficient for (detectable) autocatalytic processing into p20- and p10-like fragments. These findings correlate well with experiments in *E.Coli*, *N. Benthamiana* and human 293T cells where all three type I *Arabidopsis* metacaspases (Atmc1,2 and 3) also did not process upon overproduction (Vercammen et al., 2004). This is reminiscent with the animal field where initiator caspases, located in the upstream regions of signalling pathways, need induced oligomerization for their activation (Chen et al., 2002; Boatright and Salvesen, 2003). In these systems, zymogens of

initiator caspases are activated by proximity-induced oligomerization in which locally forced high concentrations of low-active procaspases result in oligomerization (also called induced proximity) which yields fully active caspases. Moreover, for mammalian initiator caspases this activation is independent of cleavage (Stennicke et al., 1999; Boatright et al., 2003); Donepudi et al., 2003), however it was suggested that cleavage may stabilize activity (Boatright and Salvesen, 2003). It might be possible that, analogous to the mammalian initiator caspases, **active dimerization** is sufficient and required for the activation of the Type I metacaspases, which is not achieved by mere overexpression. In that respect, processing would not even be necessary to yield active Type I metacaspases.

d Atmc4(CA)-Atmc7(CA)

Comparison of the genomic organization of all nine *Arabidopsis* metacaspases showed that the *Atmc8* gene is linked with *Atmc4*, *Atmc5*, *Atmc6* and *Atmc7* genes and this metacaspase cluster (genes *Atmc4* to *Atmc7*) originated trough a block duplication of the *Atmc8* gene that was followed by a tandem duplication (Vercammen et al., 2004). As a result, a high degree of similarity exists between the members of this cluster. Therefore, the antibody generated against Atmc7 recognizes the other metacaspases Atmc4, Atmc4 and Atmc6 as well. The corresponding transgenic lines 35S::Atmc4 to 35S::Atmc7 will be discussed together.

Figure 4 depicts transgenic protein levels of **leaf** (A) and **root** (B) material of the selected lines of 35S::Atmc4, 5, 6 and 7. For immunodetection of the Western blot a polyclonal rabbit antibody was used, raised against recombinant Atmc7 but which also recognizes the other metacaspases Atmc4 to Atmc6. Three bands could be detected: around 60 kDa, 36 kDa, and 31 kDa. The band around 60 kDa (for Atmc6: around 55kDa) probably represents the unprocessed zymogen form, which is also higher than the calculated molecular weight of the metacaspases (for Atmc4, 5 and 7 around 45 kDa, for Atmc6 around 40 kDa). The discrepancy between the theoretical molecular

weight and the observed molecular weight might be due to posttranslational modifications of the metacaspases *in vivo*. The presence of this band could be established in most overexpression lines of the wild type form, which indicates clear overexpression of the transgenic protein when compared to the wild type plants. However, in some overexpression lines of the wild type forms this full length band was not clearly or not at all detectable, as the full protein was **processed** into p20- and p10-like subunits. As such, in some overexpression lines an overproduction of the transgenic protein is observed by presence of the p20-like fragment, or even by an intermediate processed form. This intermediate form possibly represents a partially processed form of the full length. The p20-like subunit can be seen around 31 kDa, and the intermediate processed form around 36 kDa. However, in leaf tissue, detection of the p20-like fragment is masked by the presence of another (contaminating) protein which is also recognized by the antibody, however not present in root tissue. Conclusively, overexpression of type II metacaspases Atmc4-Atmc7 leads to autocatalytic processing and can hence be established by detection of one (or more) of these three bands, depending on the extent of processing. This finding is concomitant with bacterial expression of recombinant Atmc4-Atmc7, where autocatalytic processing is also observed upon its expression in *E. coli* (Vercammen et al., 2004). As known from bacterial expression of the recombinant proteins, the polyclonal antibody only recognizes the p20-like fragment and as such the p10-like fragment is not detectable. Despite clear overproduction of the wild type form, none of the tested mutant metacaspase lines (more lines were tested than depicted in Figure 4, data not shown) showed overexpression of the transgenic protein, hence an indication for a possible dominant negative effect leading to lethality.

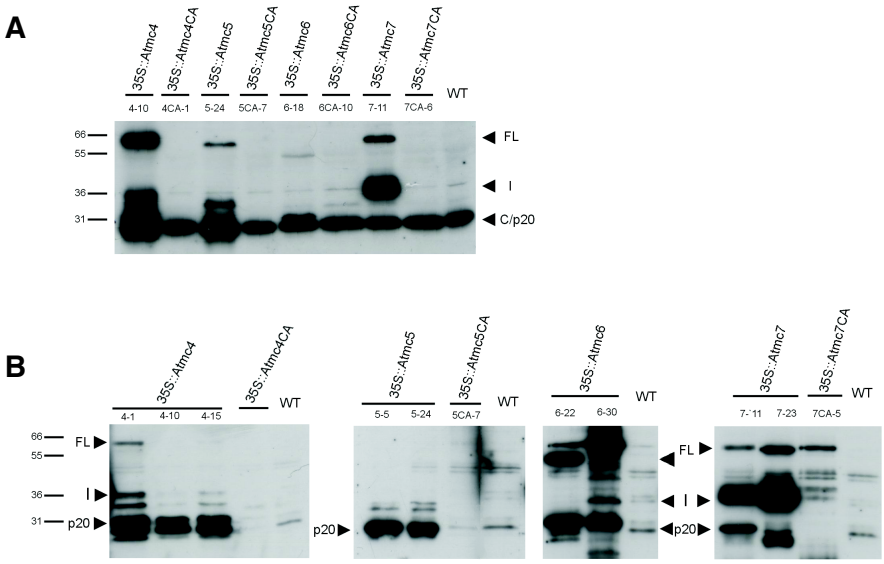

Figure 4: Overproduction of Atmc4, 5, 6 and 7 in leaves (A) and roots (B) of 35S::Atmc4-7 plants. Overproduction was shown by Western blot with antibody PS732-Atmc4.7. FL = full length (unprocessed zymogen), I = intermediate form, p20= p20-like subunit, C = contaminating band in leaf tissue

2.3.1.3.e Atmc9

Leaf and **root** material from the selected 35S::Atmc9 lines was subjected to Western analysis (Figure 5 (A) en (B)). Similar patterns were observed for root and leaf tissue, with the presence of a 36 kDa band, 23 kDa, and 16 kDa band. The 36 kDa band represents the unprocessed zymogen and the size matches exactly with the theoretical size of the proform. In accordance with bacterial overproduction of recombinant Atmc9 which leads to processing of the full length protein into p20- and p10-like subunits (Vercammen et al., 2004) the presence of the 23kDa band most probably represents the mature p20-like subunit. The smaller fragment (about 16kDa) can be representative of the small p10-like subunit, although the p10-subunit is not detectable by the antibody upon overproduction and subsequent processing of the full length protein in bacteria (Vercammen *et* al., 2004). Most probably, the smaller fragment

is a degradation fragment of the p20-subunit. Surprisingly, this autocatalytic processing could not only be observed upon overproduction of the wild type form, but also upon overproduction of mutant Atmc9 (lines 35S::Atmc9CA). This is in contrast with overproduction of the mutant form of metacaspase 9 in *E. coli* (Vercammen *et al.*, 2004), where this catalytic inactive form does not show autoprocessing upon its overproduction. A probable explanation for the observed processing in the mutant *Arabidopsis* lines is the action of endogenous metacaspase 9 (or other metacaspases / proteases) on the transgenic Atmc9CA. It is not inconceivable that the mutant metacaspase 9 can be cleaved, hence processed by endogenous metacaspase 9. Another explanation might be that the second conserved cysteine (Cys_{29}), which is positioned in spatial proximity to the catalytic cysteine (Cys_{147}) in the three-dimensional structure can replace the function of the catalytic one in case this one is blocked e.g. by replacement of an alanine, resulting in processing of the mutant protein.

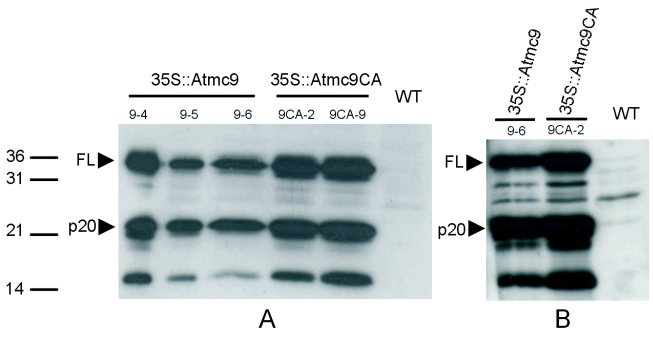

Figure 5: Overproduction of Atmc9 in leaves (A) and roots (B) of 35S::Atmc9 plants. Detection of *Arabidopsis* Atmc9 protein levels by western blot with antibody against Atmc9 (PS766-Atmc9).

Conclusively, these results suggest that type II metacaspases are able to process *in planta* upon overproduction, thereby separating a putative large (p20) and small (p10) subunit. This is similarly to the autocatalytic processing seen upon overproduction of recombinant type II metacaspases in *E. coli*, *N. benthamiana* and human 293T cells (Vercammen et al., 2004). These results are in accordance with the mechanism of

activation of mammalian executioner caspases. Zymogens of the executioner caspases are activated by limited proteolysis within their interdomain linker which is carried out by an initiator caspase or occasionally by other proteases under specific circumstances (Boatright and Salvesen, 2003). Overexpression of Type II metacaspases results in subsequent (auto)cleavage of these metacaspases, most probably due by forced high local concentrations of the proteases due to their overexpression *in planta*.

In conclusion, Type I and Type II metacaspases might use **different mechanisms of activation**, which might reflect in **functional divergence**, as related to mammalian initiator and executioner caspases. In addition, the fact that executioner caspases are activated by proteolysis by other proteases (mostly initiator caspases) while initiator caspases are activated by active dimerization is linked with the fact that initiator caspases exist as inactive monomers in the cytosol, while executioner caspases exist as inactive dimers in the cytosol (Boatright et al., 2003). Whether or not this is also the case for the metacaspases is currently unknown.

2.3.2. Metacaspase activity *in planta*

2.3.2.1. Metacaspase 4 activity in plant extracts overproducing Atmc4

As upon overexpression of the type II metacaspases and subsequent processing, the proteins are predominantly present in their processed form, we studied whether this processing was accompanied with metacaspase activity.

Biochemical studies defined the **optimal *in vitro* activity conditions** and substrate specificity for Atmc4 and Atmc9 (Vercammen et al., 2004). For Atmc4, the optimal buffer pH was 7.5-8 with the need of calcium in the buffer, while Atmc9 has an acidic pH optimum of pH5-5.5. Prominent activity of the recombinant Atmc4 towards the fluorogenic substrate GRR-AMC and FR-AMC, in the presence of calcium, was revealed (Vercammen et al., 2004). To further explore substrate specificities of Atmc4 and Atmc9 a tetrapeptide library was scanned and the tetrapeptide **Valine-Arginine-**

Proline-Arginine (VRPR) was identified as the optimal substrate for both Atmc4 and Atmc9 (Vercammen *et al.*, submitted).

To show metacaspase 4 activity *in planta* a fluorometric assay with Ac-GRR-aminomethylcoumarin [AMC] as a specific (suboptimal) substrate for Atmc4, was performed (by B. Belenghi). The GRRase activity in leaf extracts from plants overproducing Atmc4 was measured in the optimal Atmc4-activity conditions (pH7.5, addition of calcium). It can be remarked that in wild type plants there is a basal GRRase-activity, however an increased GRRase activity could be detected in the transgenic lines (Figure 6A). The increase in GRRase activity in the transgenic plants is well correlated with the transgene expression levels (data not shown). It can be assumed that the measured activity towards the Ac-GRR-AMC substrate is most probably attributed to the activity of metacaspase 4 due to the optimal Atmc4 activity conditions which are used in the experiment, however it cannot be excluded that there might be interference of other contaminating proteases which are also active towards this synthetic substrate under the used assay conditions.

Alternatively, an in-gel assay as described by (Solomon *et al.*, 1999) was performed on leaf material of the transgenic lines overproducing Atmc4 (by B. Belenghi). In this in-gel assay the proteins are first separated by native PAGE. The protein gel contains supplemental gelatin that is used as an artifical protease substrate. Active proteases, which retain their activity in the native gel, are able to digest the gelatin. Subsequent Coomassie Blue staining colours the undigested gelatine and hence protease activity is marked by white bands. Therefore, such an in-gel assay offers the advantage that the metacaspase 4 activity can be clearly separated from other contaminating proteases. In-gel assay on leaf extracts from 35S::Atmc4 lines shows an increased Atmc4 activity band when compared to extracts from wild type plants (Figure 6B). The polyclonal antibody raised against Atmc7, which also recognizes Atmc4, detects the activity band within a Western blot analysis which was performed in parallell, hereby consolidating the identity of this activity band as Atmc4 (data not shown).

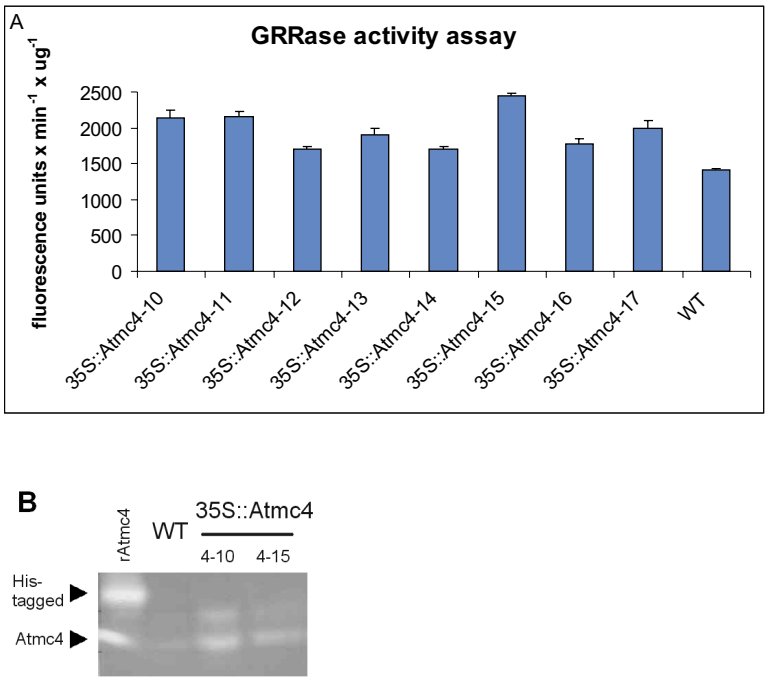

Figure 6: Metacaspase activity in transgenic plants overexpressing Atmc4 (experiments performed by Beatrice Belenghi). A) Fluorometric assay demonstrates proteolytic activity against Ac-GRR-AMC in the transgenic plants. Activity is expressed as the increase in arbitrary fluorescence units per minute per mg total protein content. B) In-gel assay demonstrates increased Atmc4 activity in the transgenic plants. The upper arrow indicates HIS tagged Atmc4 recombinant protein, the lower arrow Atmc4 activity.

2.3.2.2. Metacaspase 9 activity in plant extracts overproducing Atmc9

As described above, biochemical studies for substrate specificity and optimal assay conditions for metacaspase 9 activity revealed the tetrapeptide VRPR as the optimal synthetic substrate in an acidic environment of pH5.5 (Vercammen et al., submitted).

To show metacaspase 9 activity in plants, leaf extracts from 35S::Atmc9 and 35S::Atmc9CA plants were tested for VRPRase (optimal substrate) activity by a fluorometric assay with Ac-VRPR-AMC (performed by B. Belenghi). A clear increased activity in leaves of plants overproducing metacaspase 9 could be measured, when compared to wild type plants (Figure 7A). In addition, transgenic plants overproducing mutant metacaspase 9 also show clear increased activity towards the wild type plants.

Alternatively, an in-gel assay was performed as described for Atmc4 (performed by B. Belenghi). However, in contrast to the in-gel assay for Atmc4, after electrophoresis the gel is incubated at room temperature in an acidic environment of pH5.5. Protein extracts from both 35S::Atmc9 and 35S::Atmc9CA plants were tested. Figure 7B shows an increased Atmc9 activity band in protein extracts from 35S::Atmc9-5 and 35S::Atmc9-6 plants (Belenghi *et al.*, manuscript in preparation). Polyclonal antibodies against Atmc9 recognized this activity band within a Western blot analysis which was performed in parallell, hereby consolidating the identity of this activity band as Atmc9 (data not shown). In addition, also in extracts from 35S::Atmc9CA-2 and 35S::Atmc9CA-4, an Atmc9 activity band was detected with similar intensity, which shows that the mutant protein is active at similar levels. This finding is in accordance with the western analysis on these 35S::Atmc9CA lines which already demonstrated processing of Atmc9CA into its p20- and p10-like subunits (see Figure 5). This suggests that processing is closely linked to activity. It also indicates that once the protein is processed, the catalytic cysteine is probably not necessary for its activity. Indeed, it has already been shown that processing of Atmc9 is a prerequisite for its activity by Vercammen et al. (2004). Therefore, a mutant form of Atmc9 was created in which the P1 arginine at

the autoprocessing site was replaced by an alanine (designated R/A mutant). This R/A mutant did not show autoprocessing and did not display any activity towards a fluorogenic arginine substrate (GRR-amc) in a fluorometric assay, while the activity of the wild type protein increased gradually corresponding to the complete autoprocessing of the proform (Vercammen *et al.*, 2004). Consequently, processing of metacaspase 9, as seen in plant extracts overexpressing the wild type or mutant form of metacaspase 9, is indeed reflected in its activity.

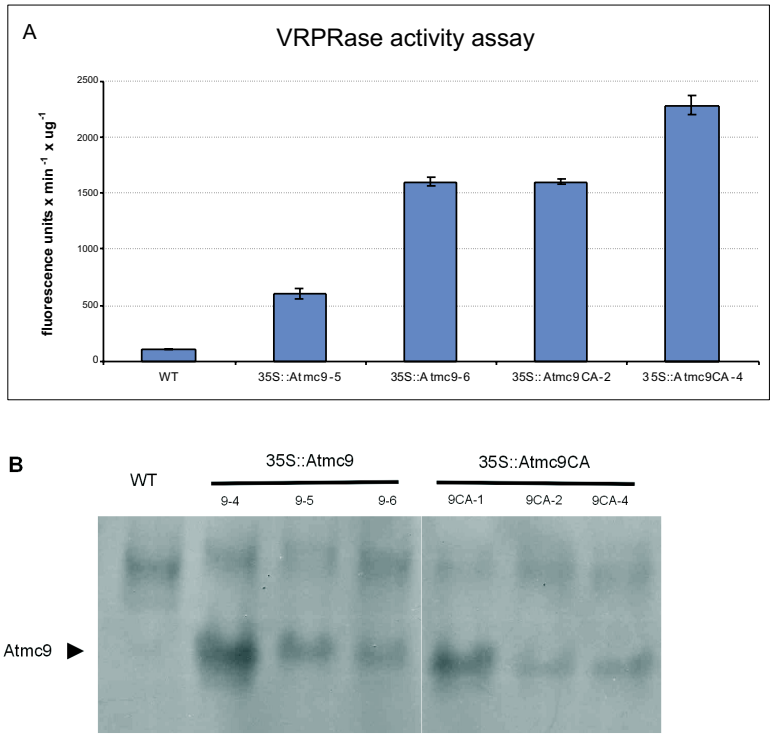

Figure 7: Metacaspase activity in transgenic plants overexpressing Atmc9 (experiments performed by Beatrice Belenghi). A) Fluorometric assay demonstrates proteolytic activity against Ac-VRPR-AMC in the transgenic plants. Activity is expressed as the increase in arbitrary fluorescence units per minute per mg total protein content. B) In-gel assay demonstrates increased Atmc9 activity in the transgenic plants, indicated by the arrow (picture was taken after conversion of the white bands to gray bands).

Despite the fact that processing is **required** for activity, processing alone is **not sufficient** to result in metacaspase activity, as there are some additional factors which influence its activity. One important factor is the pH. Interestingly, incubation of the gel at a **non-acidic pH** did not give rise to in-gel activity of Atmc9 and Atmc9CA (data not shown). Thus, activity of metcaspase 9 needs acidification of the environment. The requirement of an acidic pH has already been reported by Vercammen *et al* (2004) for the *in vitro* activity of Atmc9. This implies that Atmc9 is inactive under conditions corresponding to the physiological cytoplasmic pH. Mere overproduction and processing of the zymogen into the mature protease does not necessarily implicate protease activity but would then need additional acidification of its environment. Activation by acidification has also been observed for human caspase 3 (Roy et al., 1997). In this case, a so-called safety catch hinders both the autocatalytic maturation and the vulnerability to proteolytic activation by upstream proteases. In plants, several reports show that acidification of the cytosol is important in plant cell signaling. For instance, tracheary element formation can be regarded as a paradigm for vacuole-mediated cell death. Hereby, secondary wall thickening of differentiating tracheary elements is followed by fast vacuolar collapse, resulting in a cytosolic pH decrease due to tonoplast rupture. Interestingly, Minami and Fukuda reported previously on the induction of an acidic cysteine protease with activity toward FR-AMC substrate in Zinnia cells during differentiation into tracheary elements (Minami and Fukuda, 1995). Moreover, the activity could be blocked by TLCK, leupeptine and E-64 and less efficiently by PMSF and pepstatin, a profile somewhat reminiscent of the metacaspases (Vercammen et al., 2004).

2.4. Experimental procedures

Cloning of *Arabidopsis* metacaspases and production and cloning of C/A mutants: see Vercammen et al., 2004

Plant growth conditions

Plants were grown in Jiffy pots (Jiffy products international AS; Drøbak, Norway) in controlled environment chambers in a 16h/8h day/night regime at 21°C day/night temperature, under illumination of 100 mmol.$m^{-2}s^{-1}$ light and with a relative humidity of 60%. For *in vitro* growth, plants were grown on solid growth medium (Murashige and Skoog; Biomark Laboratories, Maharashtra, India) containing 2% of sucrose, at 21°C day/night temperature, relative humidity of 35%, a 16h/8h day/night regime and under illumination from 45-50 mmol.$m^{-2}s^{-1}$. Seeds were stratified for two to three days at 4°C before being transferred to 21°C.

Transformation, propagation and selection of *Arabidopsis thaliana* plants

The coding sequences of the metacaspases from pDONR201 were cloned into the Gateway-compatible vector pB7WG2, a plant expression vector (Karimi et al., 2002) which contains the plant selectable marker *Bar* (BASTA resistance) gene. In this vector the metacaspase is expressed under the transcriptional control of the cauliflower mosaic virus 35S promoter.

Plasmids were transformed in the *Agrobacterium tumefaciens* strain C58C1 Rif^R[pMP90] by electroporation. Four to five weeks old *Arabidopsis thaliana* plants were used for floral dip similar as described by Clough *et al.* (1998). Ecotype Colombia-0 (Col-0) was used for transformation.

Soil-grown seedlings were sprayed with 40 mg/l of BASTA (glufosinate ammonium, Riedel-de Haen; Hanover, Germany). Spraying starts 6 days after sowing and on days 9, 12, 14 and 16. T1 BASTA-resistant plants were grown and allowed to self-fertilize. T2 seeds were grown *in vitro*, on growth medium containing 2% of sucrose and 5 mg/l of BASTA, and segregation was monitored in order to determine the number of T-DNA insertion loci for every line.

Statistical determination of number of T-DNA insertion loci via segregation analysis

For a single locus line, the hypothesis of a segregation ratio $BASTA^r$:$BASTA^s$ = 3:1 is tested with the χ^2 test. Hereby, it is tested whether the observed segregation ratio correlates with the theoretical expected segregation ratio, based on Mendel's genetics. Whether or not the

hypothesis (observed segregation ratio=theoretical segregation ratio) is accepted depends on the outcome of the value of χ^2, which can be calculated in the following formula:

$\chi^2 = S_{i=1}^{m} (O-E)^2/E$.

m= number of classes (2, $BASTA^r$ and $BASTA^s$), i = 1,….., m

O = number of observed plants in class

E= theoretical expected number of plants in class

The calculated χ^2 value is retrieved from a table with probabilities, taking into account the degrees of freedom (m-1). For acceptance of the hypothesis the probability should be bigger than 0.05. With one degree of freedom χ^2 should then be smaller than 3.84.

RNA extraction and Northern blot analysis

RNA was extracted from grinded plant material in Trizol reagent (Invitrogen; Carlsbad, USA). The RNA mix is added in one and a half times the volume of the RNA. The RNA mix contains 200 ml DMSO, 4 ml 1M NaPi and 40 ml 40% glyoxal. Eight mg of the RNA is subsequently separated on a gel, containing 12.5% dH_3PO_4 and 50% triethanolamine. After transferring the RNA to nitrocellulose Hybond-N membrane (Amersham Pharmacia Biosciences, Buckinghamshire, England), the RNA is UV-crosslinked. The membrane is washed with ultrapure water and afterwards stained with 0.04% methylene blue (0.5M) and sodiumacetate (pH5.2) to check if the RNA is not degraded and if samples are equally loaded. After destaining with 0.2xSSC and 1% SDS, the membrane is washed with preheated 20mM Tris pH 8.0 at 65°C. Prehybridisation of the membrane with digoxigenin (DIG)-easy HYB (Roche, Mannheim, Germany) occurs for minimum 30°C at 50°C. Final hybridization with the denatured probe at 50°C takes place overnight. For each metacaspase the probe used in the Northern analysis was a DIG labeled probe, obtained via PCR with DIG-dUTPs on the coding sequence of Atmc3 (PCR DIG probe synthesis kit, Roche).

Protein extraction and Western blot analysis

Proteins were extracted from plant material using RIPA buffer (1% Nonidet P40, 0.5% sodium deoxycholaat, 0.1% SDS) supplemented with 10% glycerol. To 100 mg of grinded plant material approximately 300 ml of the extraction buffer is added. The homogenate was centrifuged at 14,000 rpm for 5 minutes at 4°C, this centrifugation step was repeated until the supernatans was clear. Protein concentration was determined using an adapted Lowry method (Biorad, Hercules, CA, USA) using a spectrophotometer (VERSAmax, Molecular Devices, Sunnyvale, USA). For Western blot analysis, thirty mg of proteins was separated on a 12% SDS-PAGE gel and electrophoretically transferred to PVDF membrane (Millipore Corporation, Bedford, USA) and blocked for 1h with 1% skimmilk in PBS containing 0.05% Tween 20 (PBST). First antibody

was added in PBST containing 1% skimmilk and incubated for 1 hour. After washing the membrane in PBST for one hour, the horseradish-peroxidase linked second antibody was added also for one hour. This is followed by washing in PBST for one hour. Detection was performed using the ECL detection kit according to the manufacturer's instructions (Amersham Pharmacia Biosciences, Buckinghamshire, England).

For Western blot on leaf material, three weeks-old *in vitro* grown plants were used. For Western blot on root material, roots were harvested from 10 days-old *in vitro* grown plants. Plants grown in the conditions as described above.

For the production of polyclonal antisera, three times 200 µg of the corresponding purified recombinant metacaspase were used as immunogen per rabbit (Eurogentec, Seraing, Belgium) (summarized in Table 2).

Antiserum against	Name antisera	dilution
Atmc1	PS729-Atmc1	1/5000
Atmc2	PS769-Atmc2	1/5000
*Atmc7	PS732-Atmc4.7	1//5000
Atmc9	PS766-Atmc9	1/10000

Table 2: Summary of polyclonal antisera (PS), raised against the recombinant metacaspases. *The antiserum raised against Atmc7 also recognizes the other metacaspases of the cluster (Atmc4-Atmc6).

Native in-gel assay (as described by Solomon et al., 1999)

Proteins were extracted from grinded leaf material using RIPA extraction buffer supplemented with 10% glycerol. Extraction was done as described for Western analysis**.** Twenty mg of proteins was loaded on a 12% native gel containing 0.1% gelatin and the gel was run at 4°C. After 2 washes with MES pH 5.5 (for Atmc4) or HEPES buffer (for Atmc9), the gel was incubated overnight at room temperature in Atmc9 buffer for Atmc9. Atmc9 buffer consisted of 100mM MES buffer pH5.5, 10mM dithiothreitol (DTT), 10% Sucrose, 1% CHAPS, 150mM NaCl. For Atmc4, the buffer consisted of 50mM HEPES (pH7.5), 150mM NaCl, 10% (v/v) glycerol, 100mM $CaCl_2$, 10mM DTT. After incubation the gel was stained in 0.2% Coomassie Brilliant Blue R250 and destained in destaining solution. Active proteases appear as unstained bands on a dark background.

Metacaspase activity assay with Ac-GRR/VRPR-AMC

Plant extracts were made as described above and preincubated for 15 minutes in the appropriate assay buffer (also described above for the in-gel assay), activities were normalized to the protein content. Substrate concentrations varied from 10-100mM. Synthetic substrates Ac-GRR-AMC and Ac-VRPR-AMC were synthesized by Peptides International (Louisville, KY, USA). Time-dependent release of free amido-4-methylcoumarin (AMC) was measured at excitation wavelength of 380nm and emission wavelength of 460 nm, using a FLUOstar OPTIMA plate reader (BMG Labtechnologies, Offenburg, Germany.

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Annex I: Summary of segregation and expression data of metacaspase overproducing lines.

	Segregation data					Transgene expression	
	seedlings			χ^2	Single		
Name lines	BASTA^s	BASTA^r	NG	values	locus?	NB	WB
Atmc1							
35S::Atmc1-4	1	43	6	12,12	-	Hi	Hi
35S::Atmc1-5	5	45	1	6	-	Hi	Hi
35S::Atmc1-6	**10**	**36**	**4**	**0,26**	**+**	**Mo**	**Mo**
35S::Atmc1-7	0	50	0	16,67	-	Lo	/
35S::Atmc1-8	3	46	1	9,31	-	Hi	/
35S::Atmc1-9	8	40	2	1,78	+	/	/
35S::Atmc1-10	15	33	2	1	+	/	Lo
35S::Atmc1-11	**10**	**40**	**0**	**0,67**	**+**	**/**	**Mo**
35S::Atmc1-12	**13**	**34**	**3**	**0,18**	**+**	**/**	**Hi**
35S::Atmc1-14	50	0	0	150	-	/	/
35S::Atmc1-15	18	32	0	3,23	+	/	Lo
35S::Atmc1-16	0	49	1	16,33	-	/	Hi
35S::Atmc1-17	1	43	6	12,12	-	/	Hi
35S::Atmc1-18	**10**	**39**	**1**	**0,55**	**+**	**/**	**Hi**
35S::Atmc1-19	16	34	0	1,31	+	/	/
35S::Atmc1CA-1	/	/	/	/	/	/	Lo
35S::Atmc1CA-2	/	/	/	/	/	/	Lo
35S::Atmc1CA-3	/	/	/	/	/	/	Lo
35S::Atmc1CA-4	/	/	/	/	/	/	Hi
35S::Atmc1CA-5	6	43	1	4,25	-	/	Hi
35S::Atmc1CA-6	/	/	/	/	/	/	Lo
35S::Atmc1CA-7	/	/	/	/	/	/	Lo
35S::Atmc1CA-8	/	/	/	/	/	/	Lo
35S::Atmc1CA-9	/	/	/	/	/	/	Lo
35S::Atmc1CA-10	/	/	/	/	/	/	Lo
35S::Atmc1CA-11	/	/	/	/	/	/	Lo
35S::Atmc1CA-12	6	43	1	4,25	-	/	Hi
35S::Atmc1CA-13	/	/	/	/	/	/	Lo
35S::Atmc1CA-14	/	/	/	/	/	/	Lo
35S::Atmc1CA-15	/	/	/	/	/	/	Lo
35S::Atmc1CA-16	/	/	/	/	/	/	Lo
35S::Atmc1CA-17	0	50	0	16,67	-	/	Hi
35S::Atmc1CA-18	12	38	0	0,03	+	/	Hi
35S::Atmc1CA-19	15	35	0	0,67	+	/	Hi
35S::Atmc1CA-20	/	/	/	/	/	/	Lo
35S::Atmc1CA-21	/	/	/	/	/	/	Lo
35S::Atmc1CA-22	/	/	/	/	/	/	Lo
35S::Atmc1CA-23	/	/	/	/	/	/	Lo
35S::Atmc1CA-24	/	/	/	/	/	/	Lo
35S::Atmc1CA-25	12	38	0	0,03	+	/	Hi
35S::Atmc1CA-26	/	/	/	/	/	/	Lo
35S::Atmc1CA-27	/	/	/	/	/	/	Lo
35S::Atmc1CA-28	5	45	0	6	-	/	Hi
35S::Atmc1CA-29	/	/	/	/	/	/	Lo
35S::Atmc1CA-30	/	/	/	/	/	/	Hi
35S::Atmc1CA-31	/	/	/	/	/	/	Lo
35S::Atmc1CA-32	/	/	/	/	/	/	Lo
35S::Atmc1CA-33	/	/	/	/	/	/	Lo
35S::Atmc1CA-34	11	38	1	0,17	+	/	Mo
35S::Atmc1CA-35	11	37	2	0,11	+	/	Mo
35S::Atmc1CA-36	11	38	1	0,17	+	/	Mo
35S::Atmc1CA-37	/	/	/	/	/	/	Lo
35S::Atmc1CA-38	0	49	1	16,33	-	/	Hi
35S::Atmc1CA-39	1	47	2	13,44	-	/	Hi
35S::Atmc1CA-40	9	34	7	0,38	+	/	Hi
35S::Atmc1CA-41	9	39	2	1	+	/	Hi
35S::Atmc1CA-42	5	40	5	4,63	-	/	Hi
35S::Atmc1CA-43	/	/	/	/	/	/	Lo

Atmc2							
35S::Atmc2-5	1	47	2	13,44	-	Mo	/
.35S::Atmc2-6	1	46	3	13,11	-	Mo/Lo	/
35S::Atmc2-7	**16**	**30**	**4**	**2,35**	**+**	**Mo**	**Mo**
35S::Atmc2-8	13	36	1	0,06	+	Lo	Lo
35S::Atmc2-9	**8**	**42**	**0**	**2,16**	**+**	**Hi**	**Hi**
35S::Atmc2-10	2	48	0	11,76	-	Mo/Lo	/
35S::Atmc2-11	2	43	5	10,14	-	Hi	/
35S::Atmc2-12	**14**	**34**	**2**	**0,44**	**+**	**Hi**	**Hi**
35S::Atmc2-13	0	47	3	15,67	-	Hi	/
35S::Atmc2-14	12	34	4	0,03	+	Lo	Lo
35S::Atmc2-15	7	31	12	0,88	+	Lo	Lo
35S::Atmc2-16	2	41	7	9,5	-	Lo	/
Atmc3							
35S::Atmc3-1	**13**	**35**	**2**	**0,11**	**+**	**Hi**	**/**
35S::Atmc3-2	7	37	6	1,94	+	Mo	/
35S::Atmc3-3	5	45	0	6	-	Mo	/
35S::Atmc3-4	/	/	/	/	/	Mo	/
35S::Atmc3-5	/	/	/	/	/	Mo	/
35S::Atmc3-6	/	/	/	/	/	Hi	/
35S::Atmc3-7	/	/	/	/	/	Hi	/
35S::Atmc3-8	/	/	/	/	/	Mo	/
35S::Atmc3-9	/	/	/	/	/	Mo	/
35S::Atmc3-10	/	/	/	/	/	Hi	/
35S::Atmc3-11	/	/	/	/	/	Hi	/
35S::Atmc3-12	/	/	/	/	/	Hi	/
35S::Atmc3-13	/	/	/	/	/	Hi	/
35S::Atmc3CA-1	/	/	/	/	/	Lo	/
35S::Atmc3CA-2	/	/	/	/	/	Lo	/
35S::Atmc3CA-3	/	/	/	/	/	Lo	/
35S::Atmc3CA-4	/	/	/	/	/	Lo	/
35S::Atmc3CA-5	/	/	/	/	/	Lo	/
35S::Atmc3CA-6	/	/	/	/	/	Lo	/
35S::Atmc3CA-7	/	/	/	/	/	Lo	/
35S::Atmc3CA-8	/	/	/	/	/	Lo	/
35S::Atmc3CA-9	/	/	/	/	/	Lo	/
35S::Atmc3CA-10	/	/	/	/	/	Lo	/
Atmc4							
35S::Atmc4-1	**7**	**34**	**9**	**1,37**	**+**	**Hi**	**Hi**
35S::Atmc4-2	12	38	0	0,03	+	Mo	Lo
35S::Atmc4-3	6	44	0	4,51	-	Lo	/
35S::Atmc4-4	6	39	5	3,27	+	Hi	/
35S::Atmc4-5	10	36	4	0,26	+	Hi	/
35S::Atmc4-6	12	35	3	0,01	+	Mo	Mo
35S::Atmc4-7	6	42	2	4	-	Hi	/
35S::Atmc4-8	12	37	1	0,01	+	Hi	/
35S::Atmc4-9	0	44	6	14,67	-	Lo	Lo
35S::Atmc4-10	**14**	**35**	**1**	**0,33**	**+**	**/**	**Hi**
35S::Atmc4-11	1	42	7	11,79	-	/	Hi
35S::Atmc4-12	3	40	7	7,45	-	/	Mo
35S::Atmc4-13	2	41	7	9,5	-	/	Mo
35S::Atmc4-14	8	31	11	0,42	+	/	Mo
35S::Atmc4-15	**9**	**32**	**9**	**0,2**	**+**	**/**	**Hi**
35S::Atmc4-16	10	31	9	0,01	+	/	Lo
35S::Atmc4-17	2	41	7	9,17	-	/	Mo

Atmc5							
35S::Atmc5-1	8	40	2	0,94	+	Mo	/
35S::Atmc5-2	0	49	1	16,33	-	Hi	/
35S::Atmc5-3	/	/	/	/	/	Hi	/
35S::Atmc5-4	1	49	0	14,11	-	/	/
35S::Atmc5-5	**11**	**39**	**0**	**0,24**	**+**	**Hi**	**Hi**
35S::Atmc5-6	/	/	/	/	/	/	/
35S::Atmc5-7	8	36	6	1,09	+	Hi	/
35S::Atmc5-8	8	37	5	1,25	+	Hi	/
35S::Atmc5-9	11	34	5	0,01	+	Hi	/
35S::Atmc5-10	**13**	**37**	**0**	**0,03**	**+**	**Mo**	**Mo**
35S::Atmc5-11	/	/	/	/	/	/	/
35S::Atmc5-12	9	34	7	0,38	+	Mo	/
35S::Atmc5-13	4	41	5	6,23	-	Hi	/
35S::Atmc5-14	3	38	9	6,84	-	Hi	/
35S::Atmc5-15	7	43	0	2,56	+	Hi	/
35S::Atmc5-16	6	44	0	4,51	-	Mo	/
35S::Atmc5-17	13	37	0	0,03	+	Mo	/
35S::Atmc5-18	4	45	1	7,41	-	Hi	/
35S::Atmc5-19	11	30	9	0,07	+	Mo	/
35S::Atmc5-20	6	33	11	1,92	+	Lo	/
35S::Atmc5-21	1	49	0	14,11	-	Mo	/
35S::Atmc5-22	50	0	0	150	-	/	/
35S::Atmc5-23	/	/	/	/	/	Lo	/
35S::Atmc5-24	**12**	**30**	**8**	**0,29**	**+**	**/**	**Hi**
35S::Atmc5-25	10	28	12	0,04	+	/	Lo
35S::Atmc5-26	0	50	0	16,67	-	/	Mo
35S::Atmc5-27	9	33	8	0,29	+	/	Mo
35S::Atmc5-28	13	29	8	0,79	+	/	Hi
35S::Atmc5-29	14	31	5	0,9	+	/	Hi
35S::Atmc5-31	3	36	11	6,23	-	/	Lo
35S::Atmc5-32	0	44	6	14,67	-	/	Mo
35S::Atmc5-33	2	48	0	11,76	-	/	Mo
Atmc6							
35S::Atmc6-10	8	42	0	2,16	+	Mo	Mo
35S::Atmc6-11	12	34	4	0,03	+	Mo	Lo
35S::Atmc6-12	/	/	/	/	/	/	/
35S::Atmc6-13	9	39	2	1	+	Hi	/
35S::Atmc6-14	**13**	**29**	**8**	**0,79**	**+**	**Hi**	**/**
35S::Atmc6-15	1	49	0	14,11	-	Hi	/
35S::Atmc6-16	6	40	4	3,51	+	/	/
35S::Atmc6-17	25	20	5	22,41	-	/	/
35S::Atmc6-18	**14**	**31**	**5**	**0,9**	**+**	**Hi**	**Mo**
35S::Atmc6-19	8	37	5	1,25	+	Hi	/
35S::Atmc6-20	50	0	0	150	-	/	/
35S::Atmc6-21	13	24	13	2,03	+	/	Hi
35S::Atmc6-22	13	34	3	0,18	+	/	Hi
35S::Atmc6-23	0	50	0	16,67	-	/	Lo
35S::Atmc6-24	5	21	24	0,46	+	/	Mo
35S::Atmc6-25	2	31	17	6,31	-	/	Mo
35S::Atmc6-26	5	39	6	4,36	-	/	Mo
35S::Atmc6-27	9	28	13	0,01	+	/	Hi
35S::Atmc6-28	0	50	0	16,67	-	/	Lo
35S::Atmc6-29	1	41	8	11,46	-	/	Lo
35S::Atmc6-30	**9**	**29**	**12**	**0,04**	**+**	**/**	**Hi**

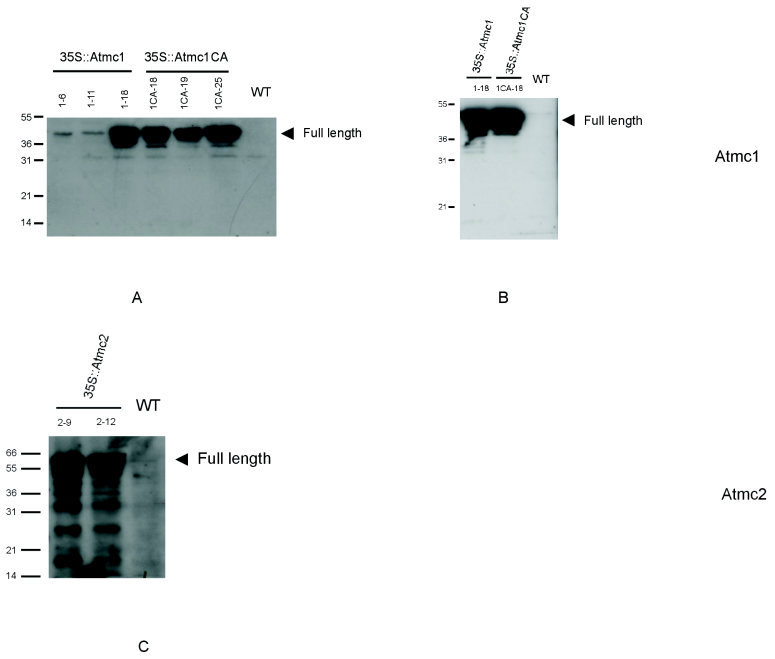
Atmc7							
35S::Atmc7-10	1	44	5	12,45	-	Hi	/
35S::Atmc7-11	**8**	**36**	**6**	**1,09**	**+**	**Mo**	**Hi**
35S::Atmc7-12	1	40	9	11,13	-	Hi/Mo	/
35S::Atmc7-13	1	46	3	13,11	-	Hi	/
35S::Atmc7-14	1	45	4	12,78	-	Hi	/
35S::Atmc7-15	1	46	3	13,11	-	Hi	/
35S::Atmc7-16	5	38	7	4,1	-	Hi	/
35S::Atmc7-17	6	41	3	3,75	+	Hi	/
35S::Atmc7-18	11	33	6	0	+	Mo	Mo
35S::Atmc7-19	/	/	/	/	/	Hi	/
35S::Atmc7-21	9	36	5	0,6	+	/	Mo
35S::Atmc7-22	6	40	4	3,51	+	/	Mo
35S::Atmc7-23	**10**	**26**	**14**	**0,15**	**+**	**/**	**Hi**
35S::Atmc7-24	1	43	6	12,12	-	/	Lo
35S::Atmc7-25	7	31	12	0,88	+	/	Lo
35S::Atmc7-26	12	29	9	0,4	+	/	/
Atmc9							
35S::Atmc9-4	**4**	**46**	**0**	**7,71**	**-**	**Mo**	**Hi**
35S::Atmc9-5	**12**	**29**	**9**	**0,4**	**+**	**/**	**Hi**
35S::Atmc9-6	**15**	**28**	**7**	**2,24**	**+**	**/**	**Hi**
35S::Atmc9-8	50	0	0	150	-	/	/
35S::Atmc9-9	50	0	0	150	-	/	/
35S::Atmc9-10	50	0	0	150	-	/	/
35S::Atmc9-11	3	41	6	7,76	-	/	Hi
35S::Atmc9CA-1	3	47	0	9,63	-	/	Hi
35S::Atmc9CA-2	**14**	**32**	**4**	**0,72**	**+**	**/**	**Hi**
35S::Atmc9CA-3	/	/	/	/	/	/	Lo
35S::Atmc9CA-4	13	23	/	/	/	/	Hi
35S::Atmc9CA-5	/	/	/	/	/	/	Lo
35S::Atmc9CA-6	/	/	/	/	/	/	Lo
35S::Atmc9CA-7	/	/	/	/	/	/	Lo
35S::Atmc9CA-8	/	/	/	/	/	/	Lo
35S::Atmc9CA-9	**15**	**35**	**0**	**0,67**	**+**	**/**	**Hi**
35S::Atmc9CA-10	/	/	/	/	/	/	Lo

Lines indicated in **bold** are transgenic lines which were upscaled to homozygous lines and / or used in further experiments.

Segregation data: Segregation of BASTA resistance (BASTA^r) trait in 35S::Atmc seedlings leads to identification of single T-DNA insertion locus lines. For each line 50 seedlings were tested and the number of seedlings showing either BASTA^r:BASTA^s trait is reported. The number of seedlings that did not germinate (NG) is also indicated in the table. The hypothesis of a segregation ratio BASTA^r:BASTA^s = 3:1 has been accepted for all these T2 lines which have a c^2 value less then 3.84 with 0.05 significance level and one degree of freedom. Single locus lines are indicated as positive (+).

Expression data: Transgene metacaspase expression levels was determined by performing Northern blot (column NB) and/or Western blot (column WB). Expression levels are indicated as low (Lo), moderate (Mo) and high (Hi). If the segregation or expression analysis was not done, this is indicated by /. If any analysis was done, the lines are not withdrawn in the table.

Annex II: Overproduction of Atmc1 in leaves (A) and roots (B) of 35S::Atmc1 and 35S::Atmc1CA transgenic lines, and overproduction of Atmc2 in leaves of 35S::Atmc2 lines (C).



For Atmc1, only the full length is visible on the western blot. For Atmc2, besides the full length protein there are some additional lower molecular weight fragments which are (most probably) not derived from (auto)processing of the metacaspase. Indeed, the number and similar intensity of the bands ("laddering" pattern) indicates aspecific degradation of the full length metacaspase, counterargumenting specific processing. In addition, most of the bands are also detected in the wild type line.

Chapter 3: Overproduction of metacaspases in *Arabidopsis thaliana* revealed minor phenotypical changes and no altered response towards a variety of stress stimuli

3.1 Abstract

Arabidopsis thaliana plants overproducing type I or type II metacaspases did not show an obvious phenotype under normal conditions. Despite the modest changes in root morphology for Atmc4, plant morphology of the transgenic plants did not change significantly, and no enhanced cell death and no obvious effect on DNA ploidy levels could be observed. In addition, upon application of both biotic or abiotic stress stimuli there was no clear enhanced or decreased sensitivity in the overexpression plants when compared to wild type plants. However, these findings do not necessarily mean that metacaspases are not involved in PCD, as there are quite some additional factors which have to be taken into account.

The ability of metacaspases to perform their function is likely to be dependent on their activity, which is not only associated with (auto)processing. Processing is required (at least for the Type II metacaspases) (Vercammen et al., 2004), however not sufficient, to give rise to an active enzyme, as there are additional levels which probably regulate the activity of the metacaspases such as the pH, the presence of inhibitors or posttranslational modifications.

Therefore, under **normal conditions**, mere overexpression is possibly not enough to induce an effect. In accordance with that, in yeast and *Trypanosoma* an apoptotic stimulus was required to activate the metacaspases and lead to (accelerated) cell death in metacaspase-overexpressing cells ((Madeo et al., 2002); (Kosec et al., 2005)). However, transgenic *Arabidopsis* plants with higher metacaspase levels did not show a decreased or increased sensitivity under any of the tested **stress conditions**. This might be due to a limiting number of substrates or the fact that the specific stress condition(s) which can result in a significant effect was not tested here.

3.2 Introduction

Previous genetic studies demonstrated that overexpression of yeast metacaspase or *Arabidopsis* metacaspases in yeast did not immediately lead to cell death. However, upon addition of an intrinsic or extrinsic apoptotic stimulus, cell death was strongly facilitated ((Madeo et al., 2002); (Watanabe and Lam, 2005)). Similarly, overexpression of a metacaspase of *Trypanosoma cruzi* accelerated stress-induced cell death (Kosec et al., 2005). It can be concluded that mere overexpression (or ectopic expression) of the metacaspases in yeast or *Trypanosoma* was not sufficient **in itself** to provoke cell death. However, an apoptotic stimulus facilitated cell death significantly, suggesting that the metacaspases required a cell death-inducing stimulus to become active.

Concomitant with that, under normal conditions we would not expect a cell death phenotype in plants overexpressing *Arabidopsis* metacaspases. Indeed, the transgenic plants did not show obvious phenotypes at first sight, at normal conditions. Easy to screen plant development parameters like normal plant growth, flower, leaf, and silique development and flowering time were followed. No aberrant development in the transgenics could be observed. Subsequently, a more thorough analysis of the overexpressing plants was performed. Leaf and root morphology of the transgenic plants, and the effect of the overproduction on cell death and cell cycle was extensively studied. In addition, the overexpressing plants were subjected to various abiotic and biotic stress stimuli to look for an altered sensitivity towards any of the tested stresses in comparison to the wild type plants., Exploring expression profiles of the metacaspases in numerous *Arabidopsis thaliana* microarray datasets involving different experimental set ups could lead to a more focussed approach to set up new experiments in order to screen for morphological changes or altered sensitivity towards specific stress stimuli due to overproduction of the metacaspases. For example, if expression of your gene of interest is significantly higher in a certain plant organ, it can be interesting to focus on this particular organ as perturbation of this gene can provoke an altered morphology. The meta-analyzer tool of Genevestigator (https://

www.genevestigator.ethz.ch) was used to study the expression profiles relative to plant organs, developmental stages and stress responses. Genevestigator comprises 1938 publicly available arrays, 113 AG (8k) and 1825 ATH1 (25k) chips. The *Arabidopsis thaliana* database involves information of a wide range of experiments, including all Affymetrix Genechip experiments, Gene Chip data of NASC arrays, ArrayExpress, AtGenExpress and GEO (Zimmermann et al., 2004) (see Chapter 1 for details). Besides Genevestigator, the expression of the metacaspases was also studied in relevant published microarray datasets such as datasets involving root development (Birnbaum et al., 2003) and cell cycle (Menges et al., 2003). In addition to publicly available data sets, different “in house” microarray datasets within the Department involving leaf development and lateral root initiation were also used.

3.3 Results and discussion

3.3.1 Overproduction of metacaspases led to modest changes in root morphology for Atmc4, however did not lead to increased cell death or altered DNA ploidy levels

3.3.1.1 Elevated metacaspase expression levels did not alter leaf morphology, however mild changes in root morphology for 35S::Atmc4 plants were observed

Analysis of expression profiles of the metacaspases in a gene expression dataset involving **leaf development**, revealed significant differential gene expression during leaf development for Atmc3 and Atmc9 (Beemster et al., 2005). Kinematic analysis on leaf primordia showed that the epidermis proliferates until day 12. Subsequently, cells then expand until day 19 when leaves reach maturity. Flow cytometry revealed that endoreduplication occurs from the time cell division rates decline until the end of cell expansion. Microarray analyses on growing *Arabidopsis* leaves defined three stages associated with leaf development: cell division (day 9-day 12), cell expansion/ cell differentiation (day 12-day 19) paralleled with endoreduplication, and mature stage. The expression of the *Arabidopsis* metacaspases during leaf development is depicted in Figure 1. In differentiating leaves and mature leaves a two-fold upregulation of Atmc3 could be detected, whereas Atmc9 is seven-fold upregulated in growing leaves (cell division and cell expansion). Therefore, leaf morphology was extensively studied for plants overproducing Atmc3 and Atmc9. The leaf size, abaxial epidermal cell number and leaf primordia size from three week old plants were determined., First, leaf blade area was measured by image analysis (De Veylder et al., 2001). Subsequently, the average cell area and number of abaxial epidermal cells was determined from drawing-tube images. From these data, the average cell size was calculated and the total number of cells per leaf was estimated as the ratio of leaf blade area and cell area (Table 1). We could conclude that plants overproducing the Atmc3 or Atmc9 gene did not differ significantly in their leaf morphology from wild type plants under these conditions (as tested by the Student T-test).

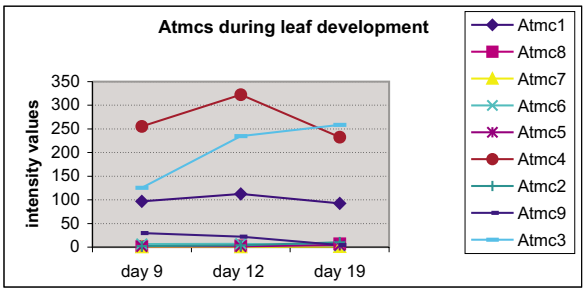


Figure 1: Expression profiles of the metacaspases during leaf development (from (Beemster et al., 2005)). Of all metacaspases, only Atmc1, Atmc3, Atmc4 and Atmc9 have present calls (detectable expression). Atmc9 goes from a present to an absent call. The fold change of the intensity values was only significant for Atmc3 and Atmc9.

		Abaxial Epidermal Cells		Abaxial Stomatal Cells	
Line	Leaf size (mm2)	Cell Size (mm2)	Estimated number	Stomatal index	Number of stomata
Col-0	18.66 ± 3.35	1878 ± 75	9737 ± 1282	26.40 ± 0.89	3566 ± 607
35S::Atmc3	15.07 ± 1.31	1763 ± 153	8786 ± 1020	23.88 ± 1.45	2874 ± 553
35S::Atmc9	19.98 ± 1.93	2122 ± 198	9798 ±1711	28.28 ± 1.40	3917 ± 845

Table 1: Abaxial epidermis cell size and cell number, and stomatal index in first leaves of Atmc3 and Atmc9 overproducing plants. All measurements were performed on 3-week old first leaves. The indicated values are means ± standard error (SE) (n=5). Student T-test (two-tailed, assuming uneaqual variances) revealed no significant differences between wild type plants and transgenic plants in any of the parameters (leaf size, cell size, number of stomatal cells and epidermis cells).

From *in silico* data it was known that Type II metacaspases are generally strongly expressed in **roots** (see Chapter 1) (Genevestigator; (Birnbaum et al., 2003)). The expression of Atmc4 appeared to be the highest in roots and promoter-GUS fusions of Atmc4 demonstrated expression almost exclusively in the trichoblasts of the roots (D. Vercammen and T. Beunens, unpublished results). In addition, in-house datasets involving lateral root initiation, revealed that Atmc7 was significantly downregulated during lateral root formation (Figure 2) (De Smet I., personal communication). In this study, pericycle cells at the xylem pole were isolated, as it is known that asymmetric

cell divisions accompanied with lateral root initiation occur at the xylem pole. Lateral root initiation was induced via a lateral root inducible system using the auxin analogue NAA. Subsequently, those isolated xylem pole pericycle cells were subjected to genome-wide microarray analysis, at 0, 2 and 6h NAA, and hereby Atmc7 was found to be downregulated five-fold in the xylem pericycle poles during lateral root initiation (Figure 2) (De Smet I., personal communication). Therefore, root morphology of 35S::Atmc4 and 35S::Atmc7 lines was profoundly analyzed. Plants were grown on vertical plates for 12 days: root length was measured and the amount of lateral roots was counted. For plants overproducing Atmc7 there was no significant difference in root length or number of lateral roots (LR)/cm when compared to the wild type plants (determined by Student T-test) (Table 2). For the 35S::Atmc4 lines, the average root length of plants overproducing Atmc4 was significantly shorter than of wild type plants: 0.68 cm for 35S::Atmc4-10 plants and 1.097 cm for 35S::Atmc4-15 plants. In addition, there were significantly less lateral roots/cm for the overexpressing plants then for the wild type plants (Table 2). These data are also visualized in Figure 3 as a box plot. Remarkably, both transgenic lines show evenly high expression levels of the transgenic protein, although the difference in root length is more pronounced in line 4-15. On the other hand, for the number of LR/cm the difference is more pronounced in the other transgenic line 4-10. Besides the mean values it is also necessary to take into account the distribution of the data, more specific of the outliers, as shown in the box plot. Therefore, the data have to be interpreted with caution. Moreover, the biological relevance of a 11% (35S::Atmc4-15) or 7% (35S::Atmc4-10) shorter root length, and of a reduction of 17% (35S::Atmc4-10) or 6% (35S::Atmc4-15) in the number of lateral roots/cm should be considered. In addition to the biological relevance also the wide distribution of the data values should be taken into account, even tough the differences between wild type and transgenic plants appeared to be statistically significant.

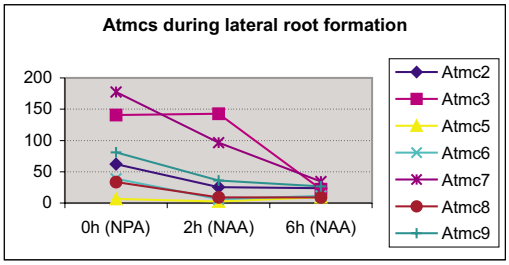


Figure 2: Expression profiles of the metacaspases (Atmc4 and Atmc1 are not included) during lateral root formation (from De Smet I.). Atmc7 was the only metacaspase which was significantly downregulated (five-fold) during lateral root formation.

35S::Atmc7

Line	root length (cm)	number of LR	LR/cm
Col-0	8.363 ± 0.183	36.8± 1.9	4.38 ± 0.9
35S::Atmc7-23	8.256 ± 0.124	35.3 ± 4.0	4.25 ± 0.17

The indicated values are means ± standard error (SE). n = 23 to

35S::Atmc4

Line	root length (cm)	number of LR	LR/cm
Col-0	9.814 ± 0.131	45.6± 1.3	4.64 ± 0.11
35S::Atmc4-10	9.134 ± 0.117	35.1 ± 0.9	3.83 ± 0.08
35S::Atmc4-15	8.717 ± 0.172	38.4 ± 1.4	4.36 ± 0.13

The indicated values are means ± standard error (SE). n = 36 to 41

Table 2: Root length and number of lateral roots of plants overproducing Atmc7 and Atmc4. For Atmc7, measurements were performed on 11 days-old roots, for Atmc4, on 12 days-old roots. LR/cm was calculated as the ratio of the number of lateral roots and total root length.

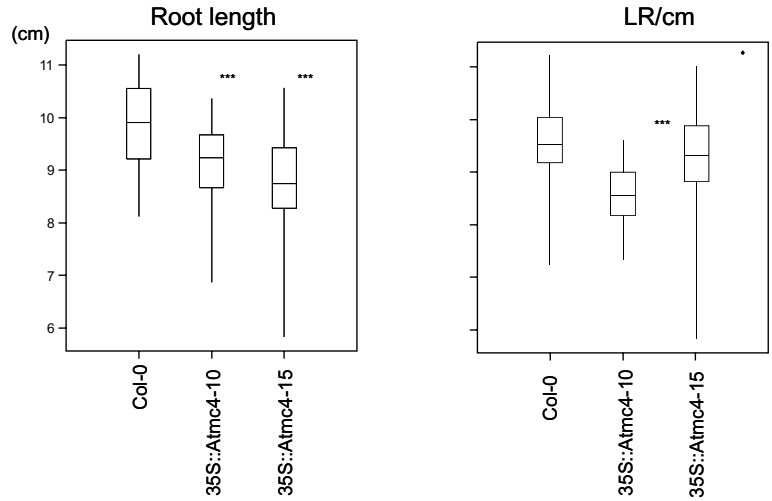


Figure 3: Box plot of root length and LR/cm of plants overexpressing Atmc4. The box plot summarizes the distribution of a set of data values. The center box comprises 50% of the data values, within the center box the median is indicated by a vertical line. The minimum and maximum values are connected with the box via a line, as such they are situated at the end of the line. Outliers are located below or above the center box. Significant differences between wild type plants and transgenic plants were determined by Student T-test; p values below 0.001 are considered as very significant and indicated with ***, p values above 0.05 are considered as not significant and indicated with ♦.

3.3.1.2 Elevated metacaspase levels did not enhance cell death or influence DNA ploidy levels

An implication of metacaspases in **programmed cell death** has been suggested, based on different studies with perturbed metacaspase expression levels in yeast, *Trypanosoma* and plants (Madeo et al., 2002); (Szallies et al., 2002); (Suarez et al., 2004). However, in those studies where metacaspases are overproduced, cell death did not occur at normal conditions and was only initiated upon application of intrinsic or extrinsic apoptotic stimuli (Madeo et al., 2002); (Watanabe and Lam, 2005); (Bettiga et al., 2004); (Silva et al., 2005); (Kosec et al., 2005). Transgenic *Arabidopsis* plants overproducing metacaspases also did not show elevated cell death at normal conditions, neither on a macroscopic level, nor on a cellular level, as established by a trypan blue assay (data not shown).

In addition, a putative role for the metacaspases in **cell cycle** has also been proposed, as yeast two-hybrid revealed an interaction between a metacaspase (Atmc3) and a cell cycle inhibitor (KRP2) (see Chapter 5). Therefore, it was interesting to study whether an effect on the cell cycle, in the transgenic plants could be detected. This was done by assessing the DNA ploidy levels in the transgenic lines. The DNA content was measured by flow cytometry on three weeks-old first leaves and cotyledons of wild type and transgenic lines. For leaves, at this stage of development, the leaf is mature: no mitotic divisions can be detected and ploidies have reached a steady-state level. In wild type leaves, the fraction of cells with a DNA content of 4C and 8C increases with leaf age at the cost of the 2C fraction because of endoreduplication, finally reaching a steady state level at the mature stage (De Veylder et al., 2001). For cotyledons, the mature state and thus steady state, is already reached at 10 days, and as more endoreduplication occurs in cotyledons when compared with first leaves, an extra peak can be observed in the cotyledon leaves with a 32C ploidy level (and in some cotyledons even 64C), which is not detected in first leaves. Figure 4 shows that there is no significant difference concerning the ploidy levels in first leaves of transgenic plants and wild type plants. In addition, overproduction of the metacaspases does not have a significant effect on the ploidy levels in cotyledon leaves when compared to the wild type leaves. It can be concluded that elevated metacaspase levels did not affect endoreduplication significantly, and hence did not alter the ploidy levels of first leaves and cotyledons.

In conclusion, despite the modest changes in root morphology for 35S::Atmc4 transgenic plants, overproduction of the metacaspases did not result in significant changes at normal conditions. Notwithstanding overexpression of the Type II metacaspases clearly resulted in **processing** of the metacaspases (see Chapter 2) and processing is a prerequisite for activity, overproduction of the metacaspases did not seem to have an effect at normal conditions. This is most probably due to the fact that processing is not sufficient for activity, and that there are additional levels which regulate the **activity** of the metacaspases.

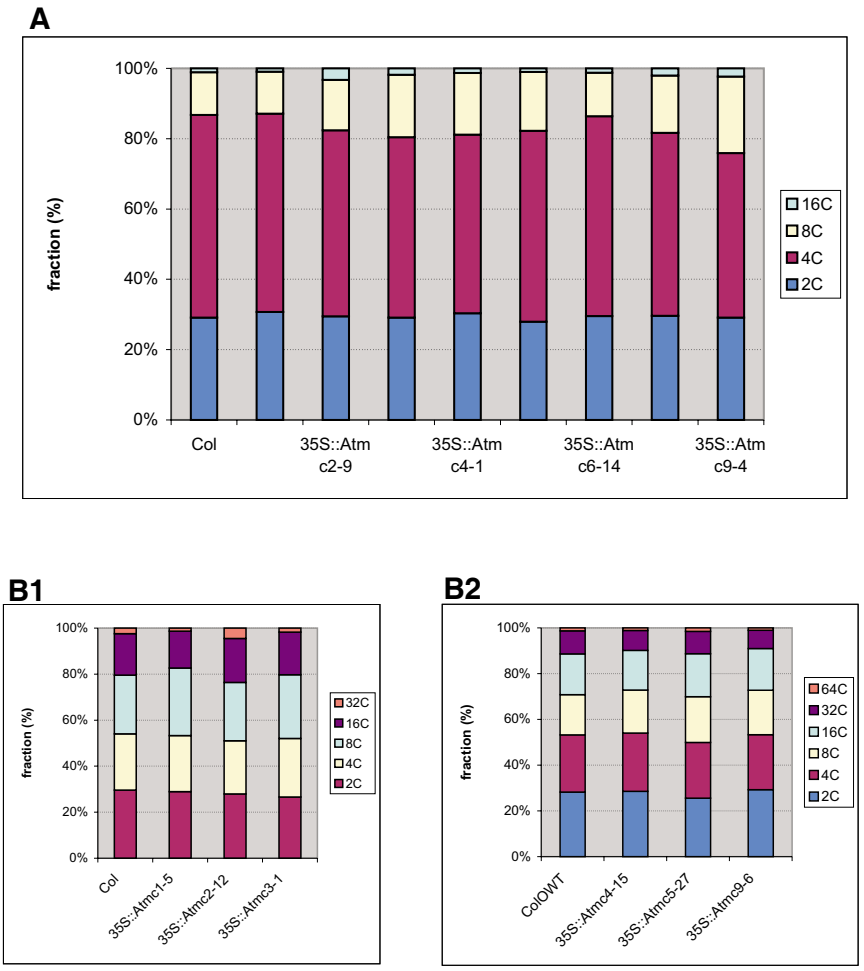


Figure 4: Ploidy level distribution of first leaves (A) and cotyledons (B; B1: cotyledons of type I MCs; B2: cotyledons of type I MCs) of 3 weeks-old wild type and plants overproducing metacaspases as measured by flow cytometry. For the cotyledons of Type II metacaspases, the average of 3 independent measurements is depicted, for cotyledons of Type I metacaspases and first leaves the data of one representative line.

Firstly, it has already been demonstrated that **pH** is an important regulating factor as for example Atmc9 needs an acidic pH to be activated and Atmc4 a neutral pH (Vercammen et al., 2004). Indeed, native in-gel assay of 35S::Atmc9 transgenic plants only resulted in activity of Atmc9 upon incubation at acidic pH (see Chapter 2), which demonstrates that processing does not equal activity. To date, for the Type I metacaspases it remains elusive whether processing is required as there are indications that active dimerization might be necessary for activation (also see Chapter 2). Mere overproduction of Type I metacaspases probably does not lead to active dimerization, therefore, an apoptotic stimulus might be required. Hence, at normal conditions no active Type I metacaspases would occur in the cell upon overproduction. Secondly, there might be **inhibitors** of the proform / processed form which prevent the proteins to be(come) active. This is also seen in case of mammalian caspases where the inhibitor of apoptosis (IAP) proteins bind and inhibit active caspase-3 and -7 (Deveraux et al., 1997; Roy et al., 1997) or can bind to procaspase-9 and inhbit its activation (Deveraux et al., 1998). It is possible that these inhibitors are only released from the metacaspases upon apoptotic stimuli, and as such hinder accidental activity of the proteases at normal conditions. Thirdly, **posttranslational modifications** might be necessary to activate or lead to an increased activity of the mature form. Or on the other hand, posttranslational modifications can also have an inhibitory effect on the enzyme activity. The latter was also seen with mammalian caspases, where nitrosylation (Li et al., 1997) and phosphorylation (Martins et al., 1998) of active caspaes inhibit caspase activity. Taken together, this indicates that mere overexpression of the metacaspases is not sufficient in itself to induce an effect under normal conditions.

3.3.2 Higher metacaspase levels do not affect sensitivity to various biotic and abiotic stress stimuli

Similarly to overexpression in yeast and *Trypanosoma*, mere overexpression of metacaspases in *Arabidopsis thaliana* did not seem to lead to an induction of apoptosis at normal conditions. In addition, no clear detectable phenotype could be observed in the transgenic plants due to overexpression of the metacaspases (except for the slight changes in root morphology for 35S::Atmc4 plants). However, in yeast and *Trypanosoma*, application of a stress stimulus was sufficient to lead to (accelerated) cell death ((Madeo et al., 2002); (Kosec et al., 2005)). Likewise, it would be interesting to test whether a similar effect would be triggered in transgenic *Arabidopsis* under certain stress conditions. Moreover, differential transcriptional regulation of plant metacaspases associated with biotic stress has also been reported. A tomato metacaspase gene was upregulated in plants infected with *Botrytis cinerea* (Hoeberichts et al., 2003), and in *Arabidopsis* all Type I metacaspases and two Type II metacaspases (Atmc4 and Atmc5) were upregulated 24 hours upon infiltration with bacterial pathogens (Watanabe and Lam, 2005). In addition, by using the Meta-analyzer tool of Genevestigator, it was found that all *Arabidopsis* metacaspases transcripts were differentially regulated under certain biotic and abiotic stress conditions (see Chapter 1). In general, besides the peach-potato aphid *Myzus persicae*-induced upregulation of all metacaspases, there is no obvious pattern of regulation shared amongst the different metacaspases upon the various stress stimuli. The respons of the metacaspases to the different stimuli is very diverse and in addition variable amongst the different metacaspases.

Therefore, various biotic as well as abiotic stress stimuli were applied to the transgenic plants and hereby it was checked if an altered sensitivity of the transgenics in comparison to the wild type plants could be observed under any of the tested stress conditions.

3.3.2.1 Transgenic plants do not respond differently to pathogen infections than wild type plants

Interestingly, Type I metacaspases carry an additional amino-terminal zinc-finger domain that is typical of plant proteins that function in the hypersensitive response (HR) pathway, such as LSD1 (Lesions simulating disease resistance-1), which is a negative regulator of HR. LSD1, a negative regulator of plant PCD, is required for correct interpretation of ROI (Reactive oxygen intermediates) or ROI-dependent signals emanating from an HR site. LOL1, LSD-One Like 1 protein, acts as a positive regulator of cell death and it is stated that LSD1 and LOL1 act antagonistically to control ROI-mediated cell death in plants. It is hypothesized that it is the balance of LSD1 and LOL1 that can regulate cell death commitment: an excess of LSD1 would antagonize the cell death machinery whereas LOL1 would activate it (Epple et al., 2003). It might be possible that the (Type I) metacaspases are also involved in this defense-related pathway.

To test biotic stress resistance in plants overexpressing metacaspases, plants were subjected to pathogen infections in collaboration with Prof. Dr. B. Cammue, University of Leuven (KUL),. Control and transgenic plants were infected with a necrotrophic fungus *Botrytis cinerea,* biotrophic virulent bacteria *Pseudomonas syringae* (DC3000) and the leaf spot necrotroph *Plectospharaella cucumerina*.

Infection of *Botrytis cinerea* was performed on transgenic lines overproducing Atmc1, Atmc2, Atmc3, Atmc5 (representative for the type II “cluster” metacaspases), and Atmc9. Leaves were sprayed with a solution containing *B. cinerea* spores with as result, damping-off from the leaves and severe rotting. Subsequently, the percentage of dead leaves was calculated for each line. No significant difference between the metacaspase overexpression lines and the control lines (Colomobia-0 (Col), and a control line containing an empty vector construct containing BASTA resistance (Pthw)) could be observed (Figure 5a). Infection with *Pseudomonas syringae* on 35S::Atmc1, 2, 3 and 5 plants occurred via a leaf dip in a *P. syringae* solution. Upon inoculation, leaves started to bleach, with gray spots. The percentage

of bleached leaves was calculated for each line. No conclusive differences in sensitivity towards *P. syringae* of the metacaspase overexpressor lines as compared to the control lines (Col, Pthw) (Figure 5b). *Plectosphaerella cucumerina* infection of 35S::Atmc1, 2, 3 and 5 plants resulted in appearance of a necrotic spot in the infected leaves. Average lesion size was determined, and no clear difference between metacaspase overproducing lines and control lines could be revealed (Figure 5c).

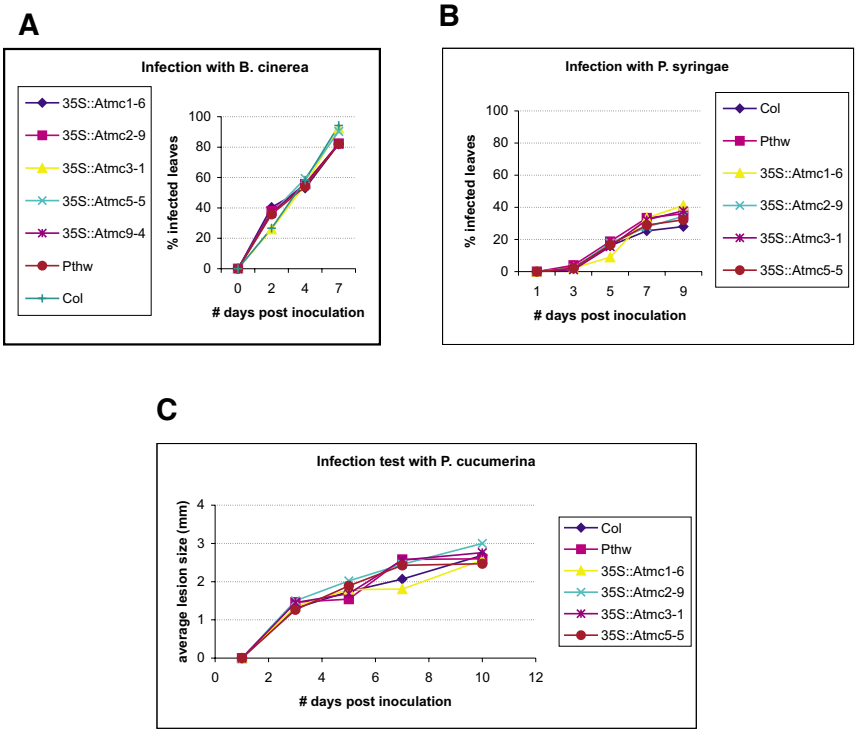


Figure 5: **Infection tests with transgenic lines using different pathogens (Centre of Microbial and Plant Genetics, University of Leuven, B. Cammue)**. A) Percentage of dead leaves upon inoculation with *B. cinerea*. Values are means of 5 independent experiments. Control lines used are wild type (Col) and control line containing empty vector construct Pthw, the latter being a control for BASTA resistance effects. B) Percentage of infected leaves upon inoculation with *P. syringae*. 150 to 180 leaves were inoculated per line. C) Average lesion size upon infection with *Plectosphaerella cucumerina*. 50 to 70 leaves were inoculated per line.

It is clear from Figure 5 that there are no significant differences in sensitivity towards any of the pathogens tested. In addition, a **Western analysis** was performed on the pathogen samples of *Botrytis cinerea* to verify whether the metacaspases were induced or repressed due to pathogen infection. Samples were taken before and 1 to 4 days after inoculation. Using a specific antibody against each metacaspase (except for Atmc4-7: use of antibody PS732-Atmc4.7), no induction or repression of the metacaspases upon *Botrytis* infection in any of the tested overexpressor lines (35S::Atmc1, 5 and 9), nor in the wild type line could be observed (data not shown). In addition, pathogen-induced samples from both *Botrytis* and *Pseudomonas* were tested for their **activity towards the fluorogenic substrates** GRR-AMC and FR-AMC. These P1-arginine specific substrates are (sub)optimal substrates for the Type II metacaspases, more specifically for Atmc4 and Atmc9 (Vercammen et al., 2004). The fluorometric assays were performed in the optimal activity conditions of Atmc4 (presence of calcium) and Atmc9 (acidic pH) (experiments performed by B. Belenghi). However, no activity could be detected, suggesting that metacaspases are not induced / activated upon Botrytis- or Pseudomonas-infection in the tested conditions. Taken together, these data indicate that metacaspase overexpressing lines do not show an increased or decreased sensitivity towards any of the tested biotic stresses. Whether or not this implies that metacaspases are not involved in biotic stress resistance, can not be concluded from mere overexpression studies (as discussed below). Therefore, it would also be interesting to see the effects of decreased metacaspase levels by using metacaspase knock-down or knock-out lines.

3.3.2.2 Transgenic plants do not show altered sensitivity to adverse conditions

To assess their performance during **salt stress**, wild type and transgenic seeds of all metacaspase-lines (35S::Atmc1-9) were germinated in the absence and presence of 100 mM NaCl. Both germination frequency and the number of first true leaf pairs (which is correlated to plant development) were monitored in 12 days-old seedlings. However, no statistically significant differences were observed between transgenic and wild type plants (data not shown).

Three weeks-old plants, both transgenic plants overproducing metacaspases (one line per metacaspase, Atmc1-Atmc9) and control plants, were exposed to a 24 hour treatment of **cold stress** (5°C, continuously) and **high light intensities** (long-day light regime). Immediately after the treatment or after an overnight recovery, no obvious differences between wild type and transgenic plants could be observed (data not shown).

Common to many adverse environmental conditions is the production of reactive oxygen species (ROS). ROS and more particularly hydrogen peroxide (H_2O_2) are produced during normal cell metabolism in plants. However, elevated ROS levels that cause **oxidative stress** are mostly associated with adverse conditions. Enhanced H_2O_2 levels have been reported during salt stress, temperature stress, UV-B irradiation and drought stress (Dat et al., 2000). Here, transgenic lines overproducing metacaspases (Atmc1-9, one line per metacaspase) were tested for their tolerance against H_2O_2 - induced oxidative stress. Therefore, leaf discs were taken from both control and transgenic plants. Subsequently, leaf discs were floated on water (control) and on a solution of 10 mM H_2O_2. Cellular injury in leaf discs exposed to exogenous H_2O_2 was assessed by measuring the conductance of the floating solution (Figuur 6). Conductance reflects the leakage of ion solutes out of the cell and hence gives an indication of membrane damage. Lipid peroxidation resulting from oxidative stress is likely to initiate this membrane deterioration. Solute conductance was measured 24h after floating on water or H_2O_2. An increase in conductance could be observed in discs floated on H_2O_2 when compared to discs floated on water, which is due to oxidative stress / cell death induced by the hydrogenperoxide. However, when compared to wild type no obvious increased or decreased sensitivity from the transgenic plants towards hydrogen peroxide could be detected (Figuur 6).

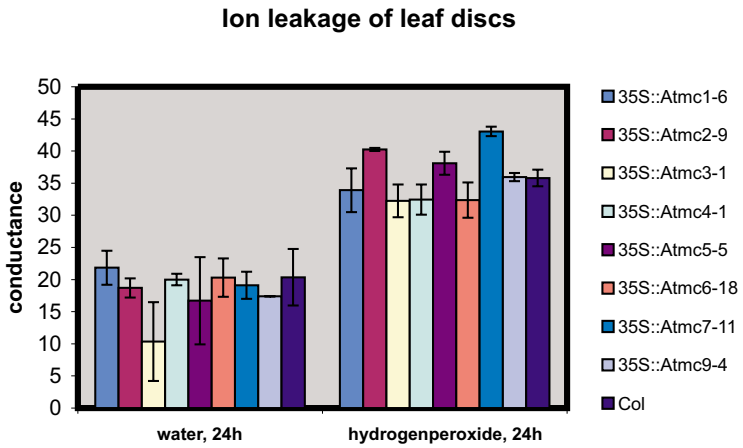


Figure 6: Ion leakage of leaf discs of wild type and transgenic plants overproducing metacaspases, floated for 24 hours on water or on H_2O_2. Ion leakage is measured as conductance of the medium. Conductance measurements represented in the figure are means of two independent experiments, standard errors are indicated as Y-error bars.

3.3.2.3 Transgenic plants overexpressing metacaspase 9 do not show changed timing of the onset of senescence

Senescence is the final stage of vegetative and reproductive development of the plant and is accompanied with a colour change in the leaves, first turning yellow and finally turning brown when they die. This visible transformation is associated with active metabolic changes that results in transfer of mobilisable nutrients, stored in the leaf during development, to other parts of the plant. The final stage of this process is leaf death, but this is delayed until all nutrients have been removed (Buchanan-Wollaston and Morris, 2000). Microarray data of programmed cell death in cell suspension cellsundergoing senescence, revealed a more than 30 fold change in expression of Atmc9, which is ten times more than the upregulation of the senescence-specific SAG12 protein (Swidzinski et al., 2002) (also see Chapter 1).

Moreover, promoter-GUS fusions of Atmc9 demonstrated that Atmc9 is highly expressed in senescing, dying plant tissues such as senescing petals and pollinated dying tissues of the silique. As such, a possible involvement of Atmc9 in senescence was studied by using the transgenic plants overexpressing Atmc9. As senescence can also be induced by dark treatments (Lim et al., 2003), a dark-induced leaf senescence experiment (as described by (Lin and Wu, 2004)) with wild type plants and plants overexpressing Atmc9 was performed. Intact plants were used to eliminate the potential effects of wounding or dehydratation, which also trigger leaf senescence (He et al., 2001). Six weeks-old green wild type and transgenic plants (pre-bolting phase) were put in the dark for 6 days. After 1 day in complete darkness, the first signs of yellowing were already visible, although weakly. With prolonged dark incubation progressive yellowing of the leaves became more and more obvious. After six days of dark treatment leaves were completely yellow. However, no obvious difference between wild type and transgenic plants were observed. Apparently, overexpression of Atmc9 does not result in a delayed or accelerated leaf senescence when compared to control plants. Although these results do not necessarily mean that Atmc9 is not involved in leaf senescence, they demonstrate that higher metacaspase 9 levels do not have an effect.

In conclusion, plants with higher metacaspase levels were not significantly affected by application of any of the tested stress stimuli. This does not necessarily mean that the metacaspases are not involved in defense-related cell death, abiotic stress resistance or senescence. It indicates that higher metacaspase levels do not have an effect as such. This might be due to the fact that the **substrates** of the metacaspases, which cleavage by the metacaspases is responsible for the effects, are not evenly highly expressed as the metacaspases and form as such the limiting factor. At last, it is not excluded that **other** (not tested) stress conditions can provoke a significant effect in the transgenic plants.

In this respect, knocking out the metacaspases might form an alternative and valuable approach to further unravel the function of metacaspases, as this could

have more drastic effects.. In addition, the identification of natural substrates and inhibitors will also provide new insight into the functions of the metacaspases (see Chapter 4-6).

3.4 Experimental procedures

Plant growth conditions

Plants were grown in Jiffy pots (Jiffy products international AS; Drøbak, Norway) in controlled environment chambers at 21°C day/night temperature, under illumination of 100 $mmol.m^{-2}s^{-1}$ light and with a relative humidity of 60%, unless mentioned differently. For *in vitro* growth, plants were grown on solid growth medium (Murashige and Skoog; Biomark Laboratories, Maharashtra, India) containing 2% of sucrose, at 21°C day/night temperature and under illumination from 45-50 $mmol.m^{-2}s^{-1}$ and relative humidity of 35 %, unless mentioned differently. Seeds were stratified for approximately two to three days at 4°C before being transferred to 21°C.

Analysis of abaxial epidermis of leaf primordia

Wild type plants and transgenic plants were grown side by side *in vitro* on solid growth medium in a 16h/8h day/night regime. At 21 days first leaves were harvested, cleared overnight in ethanol, and subsequently stored in lactic acid for microscopy. The leaves were mounted on a slide and covered. The leaf primordia were observed under a microscope fitted with differential interference contrast optics (DMLB; Leica, Wetzlar, Germany). The total (blade) area of leaf primordia were digitized with a charge-coupled device camera mounted on a binocular (Stemi SV11; Zeiss, Jena, Germany) and total area was determined with the public domain image analysis program Image J (http://rsb.info.nih.gov/ij/). Cell density was determined from scanned drawing-tube images of outlines of at least 50 cells of the abaxial epidermis located halfway from the distance between the tip and the base of the leaf primordium and halfway between the midrib and leaf margin. For each line five leaf primordia were analyzed. Subsequently, the following parameters were determined: total area of cells in the drawing (ImageJ), total number of cells and guard cells in the drawing. From these data, we calculated the average cell size and estimated the number of cells per leaf by dividing the leaf area by the average cell area.

Determination of root length and number of lateral roots

Wild type plants and transgenic plants were grown on vertical plates *in vitro* on solid growth medium in a continuous light regime. Total root length was determined from scanned images, with the public domain image analysis program Image J. The number of roots was

counted using a binocular microscope (Stemi SV11; Zeiss, Jena, Germany). For Atmc7, measurements were done on roots of 11 days old seedlings, for Atmc4 on 12 days old seedlings.

Student T-test

The t-test assesses whether the means of two groups are statistically different from each other. This analysis is appropriate whenever one wants to compare the means of two groups, for example wild type plants with transgenic plants. The formula for the t-test is the ratio of the difference between the two means or averages and a measure of the variability or dispersion of the scores (standard error). The bigger the t-value, the more the means differ from each other and the difference between the means for the two groups is different. The outcome of the result is often a P-value, which determines a significance level. In general, P>0.05 is considered as not significant, p-values below 0.01 are considered as significant, and p-values below 0.001 as highly significant. The T-test used here was two-tailed, assuming unequal variances.

Box plot

For the depiction of the data in a box plot diagram Genstat was used, a statistical analysis software tool (Payne and Lane, 2006).

Flow cytometric analysis of leaves and cotyledons

Plants were grown *in vitro* in a 16h/8h day/night light regime. Leaves were chopped with a razor blade in 300 ml of 45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM 3-(N-morpholino)propanesulfonic acid (pH7) and 1% Triton X-100 (Galbraith et al., 1991). From a stock of 1 mg/ml 4,6-diamidino-2-phenylindole (DAPI), 1 ml was added to the filtered supernatans. The nuclei were analyzed with the BRYTE HS or CyFlow flow cytometer with Win-Bryte (Bio-rad, Hercules, CA) or Flomax (Partec, Münster, Germany) software, respectively.

Biotic stress

Pathogen infections were performed on four to five weeks old plants grown *in vivo* in the University of Leuven (KUL), Centre of Microbial and Plant Genetics (Prof. B. Cammue). *Botryrtis cinerea* and *Plectosphaerella cucumerina* infection occurred by spraying with spores

(5 x 10^5 per ml) on leaves. 50-70 leaves were inoculated per line. *Pseudomonas syringae* infection was performed via leaf dip in a *P. syringae* solution (± 5 x 10^7 cfu per ml). 150-180 leaves were inoculated per line. For description of the pathogen infections see (Tierens et al., 2002).

As control lines, besides the wild type line, the Pthw line was used, which is an empty-vector containing construct (binary vector pthw142, no Gateway vector) used for control of BASTA effect.

Hydrogenperoxide-induced stress on leaf discs

Fully expanded leaves from the transgenic lines and wild type line grown for 2 weeks in a growth chamber with a long day light regime and afterwards for approximately 3 weeks in a short day light regime (8h light/16 h dark), both chambers at 21°C day/night temperature and under illumination from 100 mmol.$m^{-2}s^{-}$. Leaf discs (**4** mm diameter) were punched using a cork-bore. For H_2O_2 consumption analysis, leaf discs were floated in a 6-well plate (3 leaf discs per well, one line per well) on H_2O or H_2O_2 at a concentration of 10 mM for 24h at 21°C and constant light photoperiod. The experiment was performed in two independent experiments. Ion Leakage of the leaf discs, as a degree of oxidative stress, was measured as conductivity of the medium using a K610 conductivity meter (Consort, Turnhout, Belgium) and values are means of two independent experiments.

Salt stress and cold / high light stress experiment

For salt stress experiments, plants were grown *in vitro* in a 16h/8h day/night light regime for 12 days on MS agar plates and MS agar plates supplemented with 100mM NaCl. 276 plants per line and per condition were grown.

For cold / high light experiments plants were first grown in a growth chamber at 21°C day/night temperature in a long day light regime at illumination from 100 mmol.$m^{-2}s^{-1}$. For the treatment, 16 days-old plants were transferred to a PLC 970 Phytotron chamber (Sanyo Gallenkamp, Leicester, UK) at illumination of 1000 mmol.$m^{-2}s^{-1}$ and day/night temperature of 5 °C during 24 hours. Approximately 30 plants were used per line. Afterwards, plants were transferred back to normal conditions in the growth chamber.

Dark-induced senescence experiment

Plants were grown in a growth chamber short day light regime (8h light/16h dark) at 21°C day/night temperature and under illumination from 100 mmol.$m^{-2}s^{-1}$. At 42 days, plants were placed in a dark growth chamber (21°C) for 6 days. Approximately 40 plants were were used per line.

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Chapter 4: Building an interactome of *Arabidopsis* metacaspases via Yeast two-hybrid screening

4.1 Abstract

In this chapter, we aimed to build a comprehensive protein interactome of Type I metacaspases Atmc2 and Atmc3 by yeast two-hybrid. Identification of interactors, more particular regulators and substrates of the proteases, can provide a better insight into the putative functions of these Type I metacaspases. In addition, a putative interaction amongst members of the Type I and Type II family was also examined to investigate the possible existence of a metacaspase cascade, like is the case with mammalian caspases.

The screen for interactors of Atmc2CA and Atmc3CA (mutant forms) using an *Arabidopsis* cDNA library resulted in the identification of various potential substrates and regulators. Interestingly, many of the identified metacaspase-interactors show a homology with known interactors of mammalian caspases. Based on these interactors, hints towards putative roles for *Arabidopsis* metacaspases in different processes such as apoptosis and cell cycle progression were proposed. Yeast two-hybrid of the Type I and Type II metacaspases amongst each other, does not suggest the existence of a metacaspase cascade.

4.2 Introduction

To gain insight into the function of plant metacaspases, the identification of interactors, substrates and regulators is of indispensable help. Hitherto, the function of these metacaspases remains elusive and no substrate of the proteases is already described. In this post-genomic era, where complete genomes are being sequenced, yeast two-hybrid has proven to be a valuable technology for identifying protein-protein interactions. With the yeast two-hybrid system (Y2H) one can assess interactions between two known proteins, but also search for unknown partners (prey) of a specific protein (bait). By doing this one can establish complete protein interaction maps, so called interactomes. Protein-protein interaction mapping is extremely useful as it identifies a putative function to uncharacterized proteins (Legrain et al., 2001). Therefore, two-hybrid screens are sometimes considered as **functional screens**, since knowing the identity of interacting proteins might give a functional hint, if at least one of the partners has a known functional commitment in a well understood signaling pathway. As such, these data reveal interactions that place functionally unclassified proteins in a biological context, interactions between proteins involved in the same biological function, and interactions that link biological functions together into larger cellular processes.

Despite the fact that the Y2H system is prone to some limitations caused by different characteristics of the technique (see §4.3), it is has proven to be very successful in the past. Genome-wide yeast two-hybrid screens were used to obtain **protein-protein interaction networks** of different model organisms such as the budding yeast ((Uetz et al., 2000); (Ito et al., 2001); (Ito et al., 2000b); (Vollert and Uetz, 2004)), worm ((Walhout et al., 2000); (Li et al., 2004)), and human (Rual et al., 2005). As the large-scale two-hybrid analyses are prone to the occurrence of false negatives (see below), this system is very useful to obtain a partial coverage of protein-protein interactions within a proteome of interest, however, to obtain a complete protein interaction contiguity, alternative large-scale approaches such as Tandem Affinity Purification (Chapter 6) are necessary.

The yeast two-hybrid technique has been used for the identification of various **substrates and regulators of the mammalian caspases** as well. Using procaspase-2 as a target (bait protein), proteins controlling the function of the (pro)caspase by regulation of the maturation of the proenzyme were identified (Shirakura et al., 2005). Characterization of a novel proapoptotic caspase-2 and caspase-9 binding protein which functions as an adaptor protein of these caspases (PACAP) also occurred by using the Y2H-system (Bonfoco et al., 2001). Yeast two-hybrid assays lead to the identification of a caspase-2 isoform (prodomain of procaspase-2L) which interacts with procaspase-2L and functions as an endogenous inhibitor of the caspase cascade that antagonizes caspase activation and cell death (Droin et al., 2000). Using full length caspase-2S as a bait ISBP (Ich-1S binding protein) was isolated, which is able to partially inhibit caspase activity (Ito et al., 2000a). For the identification of aII-spectrin, a substrate of caspase-2, Y2H screening revealed that the large isoform of procaspase-2 binds to aII-spectrin and subsequent *in vitro* assays demonstrated that caspase-2 was able to cleave aII-spectrin (Rotter et al., 2004). Mostly, a modified version of the Y2H-system was used for the identification of substrates of caspases. The system was adapted to the fact that the active form of the caspases is the heterotetrameric p20/p10 form and it is hence possible that substrates or interacting proteins can escape detection in a two-hybrid approach with an unprocessed precursor as bait. In addition, to trap substrates of proteases it is advantageous to use inactive mutant enzymes since fast release of the substrate upon cleavage will be avoided and the enzyme-substrate complex will be stabilized ((Van Criekinge et al., 1998); (Kamada et al., 1998)). One of the approaches is the use of a three-hybrid system where the p20 and p10 subunits are both coexpressed as fusion proteins with the DNA binding domain. Hereby, the catalytic cysteine in the p20 subunit was mutated to serine. This system was used for the identification of p35 and CrmA as substrates for caspase-1 (Van Criekinge et al., 1998). Another alternative modified Y2H-system was also used for the identification of gelsolin (Kamada et al., 1998) and NRF2 (Ohtsubo et al., 1999), as substrates of caspase-3 and caspase-1, respectively. In these studies, both subunits were expressed separately in yeast under the same

promoters to intentionally maintain an equimolar ratio of large-to-small subunits. The main difference with the three hybrid system used by (Van Criekinge et al., 1998) is that in this case only the p10 subunit is fused to the DB, and not both subunits. In addition, a point mutation was introduced that substituted the active site to prevent proteolytic cleavage of the substrate (Kamada et al., 1998). PA28g was characterized as a caspase-7 substrate using a reversed caspase-7, a constitutive active mutant of caspase-7, in which the small subunit precedes the large subunit, as a bait. These reversed caspases were shown to undergo autocatalysis indicating that they can spontaneously fold into their active conformation (Araya et al., 2002).

Large-scale Y2H screenings already identified different **interactors of the yeast metacaspase** (YCA1; Table 1). Although these interactions are in a different organism, they can bring more insight and give a lead as yeast caspase is an orthologue of plant metacaspases and interactions are often conserved between different organisms. For mammalian caspases, a variety of intracellular polypeptides that finally lead to cell death are already identified as substrates, such as major structural elements of the cytoplasm and nucleus, components of the DNA repair machinery and a number of protein kinases. Cleavage of these substrates disrupt as such survival pathways and disassemble important architectural components of the cell, contributing to the stereotypic morphological and biochemical changes that characterize cell death ((Fischer et al., 2003); Earnshaw et al., 1999). Different studies also indicate a role for yeast caspase 1 (YCA1) in cell death ((Madeo et al., 2002); (Khan et al., 2005); (Mazzoni et al., 2005); (Bettiga et al., 2004)). The interactors which were identified in a Y2H screen with YCA1 (Table 1) are proteins involved in transcription/translation initiation, protein biosynthesis and transport. Despite the fact that to date nothing is known about the biological relevance of these interactions, it is likely that disruption of these proteins could lead to dismantling of the cell, block in DNA transcription/ translation and DNA repair, which are associated with an establishment of cell death. Therefore, it can be suggested that Y2H with YCA1 succeeded in identification of substrates. Notwithstanding, other large-scale approaches are needed to gain a full insight into the protein interaction network of the yeast metacaspase. Y2H with the

Type I metacaspases Atmc2 and Atmc3 did not lead to similar interactors of the YCA1, known to be directly involved in protein synthesis, transport or DNA transcription/ translation. However, the Y2H results of YCA1 suggest a putative implication in cell death, as is also the case for some interactors of the metacaspases (see below).

Protein ID	Genename	Description	Process	Source
YBL106c	*SR077*	suppressor of defect in the small GTPase	exocytosis	Uetz et al., 2000
YJL141c	*YAK1*	Serine-threonine protein kinase	protein amino acid phosphorylation cell growth and/or maintenance	Ito et al., 2001
YGR166w	*KRE11*	involved in biosynthetic pathway for cell wall beta-glucans	ER to golgi transport	Ito et al., 2001
YER022w	*SRB4*	subunit of RNA polymerase II holoenzyme/mediator complex	transcription from PolII promoter	Ito et al., 2002
YMR257c	*PET111*	specific translational activator for the COX2 mRNA, located in the mitochondrial inner membrane	protein biosynthesis	Uetz et al., 2000
YJR091c	*JSN1*	benomyl dependent tubulin mutant	mRNA catabolism, deadenylation-dependent	Ito et al., 2002
YDR311w	*TFB1*	subunit of TFIIH and nucleotide excision repair factor 3 complexes	transcription initiation from PolII promoter nucleotide-excision repair	Ito et al., 2003

Table 1: Interactors of yeast caspase 1 (YOR197w), identified via large-scale yeast two-hybrid screenings.

4.3 Yeast two-hybrid system: Principle and characteristics

The Y2H system is an *in vivo* method to study protein-protein interactions in *S.cerevisiae* (Fields and Song, 1989). The principle of the technique is based on the fact that many eukaryotic transcription factors (e.g. GAL4) consist of separable DNA-binding domains (DB) and activation domains (AD) (Keegan et al., 1986). To be able to activate transcription activation the two domains BD and AD do not have to be covalently joined (Ma and Ptashne, 1988). Protein X, the bait protein or protein of interest, is fused (carboxy-terminal) to the DB of the transcription factor GAL4 and protein Y, the prey protein, is fused (carboxy-terminal) to the AD of the transcription factor GAL4. Subsequently, interaction of protein X and Y functionally reconstitutes the transcription factor leading to transcription of a reporter gene containing the cognate DNA binding site (UAS). The strength of the interaction between two proteins can be correlated to the level of transcription of the reporter gene. An intrinsic limitation of the Y2H system is the potential detection of false interactions without biological significance, so called *false positives*. which is mostly due to the fact that the system relies upon the transcriptional activation of reporter genes. For example, bait proteins might activate the transcription of reporter genes above the threshold level by themselves (auto-activation), and some prey proteins might be identified in a Y2H assay in combination with a wide variety of bait proteins (sticky prey). The frequency of selfactivators can be reduced using low expression levels of the two-hybrid proteins and multiple reporter genes utilizing different promoters (Vidal and Legrain, 1999). In addition, in order to diminish background cell growth due to selfactivation of the bait protein, 3-amino-1, 2, 5-triazole (3-AT) can be added in the medium when using *HIS3* as a reporter gene. 3-AT, as a competitive inhibitor of the yeast HIS3 protein, reduces reporter gene activation due to leakage of the HIS3 reporter. In that way, basal levels of HIS3 due to leakage of the reporter, are not able to sufficiently activate the reporter gene to overcome the inhibiting effect of 3-AT (Walhout and Vidal, 1999). *False negatives* on the other hand, can be caused by different characteristics of the

two-hybrid system. First, incorrect folding and instability of bait or prey fusion proteins in the yeast cells or failure of the fusion proteins to localize to the yeast nucleus can cause the occurrence of false negatives. Second, the inability of bait and prey proteins to function as DB or AD fusion proteins. Third, the interaction between bait and prey may depend on posttranslational modifications that are absent or inappropriate in yeast cells, such as the formation of disulfide bridges, glycosylation and most commonly phosphorylation. Some new two-hybrid systems, however, try to overcome this problem by co-expressing the enzyme responsible for the posttranslational modification. Fourth, it has been reported that a number of protein-protein interactions can only be detected in the two-hybrid system upon truncation of either X or Y. Finally, using artificially made chimeric proteins always contains a potential risk. The actual conformation of the bait and/or prey might be changed due to the fusion and as a result functionalities might be altered as well. Consequently, this might lead to limited activity or inaccessibility of binding sites of the chimeric proteins. Nevertheless, the use of tagged proteins in general has proven to be very successful in many biotechnological approaches. This might be due the fact that protein domains can fold rather independently, which enables the co-existence of different, also artificially introduced, modules in the same protein (Van Criekinge and Beyaert, 1999).

Nevertheless, the Y2H system is a technique which allows a very rapid detection and subsequent cloning of interacting gene products. Indeed, one of the most appealing features of the yeast two-hybrid system is that identification of an interacting protein implies cloning of the corresponding gene at the same time. This very rapid detection of interactors makes the Y2H system a very efficient tool to set up protein-protein interaction maps. However, as a consequence of the artificial nature of the two-hybrid system, interactions should be viewed as hypotheses until they are validated in the appropriate biological system. Biological validation and/or integration of data from other sources will help to predict the biological relevance of these interactions; for example, taking into account that Y2H assays might detect interactions between proteins that are never co-localized in the cell. Bio-informatic analyses can help to elucidate the significance of an interaction by studying the transcriptional

profile of the genes of the interacting proteins, as probably these genes are co-regulated in the same biological processes. Moreover, studies with transcriptional alterations, overexpression or knock-out of the genes can also be very helpful. In addition, comparing protein-protein interactions between different model organisms can be of interest as well. It is assumed that conserved interactions between different organisms, or interologs, possess a higher chance to be biologically relevant (Walhout et al., 2000).

4.3.1 Yeast two-hybrid mating

Interactions between two known proteins can be tested in a Y2H system, using the yeast mating technique. Haploid yeast strains of opposite mating type (a and a), one containing a DNA-binding domain fusion construct, the other containing an activation domain fusion construct, can be simply fused (mated) to obtain the diploid yeast strain containing both constructs and as such bring together bait and prey proteins (Herskowitz, 1988). Yeast mating can be used to test the interaction between a great number of proteins, with the advantage of performing relatively few yeast transformations.

4.3.2 Yeast two-hybrid screening

The followed procedure to perform a yeast two-hybrid screening is depicted in Box I and illustrated in Figure 1.

· Construction of the bait vector

Genetic fusion of the bait protein to GAL4 BD using a GAL4 DB plasmid (pGBT9gate) containing a selection marker (*TRP*)

· Transformation of yeast strain with bait vector construct

Transform yeast reporter strain (HF7c), containing two reporter genes (*HIS3* and *LacZ*) under the control of a GAL4-responsive UAS, with the bait plasmid and valuate toxicity.

· Test auto-activation (false-positive test)

Test whether the bait activates transcription of the reporter gene by itself. If so, use truncated versions of the bait or supplement AT in the medium.

· Transform the prey library into the yeast reporter strain containing the bait plasmid

The bait and prey plasmid are introduced into the yeast reporter strain by sequential transformations. Introducing the prey library (*LEU* as a selection marker) into a yeast strain already containing the bait plasmid enhances the amount of double transformants and thus the screening efficiency.

· Plate transformants on selective medium

Plate transformants on medium allowing only those yeast cells to grow, which contain both bait and prey plasmid and which selects for bait-prey interaction (-Leu/-Trp/-His) medium. Optionally selection for the second reporter gene, *LacZ*, can be tested as well by performing a colony-lift-B-galactosidase assay for *LacZ* expression.

a) Perform yeast colony PCR on positive (Leu+, Trp+ and His+) colonies with prey-specific primers
   Sequencing of the PCR product gives a very rapid result of the found interactor
b) Plasmid preparation from yeast
   Transformation of both (bait and prey) plasmids in Leucine-deficient *E. coli* to specifically select for prey containing transformants
   Prey plasmid preparation
   - Sequence retained clones
   - Transform in yeast strain with bait vector to confirm the interaction in yeast

Box I: Flowchart of a Y2H screening

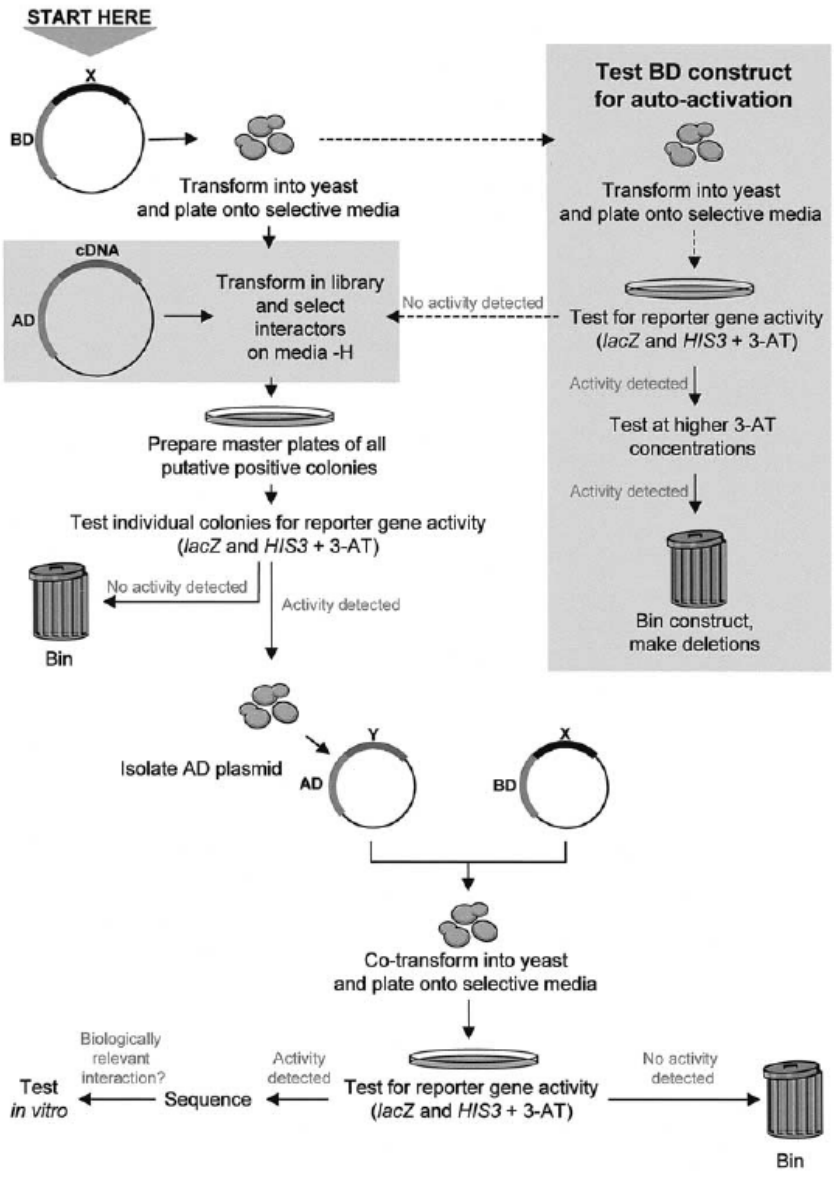


Figure 1: Illustration of a typical Y2H screen (from (Causier and Davies, 2002))

4.4 Results and discussion

4.4.1 Y2H suggests that metacaspases do not work in a cascade

Mammalian caspases are known to work in a hierarchic proteolytic procaspase activation network, which leads to a dramatic increase in multiple caspase activities once key caspases are activated (Fan et al., 2005). Figure 2 (taken from (Thornberry and Lazebnik, 1998)) depicts a simplified model of how mammalian caspases are activated in a caspase cascade. As discussed elaborately in Chapter 1, **initiator caspases** are activated by induced proximity, whereby assembly of a molecular platform in response to death stimuli is followed by recruitment and concomitant activation of procaspases to the cell death complex. This (homo)oligomerization of initiator caspases is prodomain-dependent and is indispensable for subsequent processing and activation. Once initiator caspases are activated, they can on their turn cleave and hence activate the downstream caspases, the **executioner or effector caspases**, which will finally cleave their substrates, a wide variety of intracellular proteins, which lead to the morphological and biochemical changes that characterize cell death (Nicholson, 1999).

The Type I metacaspases structurally resemble the initiator caspases, while type II metacaspases the executioner caspases. Therefore we assessed whether, in analogy with mammalian caspases, a similar cascade exists within the Arabidopsis metacaspase family. This was done by using a a yeast interaction-mating technique, which allows high-throughput testing of large numbers of individual protein-protein interactions.(Finley and Brent, 1994).

All wild type and C/A mutant versions were tested for interaction with each other within an interaction matrix. Wild type and mutant ORFs coding for all *Arabidopsis* metacaspases (Atmc1 to Atmc9, except for Atmc8) were cloned into the bait (pDEST32) and prey (pDEST22) yeast expression vectors, respectively. Yeast strain pJ69-4A was transformed with the bait vectors while the corresponding a strain was

transformed with the prey vectors. Hence mating was performed by mixing the A and a strain together. To overcome selfactivation of the bait constructs (data not shown) mated yeast colonies were grown on selective medium supplemented with 5mM 3-AT. No interaction between either the wild type or mutant constructs was detected (data not shown).

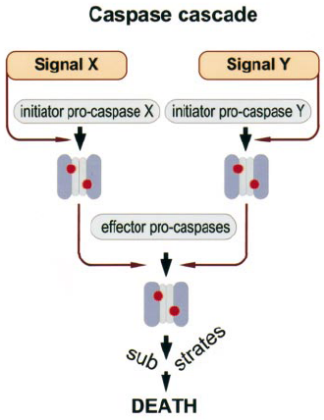


Figure 2: Model for activation of mammalian caspases through a caspase cascade (Thornberry and Lazebnik, 1998)

This does not necessarily implicate that the *Arabidopsis* metacaspases are not involved in a proteolytic cascade. Y2H is an artificial system and there might be some additional levels of regulation such as posttranslational modifications which could be essential for interaction (Van Criekinge and Beyaert, 1999). In addition, for cleavage of effector caspases by initiator caspases the active form of the latter is needed. Hence it might also take active Type I metacaspases to be able to cleave/bind Type II metacaspases. However, it might be assumed that due to the GAL4 DB which contains a dimerization domain, the bait protein is forced into a dimeric conformation. This could result in dimerized Type I metacaspases and as such possible active Type I metacaspases (activated by induced oligomerization). On the other hand, this forced dimerization might not be sufficient to lead to active proteins as there might be some additional factors which might play a role such as pH (also see Chapter 2 and 3),

which might not be optimal in yeast. Nevertheless, it is not know whether these optimal conditions would also be required in order to bind their interactors (without cleavage). In an alternative *in vitro* approach potential interaction between the different metacaspases could be assessed. In such an experiment bacterially expressed (purified recombinant) metacaspases would be used as enzymes and proemzymes (full length proteins) of the metacaspases as substrates, as was done successfully for the mammalian caspases (Van de Craen et al., 1999).

4.4.2 Identification of Atmc2 and Atmc3 interactors in yeast

To find **novel** interactions of the Type I metacaspases, a cDNA library fused to an activation domain was screened. The finding of novel interactions finally leads to the building of an interaction map and will contribute to help elucidate the putative role of the metacaspases in biological processes as cell death or others. Yeast two-hybrid screenings were performed for the Type I metacaspases Atmc2 and 3.

4.4.2.1 Yeast two-hybrid screenings with Arabidopsis *type I metacaspases AtMC2 and AtMC3.*

Y2H screening was performed with pGBTg-Atmc2CA and pGBTg-Atmc3CA as bait vectors encoding a fusion between the C-terminus of the GAL4 DNA-binding domain and Atmc2CA and Atmc3CA. Therefore, mutant (CA) versions of the full-length coding regions, of the metacaspases were used. It was chosen to perform the screenings with the mutant version of the metacaspases, as yeast already has a metacaspase itself (YCA1) and anticipated that ectopic expression of the *Arabidopsis thaliana* metacaspases could be lethal. Later reports indicated that ectopic expression of *Arabidopsis* metacaspases only induce apopotic-like cell death during oxidative stress or ageing (Watanabe and Lam, 2005). More importantly, a catalytically inactive metacaspase would prevent proteolytic cleavage of the substrate, hereby stabilizing

the enzyme-substrate complex in yeast and allowing to detect the interaction. It was reported before that this point mutation does not influence the binding capacity (Kamada et al., 1998).

Y2H screening with Atmc2CA

The bait plasmid for Atmc2CA, pGBT9gate-Atmc2CA, was cotransfected in yeast (HF7c) with prey plasmids containing an *Arabidopsis thaliana* cDNA expression library fused to the GAL4 AD. Sixty-nine His+ (supplemented with 5 mM AT to overcome self-activation of the bait plasmid) colonies were obtained by screening $1.8x10^5$ transformants. However, after retesting HIS-prototrophy (restreaking of the colonies), only 10 transformants (14.5%) were withdrawn. Nevertheless, these 10 transformants were subjected to selection of the second reporter gene (*lacZ*). This is tested by an X-gal assay, in which positive colonies turn blue caused by conversion of the X-gal substrate into a blue color. Almost all colonies (more than 95%) turned out to be positive for the activation of both reporter genes. Moreover, the strength of the interaction was tested by supplementing the medium with a higher concentration of AT (20 mM). PCR with prey-specific primers, performed directly on the yeast colonies, subsequent sequencing of the PCR product and blasting of the DNA sequence against a protein database (NCBI), directly gives information of the identity of the interactor. It should be remarked that some PCRs performed on the yeast colonies did not yield a product. Another comment, some clones were found more than once (see Table 2). Finally, upon cotransfection of the prey (library) plasmids with the bait plasmid of Atmc2CA, only one interaction clone was obtained. Results are summarized in Table 2.

	AGI gencode	Description	length protein (AA)	cDNA picked up from Y2H	#	growth 20mM AT	X-gal assay	self-activation prey	confirmation in yeast
Y2H met Atmc2CA	At3g05220	unknown protein	541	382-541:Collagen triple helix repeat	2	++	++	no	yes
	At2g40205	expressed protein/ribosomal protein L41	25	1-25	3	++	-	yes	/
	At4g00830	RNA Recoginition motif containing protein	495	365-495	1	-	ND	/	no
Y2H met Atmc3CA	At5g42270	FtSH protease, VAR1	704	579-704	1	++	++	no	yes
	At5g65430	14-3-3 like protein, GF14 kappa	248	64/74/91*-248	4	++	++	no	yes
	At5g42570	BAP31-like protein	218	100-171	1	++	++	no	yes
	At3g50630	Kip-related protein 2	209	1-209	1	++	++	no	yes
	At3g01150	Polypyrimidine-tract binding protein	399	1-114**	3	+	+	yes	/
	At5g08120	myosine heavy chain-like protein	326	1/3*-59**	2	++	++	no	yes
	At4g17080	hypothetical protein/putative phosphatidylinositol 4-P 5-kinase	471	193-275**	1	+	+	no	yes
	At5g41990	MAP kinase	563	400-563	1	+	+	no	yes
	At1g18660	expressed protein	486	121-281**	1	+	-	no	yes
	At5g58430	Leucine zipper containing protein	624	182-312**	1	++	ND	no	yes
	At1g14980	10 kDa chaperonin	98	5-98	67	++	++	no	yes
	At1g23100	10 kDa chaperonin-like	97	5-97	27	++	++	no	yes

Table 2: Summary of the results of Y2H screening with Atmc2CA and Atmc3CA as a bait. Screening with Atmc2CA and Atmc3CA as a bait was performed on histidine free medium supplemented with 5 mM AT to overcome self-activation by the bait plasmids pGBT9gate-Atmc2CA and pGBT9gate-Atmc3CA. The table indicates the protein which corresponds to the cDNA identified in the Y2H screening , with its AGI code, description and total length. In addition, the length of the cDNA clone was also given. * signifies that, if there are more than one clone for the same interactor, thus different borders. ** indicates the cDNA clone could not be sequenced till the poly A tail, so it is unclear whether the border is situated there or more downstream. The number of clones identified with the same interactor is also shown. Furthermore, the table contains information about the strength of the interaction indicated by growth of the colony on His^- medium supplemented with 20 mM AT. Specifications of reporter gene activation by selection of a second reporter gene (*LacZ*) were also given. Selfactivation of the prey was tested and finally the confirmation of the interaction in yeast was verified. Remark: cDNA clones which could not be identified, mostly due to the fact that no PCR product could be obtained, are not included in the table.

The only interactor that could be retained was a clone encoding an unknown protein (At3g05220). Pfam, a database of multiple alignments of protein domains or conserved protein regions, was used to reveal some evolutionary conserved structure which may provide a hint on the protein's function. At3g05220 contains two domains: at the N-terminal side a heavy metal associated domain; and more upstream a collagen triple helix repeat (Figure 3). Interestingly, the cDNA that was picked up from the screening spans only the triple helix repeat which is situated at AA 383-441, as the cDNA starts at amino acid 382 (until the end of the protein) (Figure 5). Proteins which contain this triple helix repeat motif are members of the collagen superfamily (Mayne and Brewton, 1993). Collagens are generally extracellular structural proteins involved in formation of connective tissue structure. Some members of the collagen superfamily are not involved in connective tissue structure but share the same triple helical structure (Myllyharju and Kivirikko, 2004).

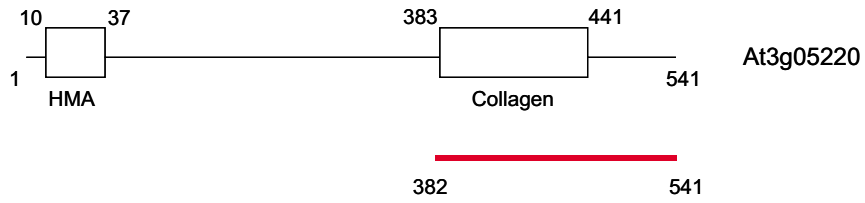


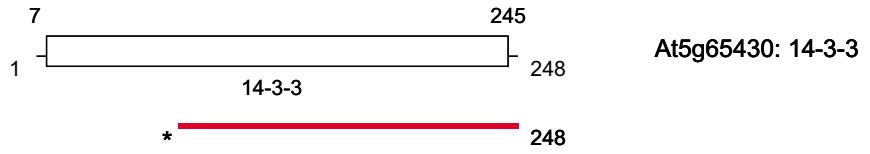
Figure 3: Schematic representation of the structural domains, identified by Pfam, in the Atmc2-interactor At3g05220, an unknown protein. The red line indicates the overlap with the cDNA clone identified via Y2H. HMA = Heavy metal associated domain, Collagen = collagen triple helix repeat.

Y2H screening with Atmc3CA

Yeast strain HF7c containing the bait plasmid pGBT-Atmc3CA was cotransfected with the *Arabidopsis thaliana* cDNA expression library. A total of 10^5 transformants were screened for their ability to grow on histidine free medium (supplemented with 5 mM AT to overcome auto-activation of the bait plasmid). 285 His^+ colonies were retested for their HIS-prototrophy and tested for their ability to activate the second reporter gene, *LacZ*, however as seen in the screening with Atmc2CA almost all colonies seemed to be both His^+ and $LacZ^+$. 173 colonies (61%) were withdrawn afterwards. Prey-specific colony PCR was performed on all colonies. However, sequencing on a small number of the obtained PCR products, identified the 10 kDa chaperonin (Cpn10) several times and as such Cpn10-specific primers were designed to screen the other PCR products with. Indeed, 67 of the 173 colonies (38.8%) were identified as the chaperonin protein. Finally, upon cotransformation of the prey and bait plasmid in yeast, 11 different interactions were confirmed. Table 2 summarizes the results.

14-3-3 protein (GF14κ) was identified four times in the Y2H screen with Atmc3CA (Table 2). Figure 4 shows the cDNA which was picked up in the screening, the corresponding protein which was identified upon blasting of the DNA sequence against a protein database (NCBI), and the protein regions identified via Pfam. 14-3-3 proteins are phosphoserine/phosphothreonine-binding proteins that regulate the activities of a wide array of proteins via direct protein-protein interactions. In animal cells, the majority of their known targets are involved in signal transduction and transcription (Aitken, 1996). In plants, they are primarily known for their regulation of the plasma membrane proton-ATPase and enzymes of carbon and nitrogen metabolism (primary metabolism) (Sehnke and Ferl, 2000). Nevertheless, an increasing number of plant signalling proteins are now being recognized as 14-3-3 binding proteins. Plant 14-3-3 proteins bind a range of transcription factors and other signalling proteins, and have roles regulating plant development and stress responses (Roberts, 2003).

A



B

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                10        20        30        40        50        60        70
           ....|....|....|....|....|....|....|....|....|....|....|....|....|
Mouse      MD---DREDLVYQAKLAEQAERYDEMVESMKKVAG---MDVELTVEERNLLSVAYKNVIGARRASWRIIS
Human      MD---DREDLVYQAKLAEQAERYDEMVESMKKVAG---MDVELTVEERNLLSVAYKNVIGARRASWRIIS
At5g65430  MATTLSRDQYVYMAKLAEQAERYEEMVQFMEQLVSGATPAGELTVEERNLLSVAYKNVIGSLRAAWRIVS

                80        90       100       110       120       130       140
           ....|....|....|....|....|....|....|....|....|....|....|....|....|
Mouse      SIEQKEENKGGEDKLKMIREYRQMVETELKLICCDILDVLDKHLIPAANTGESKVFYYKMKGDYHRYLAE
Human      SIEQKEENKGGEDKLKMIREYRQMVETELKLICCDILDVLDKHLIPAANTGESKVFYYKMKGDYHRYLAE
At5g65430  SIEQKEESRKNEEHVSLVKDYRSKVETELSSICSGILRLLDSHLIPSATASESKVFYLKMKGDYHRYLAE

               150       160       170       180       190       200       210
           ....|....|....|....|....|....|....|....|....|....|....|....|....|
Mouse      FATGNDRKEAAENSLVAYKAASDIAMTELPPTHPIRLGLALNFSVFYYEILNSPDRACRLAKAAFDDAIA
Human      FATGNDRKEAAENSLVAYKAASDIAMTELPPTHPIRLGLALNFSVFYYEILNSPDRACRLAKAAFDDAIA
At5g65430  FKSGDERKTAAEDTMIAYKAAQDVAVADLAPTHPIRLGLALNFSVFYYEILNSSEKACSMAKQAFEEAIA

               220       230       240       250       260
           ....|....|....|....|....|....|....|....|....|.
Mouse      ELDTLSEESYKDSTLIMQLLRDNLTLWTSDMQGDGEEQNKEALQDVEDENQ
Human      ELDTLSEESYKDSTLIMQLLRDNLTLWTSDMQGDGEEQNKEALQDVEDENQ
At5g65430  ELDTLGEESYKDSTLIMQLLRDNLTLWTSDMQMD------EA---------
```

Figure 4: A) Schematic representation of the structural domains, identified by Pfam, in the Atmc3-interactor At5g65430, a 14-3-3 protein. The red line indicates the overlap with the cDNA clone identified via Y2H. * indicates that different clones were picked up with different N-termini. **B) Functional conservation between plant and animal 14-3-3 proteins as shown by alignment of Arabidopsis 14-3-3κ, mouse (gi|57966) and human (gi|2117967) (both ε types) 14-3-3s (ClustalW).** Amino acid residues shaded in grey are identical residues whereas dark grey residues are similar amino acids.

It has been reported that, in *Arabidopsis*, these proteins are involved in pathogen-defence responses. Indeed, ARK2, a potential negative regulator of pathogen-related protein 1 expression and regulator of antioxidant metabolism during pathogen infection, was identified as a GF14λ-interacting protein (Yan et al., 2002). There are two features of 14-3-3 protein-protein interactions. First, interactions are regulated by the phosphorylation status of the target. Although also a variety of nonphosphorylated proteins can be bound. Second, binding to a target can affect the function of that protein, either through the stabilization of the active or inactive phosphorylated form of that protein, its absolute activity (mostly due to altered conformation), its interaction with other proteins, or its localization within the cel (Tzivion and Avruch, 2002). *Arabidopsis* contains 15 isoforms in the genome. The first *Arabidopsis* isoform was identified as part of a protein/G box complex and is therefore named “G box factor 14-3-3” or GF14. This name has been kept for all isoforms of *Arabidopsis*, and precedes the Greek letter (Rosenquist et al., 2001). The one which has picked up in the Y2H screen is the kappa isoform. There is a strong evolutionary conservation of 14-3-3s from animals to fungi and plants, which indicates that those plant and animal proteins would share common functions (Figure 4). Indeed, several *Arabidopsis* 14-3-3s can substitute for the essential 14-3-3 functions in yeast (van Heusden et al., 1996) and other substitutions in plant, yeast and animal 14-3-3s in various systems. However, there is also evidence that supports the hypothesis that there are important functional differences among the various 14-3-3s present in any multicellular organism. This is based on the fact that most higher organisms have fairly large and diverse families with the conservation of the core phosphoprotein-binding domain, however with extreme divergence at the N- and C-termini. In addition, biological phenotypes associated with altered 14-3-3 isoforms demonstrate properties that are difficult to explain in the absence of specificity. Moreover, various isoforms have different affinities for at least certain target proteins. Conclusively, the expression pattern, isoform-specific organellar presence and sequence terminal specificity suggests that the size and diversity of the 14-3-3 families is a reflection of their omnipotent roles in regulating important plant processes (Sehnke and Ferl, 2000).

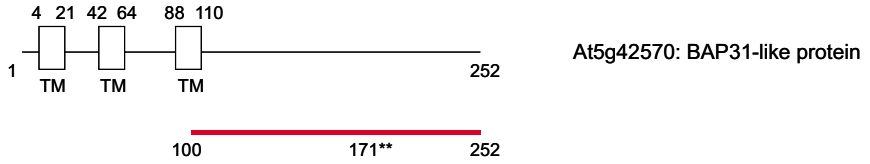
14-3-3 binding partners have found to be involved in almost every cellular processes. It is not evident to unravel a possible function of metacaspase 3 as a 14-3-3 binding protein, as this is highly dependent of the function of the metacaspase, but it is conceivable that binding to this protein regulates metacaspase activity, or alters the subcellular localization of the Atmc3. In addition, as 14-3-3 proteins are known to dimerize (Wu et al., 1997), this could result in dimerization of the metacaspases, hereby influencing the activity of the latter. Nevertheless, there is also a second possibility, namely that the 14-3-3 protein could function as a substrate of Atmc3. There is evidence from the mammalian field that the 14-3-3 proteins are substrates of the caspases. 14-3-3ε is sequestering and hereby inactivating phosphorylated Bad, a member of the pro-apoptotic Bcl2-family. In response to apoptosis, the 14-3-3ε protein is cleaved by caspase-3 which leads to the release of the associated Bad, facilitating Bad translocation in the mitochondria and its association with Bcl-X_L, counteracting the anti-apoptotic effect of the latter (Won et al., 2003). It is clear that cleavage of the 14-3-3 promotes cell death. As such, if GF14 is a substrate of Atmc3 in stead of a binding partner, the cleavage effect of GF14 will be dependent of the function of its 14-3-3 target partner and the effect of this binding at the moment of cleavage by the metacaspase, as cleavage of the 14-3-3 will lead to dissociation of the target partner. In addition, in mammalia 14-3-3 proteins seem to interact with pro-apoptotic Bax, hereby negatively regulating the activity of Bax (Won et al., 2003). An additional role for 14-3-3 proteins in the cell death process is as such established in mammalian systems. In analogy with mammalia, especially due to a strong evolutionary conservation between animal and plant 14-3-3s, 14-3-3 proteins might be a substrate of the metacaspase which cleavage could have various effects, one of them might be the induction of apoptosis.

Green Fluorescent Protein (GFP)-fusions demonstrated that the *Arabidopsis* isoform GF14k localizes to both the plasma membrane region of the cell and the nucleus (Sehnke et al., 2002), while Atmc3 localizes predominantly to the cytoplasm (Vercammen, unpublished results). This different subcellular localization does not necessarily implicate that an interaction is excluded between these two proteins as

under specific conditions there might be a change in subcellular localization of (one of)the proteins and as such colocalization under certain circumstances is still possible.

BAP31-like protein is detected once in the Y2H screening with Atmc3CA. Figure 5A shows a schematic representation of the clone identified via Y2H, the corresponding protein upon blasting of the DNA sequence, and its domains identified via Pfam. In mammalian systems, BAP31 or B-cell receptor associated protein, is an integral protein of the endoplasmic reticulum (ER) membrane and a substrate of caspase-8. p28 BAP31 is part of a complex that includes Bcl-2/Bcl-X_L, an anti-apoptotic member of the Bcl2-family, and procaspase-8. Bax, a pro-apoptotic member, does not associate with the complex; however it prevents Bcl-2 from doing so. In the absence of elevated Bcl-2 levels (upon an apoptotic stimulus) cleavage of BAP31 by caspase-8 (or a caspase-8 related caspase) at the two caspase recognition sites is stimulated. The resulting p20 fragment induces apoptosis when expressed ectopically in normal cells. Moreover, upon cleavage of p28 at the ER, this p20 fragment can direct apoptotic signals between the ER and mitochondria by calcium-mediated mitochondrial fission and release of cytochrome c, which implicates an ER-mitochondria cross-talk in this pathway (Breckenridge et al., 2003). As such, BAP31 is part of a complex in the ER that mechanically bridges an initiator caspase with the anti-apoptotic regulator Bcl-2 or Bcl-X_L. Therefore, it is possible that the p28 complex contributes to an additional regulation of procaspase-8 (or a related caspase) upon apoptotic signalling, which depends on the status of Bcl-2 within the complex ((Ng et al., 1997); (Breckenridge et al., 2002)). In addition, full length BAP31 seems to be a direct inhibitor of these caspase-8 initiated events, because mice expressing caspase-resistant BAP31 are resistant to cytochrome release in response to apoptotic stimuli. This resistance was due to association of uncleaved BAP31 with a putative ion channel protein of the ER (Wang et al., 2003). Mund and colleagues identified a novel BH3-only protein (BH3-only proteins constitute a subgroup of the Bcl3-family), called Spike, as an apoptosis inducer. Spike is localized to the ER and interacts with BAP31 at this organelle. Hereby, it inhibits the formation of a complex between BAP31 and the anti-apoptotic Bcl-X_L protein, thus promoting cell death (Mund et al., 2003).

A



B

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                10        20        30        40        50        60        70
           ....|....|....|....|....|....|....|....|....|....|....|....|....|
Mouse      MSLQWTTVATFLYAEVFAVLLLCIPFISPKRWQKVFKSRLVELVVTYGNTFFVVLIVILVLLVIDAVREI
C. Elegans MTLQWTIVAGVLYAEIAITFTLLLPWIRPTLWSKLFKSRLFTALAKHAHIYSITFGFVLFILFADGVRET
At5g42570  -------MIHLLYTVIFAEMALILLLLFKTPLRKLIILTFDRIKRGRGPVVVKTIGTTVFVVLLSSIYSL

                80        90       100       110       120       130       140
           ....|....|....|....|....|....|....|....|....|....|....|....|....|
Mouse      LKYDDVTEKVNLQNNPGAMEHFHMKLFRAQRNLYIAGFSLLLSFLLRRLVTLISQQATLLASNEAFKKQA
C. Elegans MKYNGLEGQMGRTAESDATH--HMRLFRAQRNLYISGFALLLWLVIQRIMTLLGRAAQLEAASEAAMRQA
At5g42570  VNIQRRSEDGAVLNPTDQVLA-SKHLLEAS----LMGFVLFLSLMIDRLHHYIRELRLLRKTMETAKKQN

               150       160       170       180       190       200       210
           ....|....|....|....|....|....|....|....|....|....|....|....|....|
Mouse      ESASEAAKKYMEENDQLKKGAAEDGDKLDIGNTEMKLEENKSLKNDLRKLKDELASTKKKLEKAENEALA
C. Elegans ESATKTARTLMN--------SADTN-----------EEAAGLAKQIEKLKGELKS--------ANTDRDT
At5g42570  RGFEDGKTTSGEE-------VKALG-----------EEIAALKAKIKTLESESESKGKELKGAQGETEA

               220       230       240       250       260       270       280
           ....|....|....|....|....|....|....|....|....|....|....|....|....|
Mouse      MQKQSEGLTKEYDRLLEEHAKLQASVRGPSVKKEE--------------------------------
C. Elegans LKKQSEGLQREFDRVSD---LLRDSAESKGDKKKD--------------------------------
At5g42570  LRKQADGFLMEYDRLLEDNQNLRNQLESIGHSPEGKKEVKHQINLTNIAEEMIQNLNEQMDAFKVSQLII

           ...
Mouse      ---
C. Elegans ---
At5g42570  QRA
```

Figure 5: A) Schematic representation of the structural domains, identified by Pfam, in the Atmc3-interactor At5g42570, a BAP31-like protein. The red line indicates the overlap with the cDNA clone identified via Y2H. TM= Transmembrane domain. ** indicates the PCR product could not be sequenced till the poly A tail, as only the prey-specific forward primer facing the C-terminus could be used in the sequencing reactions, so it is probable that the sequence is complete till the C-terminus, or at least the border is more downstream than has been sequenced. **B) Structural relation between BAP31 protein from mouse and *C. Elegans*, and the BAP31-like proteins from *Arabidopsis thaliana (de andere 4 Ath sequenties zou ik er nog bij aligneren)*, as shown by alignment (ClustalW).** Amino acid residues shaded in grey are identical residues whereas dark grey residues are similar amino acids.

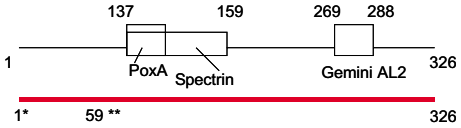
Intristingly, in the absence of a death signal, it was shown that BAP31 specifically associates with nonmuscle myosin heavy chain B and nonmuscle γ-actin, two components of the cytoskeleton actomyosin complex (Ducret et al., 2003). Consequently, BAP31 may play a fundamental role in the structural organization of the cytoplasm/ER membrane architecture in normal cell physiology. Apoptotic stimulation releases BAP31 from these motor proteins, which can lead to membrane remodelling during the execution phase of apoptosis.

In the genome of *Arabidopsis thaliana* five structurally related BAP31 genes have been identified, BAP31-like proteins. It seems that the BAP31 protein from worm and mouse are closely related, whereas the similarity between the mammalia BAP31 proteins and the related plant protein is low, however significant (Figure 5). Whether or not an analogous cell death machinery with BAP31 exists in plants, involving the Bcl2-family, remains to be investigated. Until now Bax and Bcl-2 and their relatives have not been found in plants. However, with the characterization of a bax-inhibitor (BI-1) in yeasts and plants ((Kawai et al., 1999); (Kawai-Yamada et al., 2001)), arguments for a conserved death pathway common to plants and animals have accumulated. Furthermore, in the *Arabidopsis* genome there is, besides the presence of two BI-1 homologues, a new identified family of 13 *AtBI-2* related genes. Their function remains to be elucidated but it has been suggested that these genes might represent functional equivalents of the mammalian Bcl-2 family (Lam et al., 2001). In addition, in rice and barley BI-1 has been shown to suppress fungal elicitor-induced apoptosis ((Huckelhoven et al., 2003); (Matsumura et al., 2003)) and overexpression of BI-1 in *Arabidopsis* was able to suppress bax-, hydrogen peroxide-, and salicylic acid-induced cell death (Kawai-Yamada et al., 2004). As such, BI-1 functions as an ancient suppressor of programmed cell death and is evolutionarily conserved. Moreover, the capability of animal Bcl2-like protein family to modify cell death processes in plants ((Dickman et al., 2001); (Xu et al., 2004)) points towards a common mechanism in plants and animals. Nevertheless, assuming there would be a role for Bcl2-related proteins in plants, a possible interplay between the Bcl2-family and the metacaspases should be studied. In yeast, expression of human pro-apoptotic

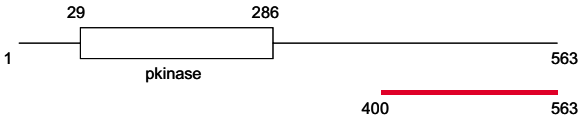
molecules (Bax) did not imply metacaspase activity as yeast metacaspase overexpression did not have an additive toxicity to Bax expression and Bax expression in a yeast metacaspase-deleted strain did not seem to be necessary for Bax toxicity (Guscetti et al., 2005). These data suggest that in yeast, the initiation of the apoptotic program and activation of the metacaspase in response to environmental signals, does not involve the Bcl2-family. However, this does not directly imply that this can be extrapolated to plants.

Finally, as BAP31 was picked up in the Y2H screen and in analogy with the mammalian field it is not inconceivable that the BAP31-like protein is a substrate of the metacaspase. The cleavage effect remains to be clarified but as another identified interactor in this Y2H-screening is a **myosine heavy chain-like protein** one can speculate that there could be a complex formation with this cytoskelet protein and BAP31, which is disturbed upon apoptotic triggering leading to membrane remodelling during the cell death process. A schematic representation of the protein and the domains within the protein, identified via Pfam, are given in Figure 6A. The corresponding cDNA that was picked op in the screening is also indicated.

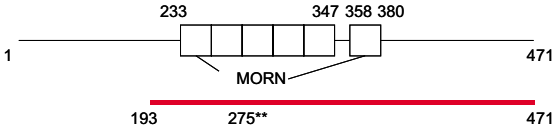
Another interactor identified in the Y2H screening is a **MAP kinase**. Only part of the cDNA of the corresponding MAP kinase protein was identified in the screening (Figure 6B). Eukaryotic serine-threonine mitogen-activated protein (MAP) kinases are key regulators of cellular signal transduction systems. MAPK activity is regulated through a MAPK cascade conserved in all eukaryotes. MAPK, the last kinase in the cascade is activated by phosphorylation, mediated by a MAPK kinase (MAPKK, MEK). This phosphorylation is in its turn activated by a MAPK kinase kinase (MAPKK, MEKK). MAPK cascades are evolutionarily conserved signalling modules with essential regulatory functions in eukaryotes. Mammals express at least four distinctly related groups of MAPKs, extracellularly-regulated kinases (ERKs), c-jun N-terminal kinases (JNKs), p38 proteins and ERK5 (Torres, 2003). Plant MAPK pathways have attracted increasing interest, resulting in the isolation of a large number of different components of MAPK cascades. All plant MAPK genes described so far belong to a single group,



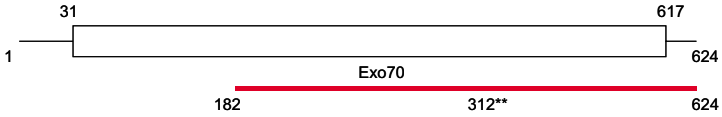
A At5g08120: myosine heavy chain-like protein



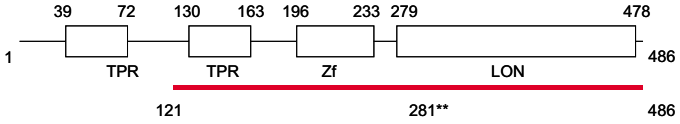
B At5g41990: MAP kinase



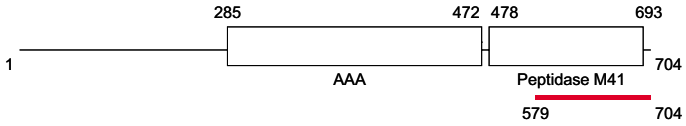
C At4g17080: PI 4-P 5-kinase



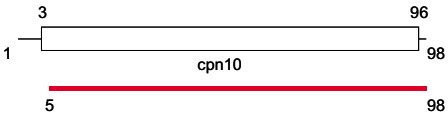
D At5g58430: Leucine zipper containing protein



E At1g18660: expressed protein



F At5g42270: FtsH protease



G At1g14980: Hsp10

the ERK subfamily. (Tena et al., 2001). The *Arabidopsis* genome encodes 23 putative MAPKs, 10 MAPKKs and 80 MAPKKKs. Plant MAPKs. In mammals, members of the ERK subfamily are mainly responsible for the transduction of mitogenic signals, but in plants ERKs have evolved in such a way to be able to transmit a broader range of stimuli. Plant MAPKs are likely to be involved in growth, development, cell cycle ((Takahashi et al., 2004); (Meskiene and Hirt, 2000)) and responses to endogenous and environmental cues. Indeed, several plant MAPKs are activated by a variety of stress stimuli including pathogen infection, wounding, temperature, drought, salinity, osmolarity, UV irradiation, ozone and reactive oxygen species ((Zhang and Klessig, 2001); (Jonak et al., 2002)). An increasing body of evidence suggests that a subset of plant responses to activation of the MAPK cascade (activated in response to biotic and abiotic stresses) is shared, such as the generation of reactive oxygen species (ROS) and the activation of early defense genes (Pedley and Martin, 2005).

Figure 6 (other side): Schematic representation of the Atmc3-interactors and their structural protein domains identified via Pfam. Overlap of the cDNA clone identified via Y2H with the corresponding protein is indicated by the red line. * indicates that different clones were picked up with different N-termini., ** indicates the PCR product could not be sequenced till the poly A tail, as only the prey-specific forward primer facing the C-terminus could be used in the sequencing reactions, so it is probable that the sequence is complete till the C-terminus, or at least the border is more downstream than has been sequenced. **A)** Pox A = Pox A repeat is found in the A-type inclusion protein of the Poxvirus family, Spectrin repeats are found in several proteins involved in cytoskeletal structure (Yan et al., 1993), Gemini AL2 is derived from Geminivirus AL2 protein. **B)** pkinase = protein kinase domain. **C)** PI 4-P 5-kinase = posphatidylinositol 4-phosphate 5-kinase, MORN = MORN repeat: Membrane Occupation and Recognition Nexus repeat is found in multiple copies in several proteins including junctophilins (Takeshima et al., 2000). However, the function of this motif is unknown. **D)** exo70 = Exo70 exocyst complex subunit. In *S. cerevisiae*, the exocyst complex is involved in the late stages of exocytosis (Hamburger et al., 2006). **E)** TPR = tetratricopeptide repeat, Zf = zinc finger, LON = ATP-dependent protease La (LON) domain. **F)** AAA = ATPase family associated with various cellular activities (Schmidt et al., 1999). Proteases of the AAA family share a conserved ATPase domain of 200-250 amino acid residues and they often perform chaperone-like functions that assist in the assembly, operation, or disassembly of protein complexes (Gottesman, 2003). Peptidase M41 = The peptidase M41 family belongs to a larger family of zinc metalloproteases and includes the cell division protein FtsH. **G)** cpn10 = 10 kDa chaperonin.

Indeed, upon activation, several plant MAPKs have been shown to translocate to the nucleus (Lee et al., 2004). In rice, a MAPK was shown to activate a transcription factor and hence regulate the expression of the PR genes (Cheong et al., 2003). Morover, the active defense mechanisms that plants can use against invading pathogens often include rapid cell death, known as the hypersensitive cell death. It was shown in tobacco that MAPK activation is probably required for tobacco cells to commit to a cell death program (Zhang et al., 2000b). As there might be a link between metacaspases and HR-cell death one could suggest that metacaspases could be a target for a plant MAPK in response to a biotic stress stimulus. To date, no substrates have been identified for any plant MAPKs, although transcription factors are most likely to be one of the putative targets. It is not excluded that Atmc3 could be a substrate of MAPKs. Phosphorylation of Atmc3 could have a regulatory function, regulating the metacaspase activity and as such its in- or activation could implicate a role in execution of the HR-process. In mammalian, caspases have known to be directly regulated by phosphorylation. Phosphorylation of procaspase-9 inhibited its protease activity (Cardone et al., 1998).

On the other hand, MAPKs could be a substrate of the metacaspase, which could play a role in inactivation of the MAPKs. Inactivation of the MAPK cascades is at least as important as activation of the MAPK as the outcome of a MAPK activation depends on the duration of its activation. MAPK inactivation processes are less well understood than the activation processes. How long a MAPK stays active can be affected by either how long the upstream kinase remains active and/or whether a MAPK phosphatase is induced. Cleavage of a MAPK by a metacaspase could possibly implicate an additional negative regulation of the MAPK activation.

In addition, in mammalia, caspase-dependent cleavage of components of the MAPK cascade has been demonstrated during apoptosis. Cleavage of MEKK has been established during genotoxin-induced apoptosis. Genotoxic agents induce activation thus phosphorylation of MEKK1 followed by cleavage of MEKK1 in a caspase-dependent manner into a smaller active kinase fragment which is a strong inducer of apoptosis. The truncated form can induce caspase activity, which in turn

stimulates more MEKK1-cleavage, as MEKK1 is itself a substrate for caspase-3. MEKK1 and caspases are thus predicted to be part of an amplification loop for increasing caspase activity during apoptosis ((Widmann et al., 1998b); (Widmann et al., 1998a)). In addition, cleavage of MEK was detected upon vitamin D_3-induced apoptosis. MEK is cleaved in a caspase-dependent manner in cells induced to undergo apoptosis by treatment with vitamin D_3. Cleavage resulted in nearly complete loss of full length MEK and subsequent removal of the MEK-ERK pro-survival signal. Finally MEKK-1 also undergoes partial proteolysis, producing fragments that exhibit constitute activity, further activating caspases and significantly enhancing the pro-apoptotic signal (McGuire et al., 2001). Interestingly, caspase-dependent cleavage of MEK has not been reported in genotoxin-induced apoptosis, which indicates that MEK cleavage is not a general phenomenon of the apoptotic program and suggests that a protease capable of cleavage is selectively induced by vitamin D_3. Hereby, it is important to note that, although a pan-caspase inhibitor can block MEK cleavage, this does not necessarily implicate that caspases are cleaved directly by caspases. However, cleavage of a MAPK has not been reported yet. Whether MAPK could be a possible substrate of Atmc3, in terms of negatively regulating the MAPK activation or during a cell death process, is a matter of assumption and needs more study to gain insight.

Another detected interactor was identified as a hypothetical protein (At4g17080), which contains a MORN repeat (Figure 6C). The MORN (Membrane Occupation and Recognition Nexus) repeat is found in multiple copies in several proteins including junctophilins (Takeshima et al., 2000). The function of this motif however is unknown. However the same protein is also identified as a putative **phosphatidylinositol (PI) 4-phosphate 5-kinase**. Members of inositol lipid kinases catalyze the synthesis of phospholipids from phosphatidylinositol. PI 4-phosphate 5-kinase, catalyzes the reaction from phosphatidylinositol 4-phosphate to phosphatidylinositol 4,5-bisphosphate (Mueller-Roeber and Pical, 2002). In animals, PIs and their derivatives operate in signal transduction pathways triggered by diverse stimuli such as growth factors, hormones, neurotransmitters and light (Berridge, 1993). The knowledge of plants however is quite limited, but there is evidence that a functional PI system

operates in plant cells, as shown by the identification of homologs of the components of the animal PI system. In addition, it has been demonstrated that PIs may participate in the regulation of cytoskeletal structures in plant cells (Yang et al., 1993) and in pollen tube growth (Kost et al., 1999). Morover, a number of reports also suggest that a wide range of signals, such as light, hormones and stress may mediate their effect through PI-dependent processes (Munnik et al., 1998) . In *Arabidopsis* cells hyperosmotic stress induced an increase in PI 4,5-bisphosphate (Pical et al., 1999).

Interestingly, phosphatidylinositol. PI 4-phosphate 5-kinase has been identified as a substrate of mammalian caspases during apoptosis. PI 4,5-bisphosphate is a direct inhibitor of initiator caspases 8 and 9 and executioner caspase 3, and as such cleavage of PI 5-kinase by caspase3 during apoptosis contributes to the abrogation of the anti-apoptotic effect of the phosphoinositides and subsequent progression of apoptosis (Mejillano et al., 2001). That there would be such a phosphoinositide regulation of metacaspases is at this stage speculative and requires more investigation.

A **leucine zipper containing protein** (At5g58430) was also identified as a candidate binding protein for Atmc3. The corresponding cDNA picked up in the screening starts at amino acid 182, till the end of the protein (Figure 6D). A leucine zipper is a configuration of a DNA-binding protein in which leucine residues on two protein alpha-helices interdigitate in zipper fashion to stabilize the protein (Wang et al., 2003). A protein contains a leucine zipper when seven leucines are present in a certain sequence. Leucine zipper containing proteins create scissors-like dimers that bind the DNA in the major groove of the DNA molecule. As leucine zipper containing proteins have the ability to dimerize, this could possibly lead to dimerization of the binding proteins, the metacaspases, as well. This may result in subsequent activation of the metacaspases. Notwithstanding the fact that upon BLAST a leucine zipper containing protein has been identified, prediction programs do not detect a leucine zipper.

Another candidate binding protein is an **expressed protein** (At1g18660). Structural analysis using Pfam could identify multiple motifs or domains. The first one is a tetratricopeptide (TPR) repeat, which can be detected two times in the protein. However, the cDNA picked up from the Y2H screening did not contain the first TPR repeat (Figure 6E). The tetratricopeptide repeat of typically 34 amino acids was first described in the yeast cell cycle regulator Cdc23p and later found to occur in a large number of proteins (Lamb et al., 1995). These TPR repeats function as scaffolds mediating protein-protein interactions, but common features in the interaction partners have not been defined. It has been proposed that TPR proteins preferably interact with WD-40 repeat proteins, but in many instances several TPR-proteins seem to aggregate to multi-protein complexes (eGoebl and Yanagida, 1991; Das et al., 1998). Interestingly, in mammalian systems Apaf-1, a WD40 containing protein, functions as a scaffold to activate procaspase-9 (Earnshaw et al., 1999). It might be speculated that we might deal with an analogous scaffold-mediated activation of the metacaspases as well. Moreover, a zinc finger can be identified, a C_3HC_4 type which is a RING finger. Zinc fingers are also known as DNA-binding motifs; most zinc finger proteins can mediate protein-protein interactions (Mackay and Crossley, 1998).Finally, an ATP-dependent protease La (LON) domain, has been characterized in the sequence of the candidate Atmc3 binding protein. The lon gene in *Escherichia coli* is a heat shock gene which encodes the ATP-dependent protease, La (Li et al., 2005). The tetrameric enzyme contains four binding sites for ATP, a DNA-binding domain, a proteolytic site, and a regulatory site that binds unfolded polypeptides. In addition, interestingly, via a psi-blast it was discovered that this expressed protein contains a certain homology with mammalian bifunctional apoptosis regulator (BAR). This protein contains among other domains, a SAM domain, which is required for its interactions with Bcl-2 and Bcl-XL, and for the suppression of Bax-induced cell death in both mammalian and yeast cells. In addition, BAR contains a Death Effector Domain (DED)-like domain responsible for its interaction with DED-containing procaspases, and suppression of FAS-induced apoptosis. In this way, BAR can bridge procaspase-8 and -10 and Bcl-2 into a protein complex. In other words, BAR can link the Bcl-2

family, governing the mitochondria-dependent apoptosis pathway or intrinsic pathway, to the extrinsic pathway, represented by the death receptors which utilize protein interaction modules such as death domains or death effector domains to recruit DED-containing proteases (procaspases-8 and 10). Therefore, BAR may represent a scaffold protein capable of bridging two major apoptotic pathways (Zhang et al., 2000a). As such, it might be speculated that the expressed protein picked up as a candidate Atmc3-binding partner functions as a scaffold protein for the metacaspase.

Another possible interactor of Atmc3CA is **FtsH protease**, namely VAR1. Figure 6F shows the structure of the protein, its consisting domains, and which part of the cDNA was identified in the Y2H screening. Filamentation temperature sensitive (FtsH) proteases are chloroplast ATP-dependent metalloproteases involved in the degradation of unassembled proteins, the repair of photosystem II (PSII) from photoinhibition, and the formation of thylakoids. In *Arabidopsis*, the family is encoded by 12 genes (*AtFsH1-12*) (Yu et al., 2004). Mutation in *VAR1 (AtFsH5)* and *VAR2 (AtFsH2)* gave rise to variegated plants with green- and white-sectored leaves. VAR1 as well as VAR2 may play an important role in degrading photodamaged subunits in photosystem II. Loss of VAR1 and VAR2 perhaps impairs the photoprotection mechanism and thylakoid development, causing leaf variegation as a consequence (Sakamoto et al., 2002). The one picked up in the Y2H is VAR1 and contains a conserved motif for ATPase and a metalloprotease, characteristic to FtsH proteins, and is targeted into chloroplasts (to date, only 4 members of the family have been found in chloroplasts: AtFsH1,2,5 and 8). A VAR1-fusion protein synthesized *in vitro* exhibited ATPase activity and partial metalloprotease activity. This protein is located to the thylakoid membrane and forms a complex with VAR2 (Zaltsman et al., 2005).

Interestingly, the *FtsH* gene of *Caulobacter crescentus* has been isolated and identified as a component of the general stress response of this organism. In *C. crescentus*, FtsH expression is transiently induced after temperature upshift and in stationary phase (Fischer et al., 2002). In addition, the *FtsH* gene of the wine bacterium *Oenococcus oeni* is involved in protection against environmental stress. The *FtsH*

gene proved to be stress responsive, since its expression increased at high temperatures or under osmotic shock (Bourdineaud et al., 2003). Even more interestingly, these *FtsH* genes seem to be related with HR in higher plants. It was reported that the chloroplast gene *FtsH* was downregulated before HR caused by tobacco mosaic virus (TMV) infection to resistant tobacco plants (Seo et al., 2000). The downregulation of the *FtsH* gene is reminiscent of the repression of photosynthetic genes in *N. benthamiana*. FtsH may be involved in the degradation of chloroplast D1 proteins that are damaged by reactive oxygen species (ROS) produced by an imbalance in the PSII reaction. Any decrease in FtsH protein concentration in TMV-infected leaves is supposed to inhibit PSII activity, this in turn causes ROS accumulation in the chloroplast, inducing HR in leaf tissue. As such, once again a link between a possible bindingspartner of Atmc3 and HR is revealed. What exactly could be a possible function of the metacaspases herein should be discovered by other means than yeast two-hybrid. Moreover, the discrepancy of an interaction between a chloroplast protein and a cytosolic protein should be considered, although a relocalization of either one or both of the proteins under certain conditions cannot be excluded.

The interactor which have been found the most in theY2H screening, no less than 67 times, is the **10 kDa heat shock protein (HSP10) or chaperonin 10 (Cpn10)** (Figure 6G). The related protein Cpn10-like protein, was detected 27 times. Heat shock proteins are produced not only in response to heat, but also in response to oxidative stress and osmotic stress. Moreover, synthesis of HSPs in plants is also linked to certain stages of development, such as embryogenesis, germination, pollen development and fruit maturation (Sun et al., 2002). Induction of HSP gene expression upon environmental stresses point to the hypothesis that these proteins play an important role in stress tolerance. Indeed, any of the stress stimuli that are capable of triggering apoptosis, such as oxidative stress and heat shock, induce the synthesis of diverse HSPs that seem to confer a protective effect against a wide range of cellular stresses. These highly conserved abundantly expressed proteins have diverse functions including protein complex assembly, trafficking, folding and targeting of

damaged proteins to the proteasome (Kiang and Tsokos, 1998). However, recent evidence in mammalian systems, indicates a direct role for HSPs in apoptosis. Although, an implication for the HSPs in apoptosis is reminiscent with its function as chaperones. Namely, the highly dynamic process of physical interaction and conformational alteration among the protein components of the cell death machinery is central to the underlying mechanism of the activation of the cell death process. Therefore, molecules such as chaperones, which can influence the assembly, transport and folding of other proteins, may directly affect the execution or inhibition of apoptotic signalling pathways (Xanthoudakis and Nicholson, 2000). Many HSPs are anti-apoptotic and directly inhibit caspase activation. HSP70 binds to apoptosis protease activating factor-1 (Apaf-1) and apoptosis inducing factor (AIF). Binding to Apaf-1 it prevents recruitment of procaspase-9 to the apoptosome, thereby inhibiting caspase-9 activation; whereas binding to AIF prevents chromatin condensation (Ravagnan et al., 2001). In contrast, HSP27, a member of the small HSP family, has been shown to bind/sequester cytosolic cytochrome c from the apoptosome and prevent procaspase-9 activation (Bruey et al., 2000). However, others demonstrate that HSP27 antagonizes apoptosis downstream of caspase-9 activation by binding to procaspase-3 and blocking its proteolytic activation (Pandey et al., 2000). In addition, the small heat shock protein aB-crystallin has been shown to inhibit the autoproteolytic maturation of the p24 partially processed (the prodomain and the large subunit) caspase-3 intermediate (Kamradt et al., 2001). Finally, a positive influence of at least one chaperone, HSP60 on the proteolytic maturation of precursor executioner caspase, procaspase-3. A direct interaction between caspase-3 and HSP60 in Jurkat T-cells undergoing apoptosis was able to substantially accelerate the maturation of procaspase-3 by different upstream activator caspases. Moreover, this effect was dependent of ATP hydrolysis, suggesting that the ATP-dependent foldase activity of HSP60 improves the vulnerability of procaspase-3 to proteolytic maturation to upstream caspases (Xanthoudakis et al., 1999). HSP60 is often found to act in combination with HSP10 (Samali et al., 1999).On the other hand, overexpression of the combination of HSP60 and HSP10, or of HSP60 or HSP10 individually protected

myocetes against apoptosis. This protection was found to be accompanied with decreases in mitochondrial cytochrome c release and caspase-3 activity and increases in ATP recovery and activities of complex III and IV in mitochondria (Lin et al., 2001). This discrepancy that overexpression of mitochondrial chaperonins (HSP60) may be different in myocytes from that seen in T cells, such as Jurkat cells, might be due to the fact that the latter are already destined to undergo apoptosis while the myocytes are induced to undergo apoptosis. In plants, a function of HSPs in cell protection and stress tolerance has also been described, which is (as in mammalian) likely to be linked to their function as a molecular chaperone, which binds and stabilizes other proteins and thereby controlles folding, oligomeric assembly, transport or disposal by degradation (Hendrick and Hartl, 1995). In *Arabidopsis*, Cpn10, which is a co-factor of Cpn60, showed approximately 50% identity to the mammalian mitochondrial Cpn10 and was found to regulate Cpn60-mediated protein folding. It was found to be expressed uniformly in various organs and was markedly induced by heat shock treatment (Koumoto et al., 1996). However, recently a function of HSP90 in disease resistance has been suggested. The cytosolic HSP90 was found to interact with RPM1, a disease resistance protein, thereby regulating RPM1 stability and its function (Hubert et al., 2003). Interestingly, a role for the Type I metacaspases in disease resistance has already been suggested (due to presence of the LSD1-like zinc fingers).

In mammalia, HSP90 was also identified as a substrate of the caspases. During radiation-induced apoptosis in breast tumor cells, HSP90 and keratins were identified as candidate substrates of apoptosis-associated proteolysis by a 2D gel electrophoresis (Prasad et al., 1998). Hereby, the insoluble network of intermediate filament proteins of epithelial cells (keratins), and the associated proteins (HSP90) constitute targets of caspase-mediated proteolysis during apoptosis triggered by ionizing radiation.

Conclusively, a possible role for cpn10 as an interactor of the metacaspase, can be a restriction of the metacaspase activity either through direct binding of the HSP to the cleaved form of the metacaspase and thereby inhibiting its activity or either through binding to the proform of the metacaspase and thereby preventing its

proteolytic maturation. Here, in this Y2H screening, the screening was performed with the full length protein or proform of Atmc3CA and thus the second explanation seems more likely. A second possibility is that the HSP is cleaved by the metacaspase and is hence a substrate. However, the cleavage effect remains unclear. Nevertheless, as the HSPs are important molecular chaperones, leading to stability, correct folding, transport, oligomeric assembly, transport or disposal of *other* proteins, its inactivation during apoptosis would be detrimental.

Although, immunogold and immunoblot analyses revealed a mitochondrial localization of the cpn10 (Koumoto et al., 1996), and Atmc3CA was shown to localize predominantly in the cytosol (D. Vercammen, unpublished results) an interaction between the two proteins cannot be excluded. As it might be possible, that upon apoptotic stimuli (or other stimuli), an altered subcellular localization of the HSPs, e.g. by rupture of the mitochondrial membrane, might take place. The reason that the interaction with cpn10 was identified 67 times (and 27 times for the cpn10-like protein) could be that the HSPs are small proteins and therefore might be more easy to pick up. In addition, it should be remarked that HSPs as small chaperonins stick to many proteins and therefore a confirmation by other methods and investigation of the functionality of the interaction is required, like it is the case for any two-hybrid interaction.

Another interactor of Atmc3CA identified in the yeast two-hybrid screening is the cell cycle regulator protein Kip-related protein 2 or **KRP2, of which the full length cDNA was picked up** (Figure 7A). Kip-related proteins are cyclin-dependent kinase (CDK) inhibitors, generally known as CKIs, and regulate the CDK activity by binding to the CDK/cyclin complex, which induces cell cycle arrest or delay of the cell cycle progression in response to intracellular or extracellular signals. CDKs are a conserved family of serine/threonine kinases which have the necessity of cyclin binding for their activity (Pines, 1994) and are essential for the G1-S and G2-M transition. The sequential and transient activation of different CDK/cyclin complexes during cell cycle progression results in the phosphorylation of a plethora of substrates. The change in

A

B

```
                10        20        30        40        50        60        70
           ....|....|....|....|....|....|....|....|....|....|....|....|....|
KRP2       MAAVRRRERDVVEENGVTTTTVKRRKMEEEVDLVESRIILSPCVQATNRGGIVARNSAGASETSVVIVRR
P27KIP1    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                80        90       100       110       120       130       140
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
KRP2       RDSPPVEEQCQIEEEDSSVSCCSTSEEKSKRRIEFVDLEENNGDDRETETSWIYDDLNKSEESMNMDSSS
P27KIP1    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~MSNVRVSNGSPSLERM

               150       160       170       180       190       200       210
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
KRP2       VAVEDVESRRRLRKSLHETVKEAELEDFFQVAEKDLRNKLLECSMKYNFDFEKDEPLGGGRYEWVKLNP~
P27KIP1    DARQAEHPKPSACRNLFGPVDHEELTRDLEKHCRDM~~~EEASQRKWNFDFQNHKPLEGKYEWQEVEKGS

               220       230       240       250       260       270       280
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
KRP2
P27KIP1    LPEFYYRPPRPPKGACKVPAQESQDVSGSRPAAPLIGAPANSEDTHLVDPKTDPSDSQTGLAEQCAGIRK

               290       300       310       320
           ....|....|....|....|....|....|....|....|.
KRP2
P27KIP1    RPATDDSSTQNKRANRTEENVSDGSPNAGSVEQTPKKPGLRRRQT~
```

Figure 7: A) Schematic representation of the structural domains, identified by pfam, in the Atmc3-interactor At3g50630, KRP2. The red line indicates the overlap with the cDNA clone identified via Y2H. CDI = CDK inhibitor domain. **B) Homology between plant KRP2 and mammalian Cip/Kip inhibitor p27Kip1 is restricted to the CDK-binding domain.** The alignment was performed with the ClustalW software.

biological activity of various substrates as a consequence of their phosphorylation status directs the unidirectional progression through the cell cycle. Activity of the cyclin/CDK complexes is regulated by a variety of mechanisms: (de)phosphorylation (Dunphy, 1994), controlled degradation of cyclin subunits (Peters, 1998), tight association of the CDK/cyclin complexes to the CKIs (Sherr and Roberts, 1995). In mammalia, two different CKI families can be distinguished, based on their shared structural features and biochemical functions: the INK4 and Kip/Cip families (Sherr and Roberts, 1999). Members of the INK4 family are characterized by the presence of multiple ankyrin-type repeats for CDK binding and only inhibit a small subset of CDKs that are primarily responsible for passage through G1. Inhibitors of the Cip/Kip family ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$) bind and inhibit a broader range of CDKs and function in heterotrimeric complexes with the CDKs and cyclins. Kip/Cip binding does not dissociate the CDK/cyclin complex, but distorts the catalytic ATP-binding center of the CDK subunit. Interspecies homology between the mammalian Kip/Cip inhibitors and the plant CKIs is restricted to a short amino-acid region (in the CDK-binding domain) (Figure 7B, also see Chapter 5), however, because of this homology, the name Kip-related proteins was suggested for the seven CKIs found in the *Arabidopsis* genome (KRP1-7) (De Veylder et al., 2001). Nevertheless, some family members are also known under other names such as ICK1 (KRP1), ICK2 (KRP2) ((Wang et al., 1997); (Lui et al., 2000)). So far, no *Arabidopsis* homologues to the INK4 inhibitors have been identified (Vandepoele et al., 2002). The functions of KRP2, the putative roles and importance of the interaction with Atmc3, a possible role for Atmc3 in the cell cycle, and the link between the cell cycle and cell death process are elaborately discussed and studied in Chapter 4.

In conclusion, yeast two-hybrid screening has delivered several candidate interacting proteins for Atmc2 and Atmc3. It is suggested that both substrates and regulator molecules of the metacaspases were picked up in the Y2H screening. Regulator molecules may control the function of the metacaspases by regulating its activity. It was suggested that some interactors may function as scaffold proteins

which contribute to the formation of a cell death signalling complex, hereby regulating the activity of the metacaspases (expressed protein At1g18660, BAP31-like protein). They are most probably interacting with the prodomain of the metacaspases, as in analogy with the mammalian initiator caspases (Ho and Hawkins, 2005). Other interacting proteins have the ability to dimerize which could result in dimerzation of the metacaspases, influencing hereby the activation of the latter (14-3-3 proteins, Leucine zipper containing proteins). Most of the interactors (both substrates and interactors) give some hints towards possible functions of the metacaspases based on analogies with the mammalian field e.g. with respect to cell death and cell cycle. The fact that for many of the metacaspase-interactors there is an analogy with the mammalian field, make the results even more promising, as this could imply a functional conservation of the metacaspases throughout different organisms. Although, it should be considered that additional experiments, like *in vitro* and *in vivo* co-immunoprecipitation, or other protein-protein interaction methods (such as Tandem Affinity Purification, see Chapter 6) are needed to confirm the interactions. Colocalization of the interacting partners at the time of interaction and biological relevance of the interaction are also important factors which need further thorough investigation. Whether or not the interactions have a biological relevance, cannot be concluded from mere Y2H-studies. Therefore, the actual function of the interaction has to be studies by functional assays. In order to achieve this, the function of the interactors has to be evaluated. Transgenic plants with perturbed levels of the interactors can help to resolve this issue. Indeed, it is possible that the interactor contains an Atmc2 or Atmc3 binding / cleavage site (which is currently not identified yet) that is simply recognized by the metacaspase, but that the interaction itself is not really biological relevant.

Considering the plethora of substrates and interactors that have already been identified for the mammalian caspases and the fact that for many of the identified substrates of the caspases the functional consequences of their cleavage are still unknown to date and have only been inferred from their normal functions, demonstrates that the field continues to evolve and that it forms a challenge to

elucidate the importance of the substrate cleavage by these proteases. Therefore, the role of metacaspase cleavage can be experimentally assessed by expressing substrate proteins that have mutant metacaspase cleavage sites or by expressing protein fragments of the caspase-cleaved products. Consequently, the identification of the first interactors or substrates of the metacaspases form just the beginning of further promising studies.

4.5 Experimental procedures

4.5.1 Y2H mating

Yeast expression vectors for bait (pDEST32, LEU selection marker, DB containing vector) and pey protein (pDEST22, TRP selection marker, AD containing vector) were provided with the Proquest yeast two-hybrid system (Invitrogen, Carlsbad, USA) and were made Gateway compatible. Both vectors contain a C-terminal fusion with the DNA binding domain or activation domain for expression of the fusion bait or prey protein respectively. Both wild type and mutant versions of the metacaspases were cloned in both vectors by Gateway recombinatorial cloning, resulting in pDEST22-Atmc1-9(CA) and pDEST32-Atmc1-9(CA). For mating purposes, yeast strain pJ69-4A and -4a (MATa *trp1-901 leu2-3,112 ura3-52 his3-200 gal4*" *gal80*" *LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*) were used (James et al., 1996) (reporter genes *HIS3* and *LacZ*). pDEST22-Atmc vectors were transformed in pJ69-4A strain and pDEST32-Atmc vectors in pJ69-4a strain, using the Lithium Acetate (LiAc) method and 0.5 mg-1 mg of the plasmid DNA (Gietz et al., 1992). Selection in a minimal dropout (SD) medium containing all amino acids except Trp (for pDEST22) or Leu (for pDEST32) (Clontech, Palo Alto, CA, USA).

Mating metacaspases versus metacaspases

For the metacaspase interactome, the Y2H mating was performed on a one-by-one basis. Yeast cells of both types were inoculated in YPD (Yeast-Peptone-Dextrose) medium (Clontech, Palo Alto, CA, USA) and grown for 6 to 12 hours. Subseqently, growth on SD-Leu-Trp medium selected for mated, diploid, colonies. These positive colonies were then tested for His-prototrophy to select for activation of the reporter gene, hence interaction between bait and prey protein. Due to self-activation of bait metacaspases the medium was supplemented with 5mM AT to overcome auto-activation.

4.5.2 Y2H screening

Bait vector pGBT9 and yeast strain HF7c (*MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3, 112gal4-542 gal80-538 LYS2::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3 URA3::GAL4*$_{17mers(3x)}$*-CYC1*$_{TATA}$*-LacZ)* were provided with the Matchmaker yeast Two-Hybrid system 2 (Clontech,

Palo Alto, CA, USA). The HF7c yeast strain contains two reporter genes: *HIS3* and *LacZ*. Yeast cells were grown at 30 °C in a complete YPD medium (Clontech) or upon transfection with bait or prey vector in SD-Trp or SD-Leu. The bait vector was made Gateway compatible in the lab (M. Karimi) to pGBT9g, and Atmc2CA and Atmc3CA were cloned into the yeast expression vector by recombinatorial cloning, resulting in pGBT9g-Atmc2CA and pGBT9g-Atmc3CA respectively. The pGBT9 gate yeast expression vector contains a C-terminal fusion with the DNA binding domain for expression of the fusion bait protein and TRP as a selection marker. The GAL4 activation domain cDNA fusion library was a gift from Csaba Koncz (Max-Planck Institut für Züchtungsforschung, Cologne, Germany) and was cloned into the vector pACT2, with LEU as a selection marker (Bhalerao et al., 1999).

First, HF7c yeast strain was transformed with bait plasmid (0.5mg-1 mg), using the LiAc method in accordance to the small transformation protocol (Gietz et al., 1992). For the screening 200 ml culture of the *Saccharomyces cerevisiae* strain HF7c, containing the bait vector pGBT9g-Atmc2CA and pGBT9g-Atmc3CA respectively (TRP as selection marker), was cotransformed with 20 mg of the library DNA (LEU as selection marker), and 20 mg herring tested carrier DNA using the lithium actetate method. To estimate the number of independent transformants, 1/1000 of the transformation mix was plated on Leu^- and Trp^- medium. The rest of the transformation mix was plated on medium to select for histidine prototrophy (Leu^-,Trp^-,His^-) supplemented with 5 mM 3-aminotriazole (Sigma-Aldrich, Bornem, Belgium). After 2 days of growth at 30°C (till 10 days), colonies were restreaked on histidine-lacking medium. His^+ colonies were tested for their ability to grow on histidine lacking medium supplemented with 20 mM AT to test the strength for the interaction. Moreover, a lift filter b-gal assay was performed to test activation of the second reporter gene lacZ (Yeast Protocol Book, Clontech Laboratories, Palo Alto, CA, USA). Subsequently, on His+ colonies a yeast colony-PCR was performed with prey-specific primers to specifically amplify the cDNA insert, using the primers 5´-ATACCACTACAATGGATG-3´ and 5´-AGTTGAAGTGAACTTGCGGG-3´. Therefore, yeast cells were lysed before in a lysis buffer (0.1 M NaPO4 buffer of pH7.4, 2.5 mg/ml Zymolase 20T, 21100 U/g (Seikagaku Corporation, Tokyo, Japan)) to break open the cells and release the DNA. The obtained PCR products were sequenced, all sequencing was done at the Department of Plant Systems Biology, and subsequently subjected to a blast against an *Arabidopsis thaliana* database (NCBI, National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov/BLAST). The program compares nucleotide given nucleotide sequences to protein (blastx) or nucleotide (blastn) sequence databases and calculates the statistical significance of matches. To analyze conserved protein regions ore protein domains which could lead to an indication in protein function, pfam was used (Bateman et al., 2002). Pfam is a database of multiple alignments of protein domains or conserved protein regions and available

on the web (http://pfam.wustl.edu). The alignments represent some evolutionary conserved structure which has implications for the protein's function. For the confirmation, first the mixture of bait and prey plasmids were isolated from yeast using the Zymoprep kit (HiSS Diagnostics GmbH, Germany, Freiburg). Subsequently, this mixture is transformed in a Leucine deficient E.coli strain (KC8) (Clontech, Palo Alto, CA, USA) via electroporation, and hence only those colonies harbouring the prey plasmid (LEU selection marker) can survive on M9 medium (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.1% NH_4Cl, 0.005% NaCl, 1mM Thiamine-HCl). Finally, the extracted prey plasmid (0.5 mg-1 mg) was used to retransform HF7c containing the bait plasmid, to confirm the interaction in yeast.

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Chapter 5: KRP2 is a substrate of a metacaspase

5.1 Abstract

Yeast two-hybrid screening revealed a strong interaction of KRP2 with Atmc3CA. To decipher the subdomain of interaction, different deletion constructs of KRP2, representing different functional domains of the protein, and the full length protein were tested for their interaction with Atmc3CA in a Y2H assay. Only the full length protein was able to interact. Recombinant Atmc9 was used as a representative of metacaspase activity and was found to be able to cleave KRP2, both *in vitro* translated KRP2 and transgenic KRP2 extracted from plant tissues. Using different deletion constructs of KRP2, it could be demonstrated that the cleavage site of the KRP2 was resided in the N-terminal domain. An *in silico* search for putative Atmc9-cleavage sites revealed that there were more than one putative cleavage sites in this N-terminal region. Moreover, the tetrapeptide sequences with the highest cleavage probability score were also detected in this domain. In addition, KRP2 was found to be colocalized with KRP2 in phloem pole-specific pericycle cells in root tissues. Taken together these data suggest that KRP2 is a likely *in vivo* substrate of Atmc3. As such, metacaspases would have a regulatory role in cell cycle progression by controlling KRP2 abundance, which in its turn regulates CDK/cycling activity.

5.2 Introduction

In plants, Kip related proteins (KRPs) have the ability to inhibit cell cycle progression in response to intra- or extracellular signals through binding to the cyclin-dependent kinase (CDK)/cyclin complex. This results in inhibition of CDK-activity, finally leading to cell cycle arrest (De Veylder et al., 2001; Verkest et al., 2005a) (also see Chapter 4). A significant homology of the KRPs to the mammalian Cip/Kip inhibitors ($p21^{Cip1}$ and $p27^{Kip1}$) was detected at the extreme C-terminal end of the protein (about 25 amino acids), so predominantly in the CDK-binding domain (De Veylder et al., 2001). Therefore, the name Kip-related proteins was suggested. In the genome of *Arabidopsis* seven KRPs have been found to date (De Veylder et al., 2001) (also see Chapter 4). Concerning the sequence similarity amongst the plant KRPs, detailed analysis allowed the identification of several sequence elements shared by different KRPs. There are only three C-terminally located motifs which are conserved in all plant inhibitors (De Veylder et al., 2001), of which the two last motifs are present in mammalian inhibitors also. In addition to the different motifs found in the KRPs, they can also be distinghuished by the presence (KRP2, KRP4, KRP5 and KRP7) or absence (KRP1, KRP3 and KRP6) of a nuclear localization signal (NLS).

The **CDK-inhibitory activity** of the KRPs was demonstrated both *in vitro*, by adding recombinant KRP to partially purified CDK complexes (Lui et al., 2000), and *in vivo*, by overexpressing diverse *KRP* genes in *Arabidopsis*. Plants overexpressing KRP2 showed a decrease of more than 50% in CDK activity which correlated with a decrease in cell division rate, resulting in leaves whose cell number is dramatically low (De Veylder et al., 2001). This decrease in cell number (more than 10-fold) is accompanied by a change in leaf morphology. Furthermore, KRP2 was shown to inhibit A-type CDKs ((Lui et al., 2000); (De Veylder et al., 2001); (Verkest et al., 2005b)) and recently besides D-type cyclins also A-type cyclins (Coelho et al., 2005). As A- and D-type cyclin containing complexes are suggested to have a role in both the

onset and progression through S-phase, a role for these KRP proteins in these stages of the cell cycle process is proposed (Verkest et al., 2005a). Similarly, overexpression of KRP1 in *Arabidopsis thaliana* also resulted in decreased CDK-activity and reduced cell number, and the transgenic plants showed an inhibition of plant growth and altered organ morphology (Wang et al., 2000).

Regarding the **function of the plant KRPs**, different roles have already been reported in literature. First, KRPs are able to integrate developmental signals into the core cell cycle machinery. For example, the mitogenic hormone auxin induces downregulation of *Arath; KRP2*, which results in re-entry of quiescent root pericycle cells into the **cell cycle** (Himanen et al., 2002). Recently, CDK inhibitors (CKI) in plants have been established to play a role in the onset of **endoreduplication**, which is an alternative cell cycle where DNA replication still occurs however not followed by mitosis and cytokinesis. The onset of the endocycle seemed to be accompanied with a decline in CDK activity ((Verkest et al., 2005b); (Joubes et al., 1999)) and KRPs have been demonstrated to control this decrease in CDK activity. Indeed, mild overexpression of KRP1 and KRP2 in *Arabidopsis thaliana* leads to increased ploidy levels ((Verkest et al., 2005b); (Weinl et al., 2005)).

In **mammalian systems**, Cip/Kip inhibitors are also suggested to play a role in **apoptosis** as they appeared to be substrates of caspases during the cell death process. In mammalia, different cell cycle regulators are already identified as targets for cleavage by caspases (Fischer et al., 2003) as hence cell cycle progression during apoptosis is arrested (Jacotot et al., 2000). Cleavage of $p21^{Cip1}$ and $p27^{Kip1}$ at $DPSD^{139}$ and $EQSD^{108}$, during apoptosis of human endothelial cells resulted in loss of the nuclear localization signal of the inhibitors. Cytoplasmic translocation of these CDK inhibitors was accompanied with a substantial reduction in their association with nuclear cyclin/cdk2 complexes, leading to a dramatic induction of cdk2 activity (Levkau et al., 1998). Subsequently, cdk2 activity is in its turn necessary for chromatin condensation and other manifestations of cell death, including cell shrinkage and loss of adhesion to substrate (Harvey et al., 2000). Although the effect of cleavage of $p21^{Cip\ 1}$ on its function is fairly well characterized, with the cleavage product being

unable to suppress apoptosis and the intact p21 playing a protective role in apoptosis, the role of p27-cleavage remains controversial. In contrast, it was shown that transfection of cleavage products protected cells from apoptosis, while stable expression of uncleavable $p27^{Kip1}$ modestly increased the sensitivity of transfected cells to drug-induced apoptosis (Eymin et al., 1999).

Lately, arguments have augmented to believe that caspases are not only killers, but may also be involved in cell differentiation, spreading and cell proliferation. **Caspases are required for cell cycle progression**, as they might confer additional checkpoints to assure that only healthy cells will complete the cell cycle. In that sense, activation of caspases during cell cycle results in the cleavage and inactivation of proteins that act as negative regulators of the cell cycle machinery or activation of other cell cycle-regulatory proteins (Los et al., 2001). An apparently novel non-apoptotic caspase-like activity has been described, which cleaves $p27^{Kip1}$ at $DPSD^{139}$ and was demonstrated to regulate the expression of $p27^{Kip1}$ in non-apoptotic lymphoid cells. Inhibition of this caspase-like activity *in vivo* promotes an accumulation of full length $p27^{Kip1}$, as well as a decrease in cell proliferation. This caspase-like activity was not inhibited by the broad spectrum caspase inhibitor z-VAD-fmk and furthermore none of the human caspases (1-10) were able to cleave the $DPSD^{139}$ substrate *in vitro*. Based on this, the protease was catalogued as a protease with novel caspase-like activity, designated as KIPase (Medina-Palazon et al., 2004). Conclusively, KIPase is a currently unidentified caspase-like enzyme (whether or not it is a member of the caspase family remains unclear until now), which regulates the abundance of $p27^{Kip1}$ in a proliferation-dependent manner. It has been suggested that paracaspase, a distant relative of the caspases, could be a candidate gene for KIPase (Medina-Palazon et al., 2004). In addition, it was discovered that caspases were required for proliferation of primary human T-cells *in vitro*, which favoured the role of caspases in cell proliferation ((Alam et al., 1999); (Kennedy et al., 1999)). It was demonstrated that T-cell receptor activation leads to activation of an upstream caspase (most likely caspase-8) but not to apoptosis. The apoptosis-independent function of the caspases is associated with selective substrate processing, leaving the other vital proteins

intact. Selective substrate processing might be regulated by activation of anti-apoptotic factors, via a compartimentalized activation of caspases, through the existence of scaffold proteins or a different accessibility of cleavable substrates, or through limited activity of caspases during apoptosis-independent functions (Schwerk and Schulze-Osthoff, 2003).

The involvement of CDK inhibitors in apoptosis and caspases in cell proliferation in mammalian systems, illustrates the **connectivity between cell cycle and cell death process**. Intriguingly, in plants a role for KRPs in cell death has also been suggested, as misexpression of KRP1 in *Arabidopsis* trichomes induces cell death (Schnittger et al., 2003). However, this phenotype seemed to be linked to the developmental program of trichomes, because for other cell types no cell death fenotypes have been observed upon KRP overexpression. Furthermore, currently it is unclear whether the observed trichome cell death phenotype is linked with cell death directly being activated as a consequence of a discrepancy between DNA content, due to a compromised endoreduplication program, and cell size. The fact that by means of Y2H, KRP2 is identified as a potential interactor, more particular as a potential substrate for a metacaspase, which is a distant related caspase, can also point towards a putative role for the metacaspases in the cell cycle process and provide a link between the genetic networks that govern cell-cycle progression with those that govern cell death.

In this chapter, a potential link with the cell cycle was investigated by assessing the interplay of the metacaspases with KRP2 and in extenso by assessing the interaction of *Arabidopsis* metacaspases with 92 core cell cycle proteins by yeast two-hybrid.

5.3 Atmc3 only interacts with the full length KRP2 protein

Yeast two-hybrid screening revealed a strong interaction of KRP2 with Atmc3CA (also see Chapter 4, Table 2). From this screening, a full length cDNA clone of the KRP2 was picked up (Chapter 4, Figure 7A).

To elucidate the **specificity** of this interaction, Atmc3CA was also tested for its interaction with other members of the plant KRP family. A yeast two-hybrid was performed with KRP5, KRP6 and KRP7 as fusion prey proteins (De Veylder et al., 2001). Specificity of Atmc3CA to KRP2 was shown as no other members of the KRP family revealed interactions (data not shown).

To delineate the **domain of interaction** we assessed the interaction of Atmc3 with different deletion constructs of KRP2 via Y2H (Figure 1A). The KRP2 full length protein consists of 209 amino acids (AA): an N-terminal domain, starting from AA1 to AA110 and a C-terminal domain, from AA110 to AA209. Currently, the function of the N-terminal domain remains unclear. However, the C-terminal part of the KRP2 protein is known to be sufficient for interaction with CDK and cyclins (Lui et al., 2000). This C-terminal region can be subdivided into a cyclin binding domain (residues 110-190) and a CDK binding domain (residues 170-209). It was demonstrated that both domains are necessary for CDK inhibition (A. Verkest, non published results). Based on this, different deletion mutants were constructed and available in the Department (A. Verkest, unpublished results): a mutant only containing the N-terminal part of the protein (KRP2N), one containing the C-terminal region (KRP2CK), and one with or the cyclin (KRP2C) or CDK binding domain (KRP2K). Results are depicted in Figure 1B. Interaction with Atmc3CA could only be demonstrated with the full length KRP2 protein. None of the domains interacted with Atmc3CA. This indicates that the full length protein is necessary for interaction, or that the caspase bindingssite is located at a region overlapping different domains.

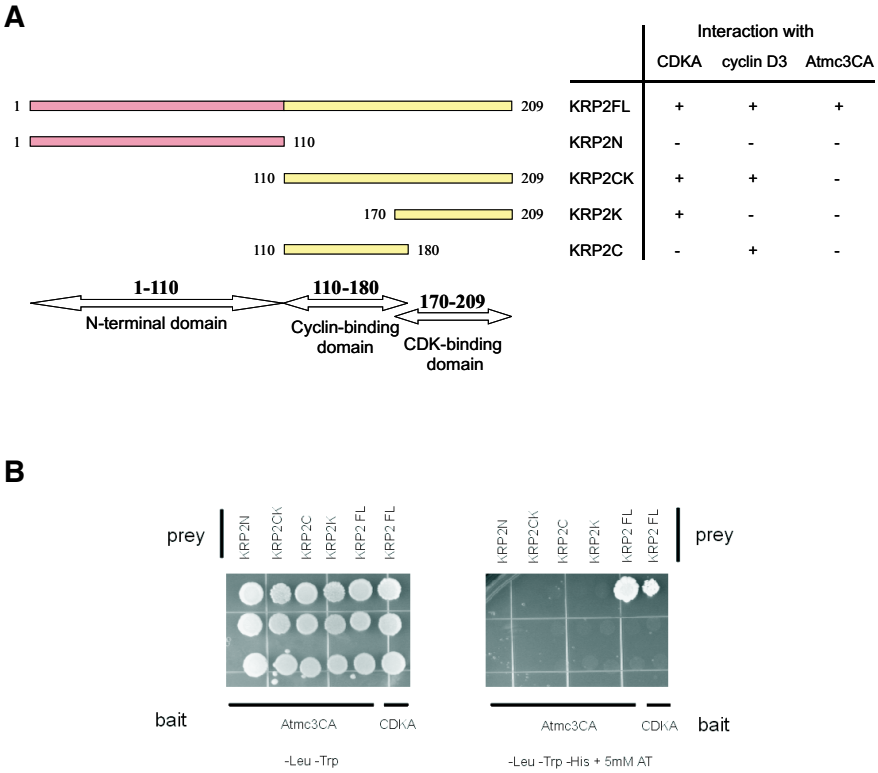


Figure 1: Yeast two-hybrid of Atmc3CA with KRP2 full length and different deletion mutants. A) Scheme of constructs. Data of interaction of KRP2 FL/mutants with CDKA en CyclinD3 are provided by A. Verkest. **B)** Interaction of KRP2 FL with Atmc3CA, not with the deletion mutants. The interaction of KRP2 FL and CDKA was used as a positive control.

If the interaction is dependent on the full length KRP2 protein this implies that also the N-terminal region is of importance. Currently, there is little known about the exact function of the N-terminal domain. It has been suggested that, despite the fact that the major CDK and cyclin binding domain of the KRPs have been mapped at the C-terminal domain (Wang et al., 1998), the additional motifs found in the N-terminal part of the KRPs could help to determine the specificity toward different cyclin/CDK complexes or interaction with other unknown proteins (De Veylder et al., 2001).

In addition, the fact that N-terminal plant CKI sequences are so highly diverse could point towards additional functional implications, imply different specificity towards CDK/cyclin complexes or different regulatory mechanisms. Indeed, the N-terminal region of KRP1 of *Arabidopsis* was suggested to *negatively regulate CDK inhibitory (CKI) function* as an increase of yeast two-hybrid interaction of KRP1 with CDKs and cyclins could be observed upon deletion of the N-terminal region (Wang et al., 1998). This was due to an increased affinity of the CDK-inhibitor for the CDK/cyclin complex. Similarly, immunoprecipitation of both full length KRP2 and the N-terminal deletion mutant (KRP2CK) with CDKA-specific antibodies demonstrated that the deletion mutant of KRP2 showed a higher affinity for CDKA than the full length protein (A. Verkest, unpublished results). A similar deletion resulted in an enhancement of the phenotype upon KRP1 overexpression in *Arabidopsis* (Schnittger et al., 2003). The N-terminus could also possibly function to *regulate the stability* of the KRP proteins. Indeed, KRP2 proteins are very unstable and their degradation depends on the proteasome (Verkest et al., 2005b). Similarly, removal of the N-terminal region enhances the KRP1 protein level (Zhou et al., 2003).

5.4 KRP2 is an *in vitro* substrate of metacaspase 9

We assessed whether KRP2 is a substrate of metacaspase 3. To test whether KRP2 is cleaved by the metacaspase, active recombinant metacaspase was needed. Therefore, recombinant His-tagged *Arabidopsis thaliana* metacaspase 3 was purified from *E. coli*, both a full length (data not shown) and a prodomainless version (Figure 2A). The prodomainless version of Atmc3 is the full length protein without the prodomain, and it was anticipated that this form would be more prone to (self)activation as only separation of the two subunits should still occur in order to autoprocess. However, as there was no antibody available against the Atmc3 protein, autoprocessing could not be checked. Fluorometric assays were performed at different conditions (pH, buffers) (Table 1, experimental procedures) to test cleavage of different synthetic fluorogenic P1-Arginine/Lysine substrates. However, despite several attempts, we were unable to produce active recombinant metacaspase 3. Therefore, we decided to use active recombinant metacaspase 9 (Vercammen et al., 2004)), as a representative of metacaspase activity. As Atmc9 did not seem to interact with KRP2 in a Y2H assay, we should consider Atmc9 only as a tool for Atmc3, using the P1-Arginine/Lysine cleaving properties of the metacaspase family.

5.4.1 Recombinant metacaspase 9 does not cleave recombinant KRP2

Recombinant metacaspase 9 (rAtmc9) was tested for its ability to cleave recombinant KRP2 (rKRP2) *in vitro*. Therefore, rKRP2 was incubated with rAtmc9 in a KRP2 : Atmc9 ratio of 1:10 to have an excess of rAtmc9. Before addition to the recombinant KRP2, the rAtmc9 was pre-activated by an acidic pH and application of DTT, which were both provided by incubation of rAtmc9 in the appropriate buffer (Vercammen et al., 2004). These conditions would allow rAtmc9 to bear its maximal activity (D. Vercammen, oral communication, unpublished results). The experiment

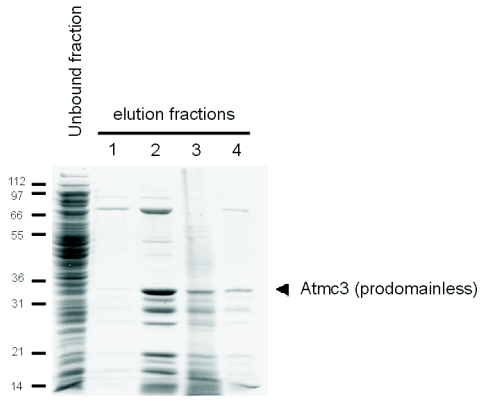


Figure 2: Purification of His-tagged Atmc3 from *E. coli*, prodomainless version. A western blot probed with anti-His was performed to validate the identity of the purified protein. The blot shows clear purification of the recombinant prodomainless Atmc3 protein in the second, third and fourth elution fraction (molecular weight of the prodomainless Atmc3 around 34kDa). First lane: unbound fraction from the TALON His-tag affinity resin, next lanes: elution fractions.

pH	4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9
buffer	MES, Tris, Acetic acid (at all pH)
addition of	glycerol, calcium, sodium citrate, sodium chloride, dimerization compounds
tested substrates	GRR, GKR, FR, FK

Table 1: Different conditions used in order to screen for activity from the recombinant protein in a fluorometric assay with different fluorogenic substrates. Different buffers, at different pH were used in the activity assays as well as the addition of certain compounds. As substrates P1-arginine/lysine substrates were tested such as GRR = glycine-arginine-arginine, GKR = glycine-lysine-arginine, FR = phenylalanine-arginine and FK = phenylalanine-lysine. However, no clear activity was detected towards any of the tested substrates, in any of the tested conditions (data not shown).

was performed in three experimental set ups. The first experimental setup was mere incubation of the two recombinant proteins, the second was in presence of an *Arabidopsis* cell suspension culture LMM1 protein extract, to provide additional cofactors that may possibly be necessary for KRP2-Atmc9 catalysis. Finally, the third setup was in the presence of an ATP generating system, consisting of phosphocreatine and a creatine kinase, to allow phosphorylation events (Figure 3). In the three cases, natural degradation of the KRP2, due to instability of the KRP2 protein, was compared with the effect of addition of the recombinant Atmc9. Cleavage of rKRP2 was assessed by western blot analysis with anti-KRP2 antibody. In none of the conditions KRP2 cleavage was detectable. This shows that under these specific *in vitro* conditions rKRP2 is not cleaved by rAtmc9. This can be explained by the fact that there might be no correct folding of the recombinant protein(s) which could hinder an interaction between the two proteins, or some additional cofactors required under these conditions are still missing, or that the *E. coli* purified recombinant proteins might lack some important posttranslational modifications necessary for their interaction (Garcia et al., 2004).

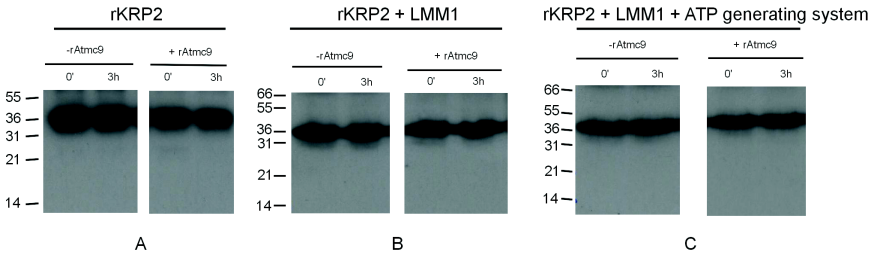


Figure 3: In an *in vitro* situation no cleavage of the recombinant KRP2 (36kDa) by the recombinant active Atmc9 (rAtmc9) can be detected. Samples were subjected to immunoblotting with anti-KRP2. A) Addition of rAtmc9 to rKRP2 B) in presence of the *Arabidopsis* cell culture LMM1 C) in completion with an ATP generating system.

5.4.2 An *in vitro* transcription / translation KRP2 product is cleaved by rAtmc9 in the N-terminal domain

The KRP2 full length and deletion constructs (KRP2N or N-terminal domain, KRP2CK or C-terminal domain, KRP2C or cyclin-binding domain) (Figure 1A) were synthesized *in vitro* using cytoplasmic extract of rabbit reticulocyte lysate in an *in vitro* transcription-translation (TnT) reaction, which starts from the DNA template. Besides the full length protein, deletion mutants were included in order to decipher subdomains in which the potential catalytic sites resides. The advantage to synthesize proteins in rabbit reticulocyte lysate is that there is a higher chance on correct folding of the protein when compared to recombinant proteins purified from *E. coli*. Additionally, the rabbit reticulocyte lysate may contain a variety of post-translational processing activities, including acetylation, isoprenylation and some phosphorylation activity, which can provide secondary modifications of the proteins ((Zhang and Ling, 1995); (Nakamura, 1993); (Martin et al., 1997)). This in contrast to proteins expressed in *E. coli*, an *in vivo* expression system which does not allow posttranslational modifications. Subsequently, rAtmc9 was pre-activated at the optimal conditions to induce its autocatalytic processing, and active rAtmc9 was added to the *in vitro* translation mixture. As a negative control inactive rAtmc9CA was added in parallel. rAtmc9CA was shown to be completely inactive in the optimal Atmc9-activity conditions (pH, buffer) against P1-arginine / lysine substrates because of the mutation in its catalytic center (Vercammen et al., 2004).

After 30 minutes of incubation of the translation mixture with the rAtmc9 / rAtmc9CA results were compared by Western blot using the KRP2 antibody (Figure 4). The full length KRP2 protein with its apparent mass of 36kDa, was cleaved upon addition with rAtmc9, however no cleavage occurred with the non-active rAtmc9CA. After 30 minutes of incubation of the N-terminal domain of the KRP2 (KRP2N) with rAtmc9 the 22 kDa fragment had disappeared, but with the mutant metacaspase 9 no cleavage of the fragment was observed. It should be noted that cleavage products were not visualised in either the full-length or KRP2N digestion. This is likely a result

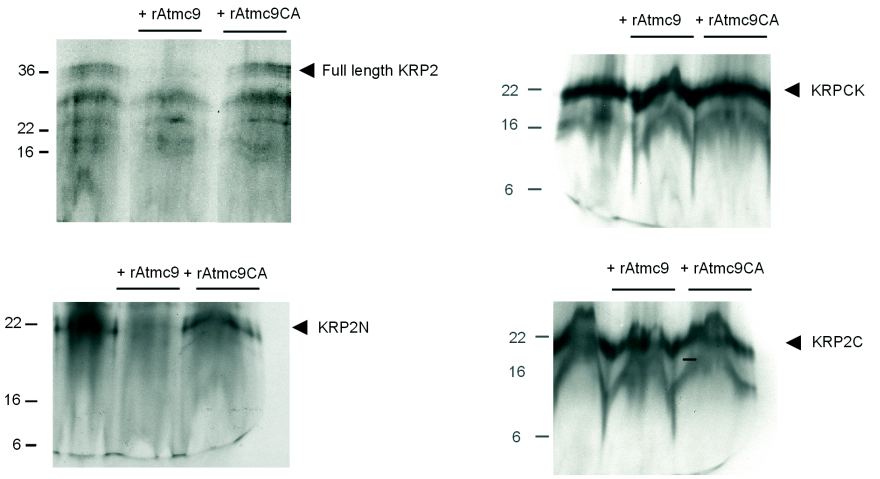


Figure 4: The full length KRP2 protein and the N-terminal domain of KRP2 (KRP2N) are cleaved by metacaspase 9. The full length protein and different deletion mutants of the KRP2 protein (KRP2N, KRP2CK, KRP2C) were produced in an in vitro transcription-translation (TnT) reaction and subsequently tested for cleavage by rAtmc9 and rAtmc9CA (incubation time of 30 minutes). First lane: TnT input reaction of KRP2 FL or deletion mutants (time = 0').

of the fact that cleavage fragments are too small to be visualised by gel electrophoresis under these conditions, possibly because of multiple cleavage sites residing within this domain, or that the cleavage fragments are degraded directly for example by the Ubiquitin-proteasome pathway (Hatfield and Vierstra, 1989). The other deletion mutants (KRP2C and KRP2CK) were not cleaved by the rAtmc9, which demonstrates that there is no cleavage site within the C-terminal domain of the KRP2 protein, neither in the CDK-binding nor the cyclin-binding domain. This experiment shows that the rAtmc9 cleavage site of the KRP2 resides in the N-terminal domain of the protein. The fact that the KRP2 FL and KRP2N are cleaved by rAtmc9 and not by the inactive mutant rAtmc9CA shows that cleavage of KRP2 by rAtmc9 is not due to the activity of residual bacterial proteases, present after purification, however to the activity of rAtmc 9 itself.

Reasons for cleavage of KRP2 by rAtmc9 in the *in vitro* translation system as opposed to the *in vitro* situation *strictu sensu* can be explained by proper folding of the recombinant KRP2 in the TnT system. This folding of the protein might enable binding of the rAtmc9 and hence cleavage. Alternatively, interactions between these proteins might be dependent on their requirement for additional secondary modifications which do not occur in the *in vitro* situation.

5.4.3 The N-terminal domain of the KRP2 harbours more than one predictable Atmc9-cleavage site

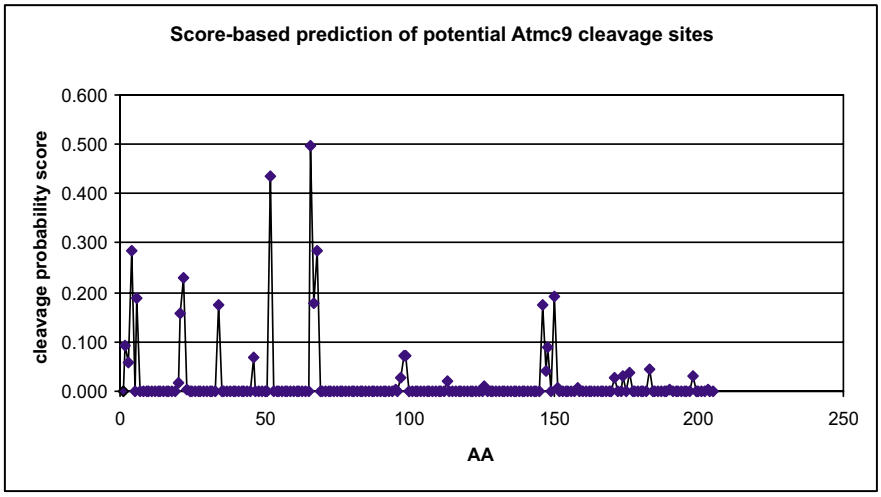
Previous data have shown that the cleavage site(s) of KRP2 by rAtmc9 is situated in the N-terminal domain, thus between AA 1 and AA 110. In parallel research, substrate specificity of rAtmc9 was explored by scanning tetrapeptide (P4-P1) positional synthetic combinatorial libraries (Harris et al., 2001) (Vercammen et al., publication submitted). Hereby, information was achieved on the ability of Atmc9 to cleave a given tetrapeptide sequence. All tetrapeptide sequences were assigned a certain value which reflects the probability of the sequence to be cleaved by Atmc9, the cleavage probability score. As Atmc9 is an Arg / Lys-specific protease, only P1-Arg or P1-Lys tetrapeptides will have a value that does not equal zero. Valine-Arginine-Proline-Arginine (Val-Arg-Pro-Arg or VRPR) was assessed as the optimal tetrapeptide substrate for Atmc9, and has a value that equals 1.000. As such, the closer the value is to 1.000, the higher the probability that the tetrapeptide sequence is a possible cleavage site. However, we do not know from which probability score a tetrapeptide sequence can be considered as a possible Atmc9 cleavage site. In addition, it must be stressed that such a probability score only gives an idea of the probability for a tetrapeptide to be a Atmc9-cleavage site, however, without taking into account the three dimensional structure of the protein.

A score-based prediction was made for the complete KRP2 protein sequence, and values are depicted in a graphic (Figure 5A). Figure 5A depicts the cleavage probability score at every position of the KRP2 protein sequence. All possible

metacaspase cleavage sites located within the N-terminal domain are represented in the table (Figure 5B), and represented in the KRP2 sequence (Figure 5C). Conclusively, the tetrapeptide with the highest cleavage probability score is VIVR, starting at AA 66. As there is more than one probable metacaspase cleavage site according to the prediction, and as there are no cleavage fragments detected after cleavage of the N-terminal domain of the KRP2, it is very likely that the N-terminal domain is cleaved multiple times by the metacaspases.

It is important to be aware that the Atmc9 cleavage probability prediction that has been made for the KRP2 protein sequence, cannot be extrapolated as such for Atmc3, which is the binding interactor of the KRP2 protein in the Y2H system. In parallel research, substrate specificity was only explored for two Type II metacaspases, namely for Atmc9 and Atmc4, however not for Type I metacaspases (Vercammen et al., publication submitted). For Atmc9 and Atmc4 it was demonstrated that both proteases have a clear specificity for basic P1 residues, regardless of the other positions of the tetrapeptides, with a clear preference for Arg above Lys. For both proteases the optimal substrate was identified as VRPR. However, as Type I and Type II metacaspases might have different substrate specificities, this substrate specificity cannot be extrapolated as such to Atmc3, a Type I metacaspase. Most probably the Type I metacaspases are also P1-Arg/Lys-specific proteases, as there is a conserved Asp-residue amongst all members of the metacaspase family which is suggested to coordinate the P1 residue. However it is not self-evident that the P2-P4 specificities are similar, and that for instance VRPR would also be the optimal substrate for Atmc3. On the contrary, there are arguments to assume that a similar substrate specificity between Type I and Type II metacaspases exists. An identified substrate of Atmc9 is Atserpin (Vercammen *et al.*, publication submitted). In addition, this Atserpin was also isolated by Tandem Affinity Purification with Atmc1, which is a Type I metacaspases (see Chapter 6). This suggests that there is a similar binding/cleavage site or similar substrate specificity (at least for the P1 position) for both Types of the metacaspases.

A



B

position in the protein sequence	tetrapeptide sequence	cleavage probability score
AA 4	VRRR	0,285
AA 6	RRER	0,188
AA 21	TVKR	0,159
AA 22	VKRR	0,23
AA 34	VESR	0,176
AA 46	ATNR	0,067
AA 52	IVAR	0,434
AA 66	VIVR	0,496
AA 67	IVRR	0,179
AA68	VRRR	0,285
AA 97	EKSK	0,027
AA 98	KSKR	0,072
AA 99	SKRR	0,073

= three optimal tetrapeptides in the KRP2 sequence AA1-110

= tetrapeptide cleavage sites which would destroy NLS

Figure 5: Prediction of the Atmc9-cleavage sites within the KRP2 sequence. A) Atmc9-cleavage probability score of every AA of the KRP2 sequence. **B)** Tetrapeptide sequences within the KRP2 sequence, suitable for Atmc9 cleavage. **C)** (next page) Representation of these tetrapeptide sequences within the KRP2 sequence. Besides the potential metacaspase cleavage sites, the possible phosphorylation sites are also indicated. In addition the NLS, the different domains of the KRP2 and the three C-terminal located motifs conserved amongst all plant KRPs are also shown.

C

MAAVRRRERL VVEENGVTTT TV**KRRK**MEEI VDLVESRIIL SPCVQATNRG

GIVARNSAGA SETSVVIVRR RDSPPVEEQC QIEEEDSSVS CCSTSEEKSK

RRIEFVDLEE NNGDDRETET SWIYDDLNKS EESMNMDSSS VAVEDVES RR

RLRKSLHETV KEAELEDFFQ VAEKDLRNKL LECSMKYNFD FEKDEPLGGG

RYEWVKLNP

KRRK = nuclear localization signal

= possible metacaspase cleavage sites within the N-terminal domain

= possible phosphorylation site

1 = conserved motif 1

2 = conserved motif 2

3 = conserved motif 3

= N-terminal domain (KRP2N)

= C-terminal domain (AA110-190) (KRP2C)

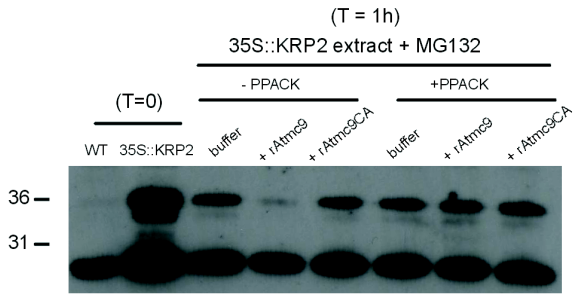
= C-terminal domain (AA170-209) (KRP2K)

5.4.4 The KRP2 protein is cleaved by the rAtmc9 protein in plant extracts

To determine whether KRP2 could be cleaved by Atmc9 under other conditions, plant extracts were subjected to incubation with rAtmc9. Therefore, protein extracts were derived from plants overexpressing KRP2 as the KRP2 is a very low abundant protein and hence its detection in wild type plants is difficult. The protein extract was incubated for one hour, with only Atmc9 buffer (pH5.5), pre-activated rAtmc9, or inactive rAtmc9CA (Figure 6A). Each time this occurred in the presence of the proteasome inhibitor carbobenzoxyl-leucinyl-leucinyl-leucinal (MG132) to increase stability of the KRP2 protein as MG132 delays natural degradation of the KRP2 protein, by inhibiting proteasomal degradation (Verkest et al., 2005b). Indeed, Western blot with anti-KRP2 showed Atmc9-dependent cleavage of the full length of the KRP2 protein (apparent mass of 36kDa) as upon incubation with rAtmc9 the amount of full-length protein wasgnificantly reduced. Under the same conditions, incubation with rAtmc9CA resulted in no significant reduction in the amount of full length protein. However, it should be noted that when compared to the amount of protein that was initially added (KRP2 overexpressor protein extract at T=0, Figure 6A), one hour of incubation with rAtmc9CA (or buffer) led to a decrease in amount of FL KRP2 as well. This is most probably due to natural degradation of the KRP2 by the proteasomal pathway, which shows that inhibition by the MG132 protein is not sufficient to inhibit natural degradation of the KRP2 completely. Nevertheless, MG132 clearly delays the degradation process (results not shown). In a second experimental setup besides the proteasomal inhibitor MG132, an inhibitor for rAtmc9-activity was also added, PPACK or Ac-Phe-Pro-Arg-CMK (D. Vercammen, unpublished results) (Figure 6A). Western analysis with anti-KRP2 demonstrated that addition of PPACK clearly inhibited the rAtmc9-dependent degradation of the KRP2, while addition of PPACK had no effect upon incubation of the KRP2 overexpressing extract with the Atmc9 buffer or with the rAtmc9CA.

This conclusively shows that rAtmc9 is able to cleave KRP2 in plant extracts, suggesting that KRP2 is an *in vivo* substrate of Atmc9. It should be noted that even though an experimental incubation time of one hour was chosen, rAtmc9-dependent KRP2 degradation occurs significantly faster. In a time course experiment it was demonstrated that as little as 15 minutes incubation time of the plant extract with the rAtmc9 is sufficient for complete degradation of KRP2 (Figure 6B).

A



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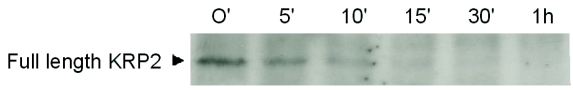

Figure 6: Cleavage of KRP2 by rAtmc9 in plant extracts. A) PPACK, an Atmc9-inhibitor, is able to delay rAtmc9-dependent KRP2 degradation. Overexpression of KRP2 in the 35S::KRP2 plant extracts is assessed by clear expression of the full length protein (36 kDa) when compared to the wild type plant extract. Incubation of the 35S::KRP2 extract (in the presence of the proteasome inhibitor MG132) during one hour with Atmc9-buffer, rAtmc9 or rAtmc9CA protein; with or without the addition of rAtmc9-inhibitor PPACK. **B) Time course experiment of rAtmc9-dependent cleavage of KRP2 demonstrates that 15 minutes is sufficient for complete degradation of KRP2.**

These results demonstrate that KRP2 can be cleaved by rAtmc9 both in a reticulocyte lysate with *in vitro* translated KRP2, and in the plant extract. Degradation of KRP2 by rAtmc9 was simply employed as a predictive tool to identify possible KRP2 cleavage sites by other members of the metacaspase family. This choice was made based on an inability to activate recombinant Atmc3.

Given that KRP2 can be cleaved by at least one member of the metacaspase family we can anticipate that KRP2 is a likely proteolytic substrate for the Y2H identified Atmc3. Based on this assumption, KRP2 proteolysis needs to be placed in a **biological context**. Although speculative, it is hypothesised that KRP2 proteolysis could be a form of post-transcriptional regulation whereby KRP2 cleavage is a regulatory mechanism controlling KRP2 abundance, and hereby regulating its activity.

This could have implications on **cell cycle progression**, as KRP2 is a direct inhibitor of CDK/cyclin complexes. Cleavage of KRP2 could result in its inactivation. This inactivation could be due to cleaving off the nuclear localisation signal (NLS) which is resided in the N-terminal domain. Consequently, progression through the cell cycle is followed due to the inability of the KRP2 protein to bind to CDK/cyclin complexes caused by its altered subcellular localization (cytoplasmic translocation). In that respect, metacaspases would be required for cell cycle progression, as they might confer additional checkpoints to assure that only healthy cells will complete the cell cycle. This would present a possible regulatory role of the metacaspases for CDK/cyclin activation via KRP2. It would then be expected that the activity of metacaspases during cell cycle results in the cleavage and inactivation of proteins that act as negative regulators of the cell cycle machinery, such as KRP2. Such a role has already been established for mammalian caspases where caspases regulate the abundance of $p27^{Kip1}$ in a proliferation-dependent manner in human non-apoptotic lymphoid cells (Medina-Palazon et al., 2004). On the other hand, cleavage of KRP2 could also result in activation of the CDK inhibitory activity of KRP2 as upon Atmc9-dependent cleavage the N-terminal domain (or part of the domain) is cleaved off, and for some members of the KRP family (KRP1, KRP2) the N-terminal region is known to have a negative regulatory effect on CDK inhibitory activity ((Wang et al.,

1998); (Schnittger et al., 2003); A. Verkest, unpublished results). Whether cleavage of the KRP2 would result in its activation or inactivation has to be elucidated, and is further discussed in §5.5.

Since KRP2 has recently been found to control the exit from the cell cycle and onset of endoreduplication, it can also be suggested that metacaspases could also play a role in another aspect of cell cycle. As it has been demonstrated that endoreduplication is accompanied with a KRP2-dependent decline in CDK activity ((Verkest et al., 2005b); (Joubes et al., 1999)), a role for the metacaspases in regulating the abundance of the KRP2 and as such **controlling the onset of endoreduplication** can be suggested. As endoreduplication is commonly correlated with differentiation, metacaspases could hence be important in the differentiation process.

Another putative cleavage effect of KRP2 could be the **induction of apoptosis**. This assumption is based on loss of NLS upon metacaspase-dependent cleavage, which would lead to a substantial reduction in the association with nuclear cyclin/CDK complexes accompanied with a dramatic induction of CDK activity. In mammalia, cleavage of $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ mediates apoptosis through cleavage by caspases (Levkau et al., 1998). In addition, cdk2 activity is necessary for chromatin condensation and other manifestations of cell death, including cell shrinkage and loss of adhesion to substrate (Harvey et al., 2000).

Interestingly, the KRP2 protein at its full length is approximately 36kDa. However, in plant extracts an additional fragment around 27 kDa can also be detected. As this fragment can only be recognized by the polyclonal antibody generated against a peptide containing the last 20 C-terminal AA of the KRP2 and not by an antibody generated against the HIS tagged recombinant KRP2, it is assumed that this is a C-terminal degradation fragment derived from the KRP2. This C-terminal fragment is until now only detected in plant extracts. Thus, protein extract from a KRP2 overexpressing plants shows, besides the KRP2 FL protein, also the C-terminal cleavage product. Interestingly, when this KRP2 overexpressing extract is put on gel together with the *in vitro* translated Atmc9-cleavage product of the KRP2 it seems that the Atmc9 C-terminal cleavage fragment has the same apparent mass as the

degradation fragment in the plant extract (data not shown). This suggests that this degradation fragment could be a result from cleavage by the metacaspases. Intriguingly, subcellular fractionation pointed out that the C-terminal degradation fragment is a cytoplasmic fragment, while the full length protein resides in the nucleus. This is concomitant with the hypothesis that cleavage of the metacaspases which occurs in the N-terminal domain destroys the NLS and leads to a cytoplasmic localization, which provides an additional argument that this C-terminal fragment could be due to cleavage by a metacaspase.

5.5 Atmc3-promotor::GFP:GUS plants show a phloem-specific expression

As gene expression in specific tissues of the plant or expression under specific conditions can often give some useful information on the function of a protein, Atmc3-promotor::GFP:GUS lines were produced to analyze *Atmc3* expression. Hereby, a GFP:GUS cassette (coding for Green Fluorescent Protein and b-glucoronidase respectively) was put under control of the promoter region of Atmc3 (1500 base pairs upstream of the start codon), and a binary vector was constructed. Ten primary transformants were retained and expression analysis was performed on at least two independent single-locus T2 lines. Figure 7 shows expression analysis of *Atmc3* by GUS staining of the Atmc3-promotor::GFP:GUS line, which is representative for the other lines as well. Expression analysis of the flower, leaf, stem and root shows expression of *Atmc3* predominantly in the phloem tissue (Figure 7A). To analyze the expression of metacaspase 3 in the root in more detail, transverse sections of the root were made, and hereby expression in the phloem cells and pericycle cells at the phloem poles in the root could clearly be demonstrated (Figure 7B).

Interestingly, expression of the KRP2 gene has also been analyzed in detail in root tissues by an in situ hybridisation of *Arabidopsis* and radish roots (Himanen et al., 2002). The *KRP2* gene was also found to be expressed in pericycle cells opposite the xylem pole, which is the site of developing root primordia. It was demonstrated that KRP2 plays a significant role in the regulation of lateral root induction, blocking the G1-S transition and hereby inhibiting lateral root formation. More specifically, upon auxin treatment KRP2 was transcriptionally downregulated, leading to a progression in the G1-to-S transition and subsequent lateral root formation ((Himanen et al., 2002); (Himanen et al., 2004)).

Given that Atmc3 is localized in the same cells as KRP2 and that Atmc3 interacts with KRP2 in a Y2H assay it is hypothesized that KRP2 is a likely substrate of Atmc3. Further support from this hypothesis comes from the finding that at least one member of the metacaspase family, (recombinant) metacaspase 9, has an ability to cleave both in vitro translated KRP2 and KRP2 extracted from plant tissues.

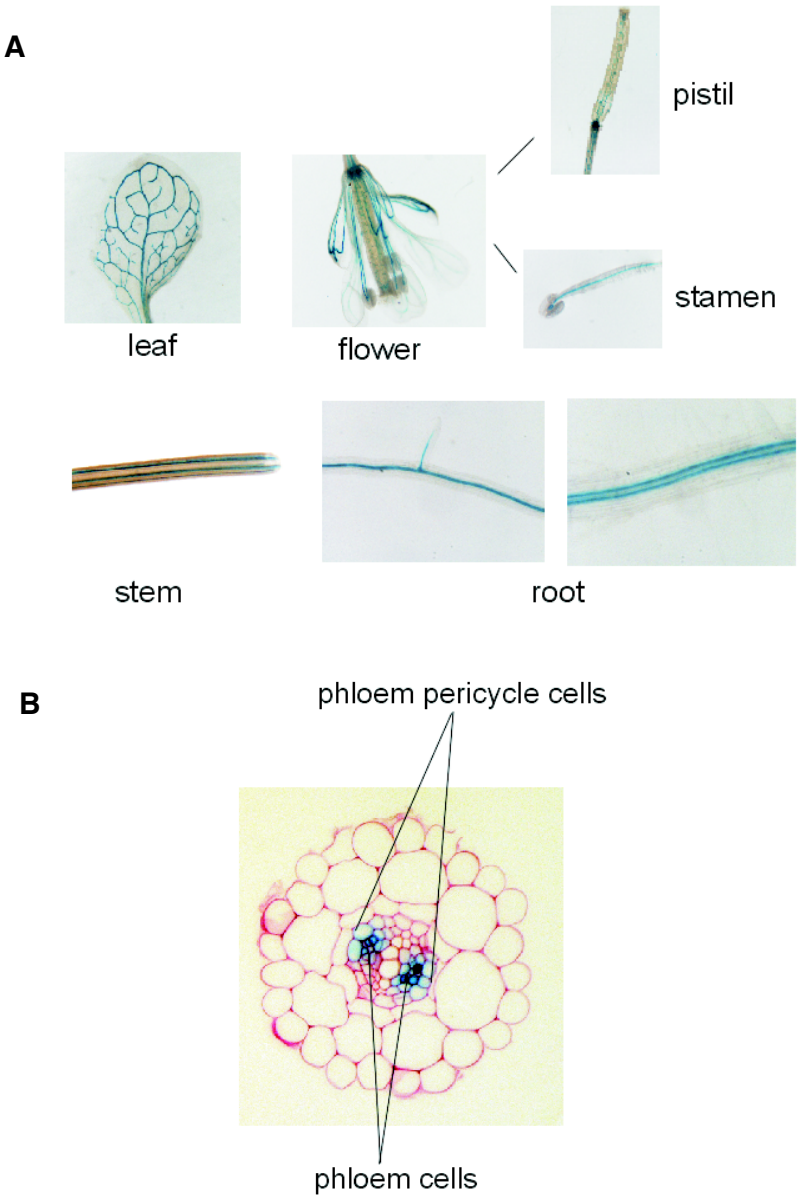

Figure 7: A) Atmc3 is predominantly expressed in phloem tissue in leaf, flower, stem and root as demonstrated by GUS staining of Atmc3-prom::GFP:GUS line. B) Transverse section of the root revealed an expression of Atmc3 specifically at the phloem poles, in the phloem cells and pericycle cells.

Based on this, we can speculate a functional link between metacaspases and KRP2. Although this link is speculative, additional experiments need to be performed to elucidate the exact function of metacaspases in KRP2 posttranslational regulation. Before additional experiments are performed, active recombinant metacaspase 3 is needed. Therefore, optimal Atmc3-activity conditions and the ideal substrate specificity for the Atmc3 should be elucidated in order to be able to purify active rAtmc3. Subsequently, we should be able to confirm the cleavage sites which were highlighted by metacaspase 9-dependent proteolysis. It should be noted that expression within the plant of metacaspase 3 does not mean activity of the Atmc3 as such. This could be due to the fact that the presence of inhibitors and zymogen-activation of the Atmc3 are important factors which regulate the activity of Atmc3-expression (also see Chapter 2 and 3), and an extra stimulus might be needed to provoke an activation of the Atmc3 protein by releasing inhibiting factors or inducing maturation of the metacaspase protein.

Given the hypothesis that we believe that KRP2 is proteolytically inactivated by Atmc3, there is a contradiction in terms of cell cycle regulation based on the expression of both KRP2 and Atmc3. As both are localized in pericycle cells, we would have anticipated that if Atmc9 has an ability to inactivate KRP2 then cell cycle would be reinstated and hence lateral roots would be initiated. However, this is not the case as these phloem pole-specific pericycle cells are arrested in G1-S. If an alternative hypothesis would be proposed where cleaving off the N-terminal domain (or part of) of the KRP2 can activate the CDK inhibitory activity of KRP2, then we would anticipate that those phloem-pole specific pericycle cells would indeed be arrested in G1-S. Evidence for an increased inhibitory activity of KRP proteins upon deletion of the N-terminal domain comes from (Wang et al., 1998) which have shown that deletion of the N-terminal domain of KRP1 resulted in an increased affinity for CDKs and cyclins as shown by an increased Y2H interaction of KRP1 with CDKs and cyclins. In addition, a similar deletion led to an enhancement of the phenotype upon KRP1 overexpression in *Arabidopsis* (Schnittger et al., 2003). Recently, this enhanced affinity for CDKA was also shown for the KRP2 N-terminal deletion mutant (KRP2CK) in an

immunoprecipitation assay (A. Verkest, unpublished results). Furthermore, transgenic plants overproducing the N-terminal deletion mutant showed a higher percentage of plants with a strong phenotype when compared to transgenic plants overexpressing the KRP2 full length protein (A. Verkest, unpublished results). Based on these studies, this needs to be confirmed with regards to the exact Atmc3-cleavage product of KRP2. Transgenic plants overexpressing the cleavage product of KRP2 (expressing no endogenous KRP2) can be used to verify whether they display a decreased CDK activity or altered cell cycle progression. Furthermore, the effect of a mutated Atmc3-cleavage site in the full length KRP2 protein (uncleavable KRP2 FL) on the protein's inhibitory function, and hence cell cycle progression should be examined.

5.6 Y2H of type I metacaspases Atmc2CA and Atmc3CA with the core cell cycle genes did not reveal interactions in yeast

To elucidate a putative role for the (Type I) metacaspases within the cell cycle, a Y2H mating of Atmc2CA and Atmc3CA with the core cell cycle genes was performed. In our Department of Plant Systems Biology, in the group of Functional Genomics (Dr. P. Hilson) yeast strains containing the core cell cycle genes as bait (pDEST32) and as prey (pDEST22) were available in the pJ69-4a and pJ69-4A strain respectively. A list of the 92 tested core cell cycle genes is shown in Figure 8A. Amongst these core cell cycle genes are included the different members of the KRP family, different types of CDKs (type A to type F) and cyclins (type A, B, D, H and T), WEE1 kinase, an inihibitor of G2-M transition, and E2F (a, b and c) and DP (a, b) genes, both transcription factors which lead to transcriptional activation of S phase specific genes. Moreover, also included are DEL genes (1, 2, 3) which are DP-E2F-like genes and inhibitors of the DNA endoreduplication cycle, CKS, cyclin dependent kinase subunits which act as docking factors for both positive and negative regulators of CDKs, and RB, the retinoblastoma protein which is phosphorylated upon mitogenic stimulation and subsequently releases the previously associated E2F/DP transcription factors to allow G1-S transition.

Mating was performed in both directions, once the metacaspases as bait and the cell cycle genes as prey and once the metacaspases as prey and the cell cycle genes as bait. Using the cell cycle genes as bait, self-activation of the cell cycle genes must be taken into account. However, a list of bait-selfactivating cell cycle genes was available and could be subtracted from the interacting list at the end. On the other hand, concerning the bait metacaspases, self activation was overcome on medium lacking histidine supplemented with 5mM AT. Y2H mating was done on medium lacking histidine supplemented with 10 mM AT and 40 mM AT (Figure 8B). Upon mating of Atmc2CA and Atmc3CA against all the core cell cycle genes (in both directions), possible candidate interacting protein pairs were tested in a one by one

mating. However, upon one by one confirmation and subtraction of all the self activation proteins, no interaction could be obtained.

It has to be remarked that the interaction between Atmc3CA and KRP2 could not be confirmed in this Y2H, as the KRP2 as a bait protein in the pDEST32 vector is still self activating on -His medium supplemented with 80 mM AT, self activation can only be overcome on 100 mM AT. However, selection on -His supplemented with 100 mM AT is considered as not useful, as the selection is too strong and most biological relevant/significant interactions would be missed anyway. In the other way, with KRP2 as a prey protein, no interaction could be detected. However, it is seen in many cases, that interactions can only be shown in one direction. Moreover, it is also not exceptionally that certain interactions cannot be repeated in other Y2H systems using other bait/prey vectors and other yeast strains.

Whether these Y2H mating results reflect that both Atmc2CA and Atmc3CA are not directly implicated in the cell cycle, would be a premature conclusion, as this would only be based on this Y2H mating. In addition, as it was already previously mentioned, the Y2H technique is a valuable technique, but it has to be taken into account that the technique is also prone to some limitations, which might be responsible for missing interactions. For example, it is possible that secondary modifications are necessary to establish an interaction between two proteins, or that the interaction is indirect whereby binding of an intermediate protein is needed. In addition, it is a technique for detection of interactions in yeast and not in plants. Conclusively, a direct intereraction between Atmc2CA and Atmc3CA and the core cell cycle genes in yeast cannot be detected. For the rest, no further conclusive results can be drawn from this Y2H mating experiment.

A

CDKB2;1	CYCA1;1D	CYCA3;1	CYCB2;1D	CYCD6;1	DEL2	E2Fb	KRP5	WEE1	PLP6
CKS1	CYCA2;1	CYCA3;1D	CYCB2;2	CYCH;1	DEL3	E2Fb-BC010	KRP6	CYCC2	PLP7
CKS2	CYCA2;1D83	CYCA3;2	CYCB2;3	CYCT	DPa	E2Fc	KRP7	INT62-1	PLP8
CKS-BC001	CYCA2;2	CYCA3;2D	CYCD2;1	DEL1	DPa-BC121	GPTv2	KRP-BC004	PLP1	GUS
CKS-BC011	CYCA2;2D	CYCB1;1	CYCD3;1	DEL1-BC059	DPa-BC60	KRP1	KRP-BC078	PLP2	2D7
CKS-BC098	CYCA2;3	CYCB1;3	CYCD3;2	DEL1-BC062	DPb	KRP2	KRP-BC112	PLP3	
CKS-BC103	CYCA2;3D	CYCB1;4	CYCD4;1	DEL1-BC068	E2Fa	KRP3	KRP-BC23	PLP4	
CYCA1;1	CYCA2;4D	CYCB2;1	CYCD5;1	DEL1-BC072	E2Fa-BC009	KRP4	RB	PLP5	

B

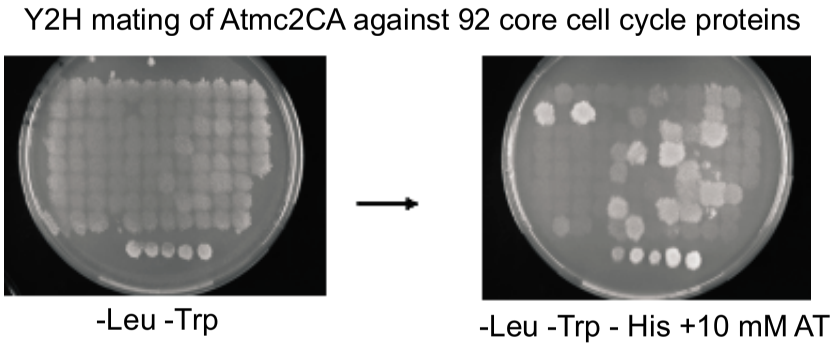

Figure 8 A) List of the 92 tested core cell cycle genes which were tested for their interaction with Type I metacaspases Atmc2CA and Atmc3CA. (DN = dominant negative). GUS = β-glucuronidase gene which functions as a negative vector control. The order of the list of the genes in the table represents the exact postions as used in the mating. **B) Y2H mating of Atmc2CA against the 92 core cell cycle genes**. Upon mating of the metacaspase with the cell cycle genes in rich medium YPD, the plate is replica-plated to SD-Leu-Trp for selection of both fusion proteins (BD-bait and AD-prey proteins), and afterwards to medium lacking His and supplemented with the appropriate AT concentration, for selection of interacting proteins.

5.7 Experimental procedures

5.7.1 Yeast two-hybrid of Atmc3 with KRP2 FL, different deletion constructs of KRP2 and other members of the KRP family

Prey vectors containing the KRP2 FL, and the different KRP2 deletion mutants were cloned into the AD-containing Gateway compatible prey vector pGADg424, harboring -Leu as a selection marker and were provided by A. Verkest, cell cycle group of the Department of Plant Systems Biology. Atmc3 was cloned into the Gateway compatible bait vector pGBTg as described above. For the Yeast two-hybrid, the Hf7C yeast strain was used and a sequential cotransformation was performed using the LiAc method in accordance to small scale transformation protocol. First, the Hf7C yeast strain was transformed with the bait protein Atmc3CA using the Lithium Acetate-based small scale transformation protocol and 0.5mg-1 mg of the bait plasmid (Gietz et al., 1992). Secondly, the yeast strain containing the bait plasmid Atmc3CA was super-transformed with the appropriate prey vector (0.5mg-1 mg), also using the LiAc-based small scale transformation protocol. Transformation mixture was plated out on selective medium SD-Leu-Trp and hence colonies were streaked on -His medium supplemented with 5mM AT to overcome self activation of the bait protein pGBTg-Atmc3CA.

5.7.2 Cleavage assays of KRP2 by rAtmc9

Production of rAtmc3, rAtmc9, rAtmc9CA and rKRP2

Atmc3 cDNAs, both full length and prodomainless, were cloned into the bacterial expression vectors pDEST17 via Gateway recombination. For construction of full length Atmc3 cDNA (ORF) can be referred to (Vercammen et al., 2004). Construction of prodomainless cDNA occurred by PCR on the full length cDNA with a forward primer annealing downstream of the prodomain and the reverse primer for Atmc3 (5'-TCAACAGATGAAAGGAGCGTTGG-3'). Subsequently bacterial expression vectors were transformed into the *E. coli* BL21(DE3) pLysE strain. Production was induced for 24 hours with 0.2 mM isopropyl 1-thio-b-D-galactopyranoside (IPTG). Cultures were harvested and resuspended in natrium phosphate extraction buffer pH 7.5 supplemented with 300mM NaCl. This was followed by sonication of the cell cultures (four times 15 seconds, with each time an interval of 15 seconds). Upon centrifugation, the supernatant was mixed with the TALON cobalt affinity resin (DB biosciences, Franklin Lakes, NJ, USA) and incubated overnight at 4°C. After two washing steps, the beads

were loaded on a column and two more washing steps were performed. Elution of the bound (His-tagged) proteins occurred with the extraction buffer supplemented with 150 mM imidazole. Activity assay were performed using different buffer conditions and substrates as show in Table 1. Recombinant Atmc9 and Atmc9CA proteins were also purified using metal ion affinity chromatography (TALON) (Vercammen et al., 2004). Both proteins were pre-incubated (pre-activated in case of Atmc9 protein), prior to usage in cleavage assays, in Atmc9 buffer (described in Chapter 2) at 30°C for at least 15 minutes, to allow processing to occur (in case of Atmc9). Recombinant His-tagged KRP2 was expressed and purified as described by (Verkest et al., 2005b).

***In vitro* degradation assay: incubation of rKRP2 with active rAtmc9**

For the *in vitro* experiment, a 10:1 ratio of rAtmc9:rKRP2 was used. Per reaction, 100 ng KRP2 and 1 mg pre-activated rKRP2 were co-incubated in the presence of a final concentration of 10 mM DTT. In case of addition of the *Arabidopsis* cell suspension culture LMM1 protein extract (extraction in Atmc9 buffer supplemented with 1mM oxidized glutathione) 100 mg extract per reaction was used, and for the ATP regenerating system a final concentration of 35 mM phosphocreatine, creatine kinase (50 mg/ml) and ATP (1 mM) (Promega, Madison, WI, USA). The reaction mixtures were incubated at 30°C for 30 minutes, one and three hours. As a control time point zero (immediate boiling in loading dye after addition of reaction components) was put on gel together with the time course samples. Reaction mixtures were stopped by the addition of SDS sample buffer.

***In vitro* transcription/translation (TnT) of KRP2 full length and different deletion mutants of KRP2 and cleavage assay with the different forms of TnT KRP2**

KRP2 FL and different mutant forms were cloned into the pET19b vector backbone (Novagen, EMD biosciences, Darmstadt, Germany) for *in vitro* transcription translation, which contains a T7 transcription promoter and an N-terminal His tag (6xHis) (vectors provided by A. Verkest). PCR products, starting from the T7 promotor until the stop codon of the KRP2 FL/ mutants, were obtained by PCR amplification with a T7 promotor primer and with specific reverse primers for KRP2 AA1-110, KRP2 AA110-190 and KRP2 AA110-209 respectively. Information about the primer set sequences can be given upon request. The T7-containing PCR products were subsequently used as DNA templates for coupled transcription/translation in the TNT T7 quick coupled transcription/translation reticulocyte lysate system (Promega, Madison, WI, USA). Correct expression of the proteins by the TnT system was checked by PAGE. Per reaction 0.5 mg pre-activated rAtmc9 and rAtmc9CA were added to approximately

one third of the reaction volume (with an estimated amount of protein around 100 ng) of the TnT expressed KRP2 FL and mutant forms, and were incubated in the Atmc9 buffer (final concentration of 10 mM DTT) during 30 minutes at 30°C. Reactions were stopped by addition of SDS sample buffer. For time point zero TnT expressed fragments were put on gel (without addition rAtmc9/CA).

Cleavage of KRP2 in plant extracts

Per reaction 50 ng of rAtmc9/rAtmc9CA was added to 30 mg of protein from KRP2 overproducing plant extract (RIPA buffer: 1% Nonidet P40, 0.5% sodium deoxycholaat, 0.1% SDS) in Atmc9 buffer (final concentration 10 mM DTT) and in the presence of 100 mM of the proteasome inhibitor carbobenzoxyl-leucinyl-leucinyl-leucinal, MG132 (Affinity Research, Exeter, UK). Incubation during one hour and arresting the reaction by addition of SDS loading buffer. The Atmc9-inhibitor PPACK (Schmaier et al., 1992) was added to the reaction mixtures at a final concentration of 1 mM.

Purification of KRP2 antibody

The KRP2 antibody was raised against a peptide of 20 amino acids located in the C-terminal domain of the KRP2 protein [(C)-FEKDEPLGGGRYEWVKLNP, with C indicating an extra Cys] (Verkest et al., 2005b). The antiserum was purified in two steps. For the first purification a Protein A Sepharose CL-4B matrix (Amersham Biosciences, Buckinghamshire, England) was used to bind immune complexes, according to the manufacturer's instructions. Bead-bound fractions were used for a second purification. For the second purification, the recombinant His tagged KRP2 protein was coupled to cyanogen bromide (CnBr)-activated Sepharose 4B (Amersham Biosciences, Buckinghamshire, England) at a concentration of 5 mg/ml according to the manufacturer's instructions. Protein A sepharose purified KRP2 serum was incubated with the CnBr-coupled recombinant KRP2 protein on a rotating wheel for 2 hours at 4 °C. Subsequently, the bead-bound fraction was washed five times (10 mM Tris pH7.5, 0.5M NaCl) and collected after elution with (0.1 M glycine pH3.0).

Production of promotor::GFP:GUS *Arabidopsis* plants and GUS assays

Promotor regions of all metacaspases (till 1500 base pairs upstream of start codon) were cloned upstream of a GFP:GUS cassette into the binary vector pBGWFS7 (Karimi Mansour). Binary constructs were transformed in *Agrobacterium tumefaciens* C58C1Rif^{R} [pMP90] and subsequent *Agrobacterium*-mediated floral dip transformation of *Arabidopsis*

thaliana resulted in transgenic Atmc3-promotor::GFP:GUS lines. Ten primary transformants were retained per construct, hence T2 segregation analysis resulted in selection for single locus lines and subsequently homozygous lines were produced.

Histochemical stain for GUS reporter activity was carried out by incubating seedlings in 90% acetone for 30 minutes at 4°C followed by a rinse in the base buffer (0.1 M Tris.HCl, 50 mM NaCl, pH 7.0) and subsequent incubation overnight in the staining buffer (base buffer plus 2.0 mM $K_3Fe(CN)_6$ and 1 mg/ml 5-Bromo-4-Chloro-3-Indolyl-a-D-Glucuronide, X-Glu) (ImmunoSource, Halle, Belgium) at 37 °C. Seedlings were then transferred to the base buffer to terminate the reaction at 4°C. GUS assays shown in this Chapter were performed on segregating T2 lines (at least 2 independent lines were observed). Samples mounted in lactic acid (Acros Organics, Geel, Belgium) were observed and photographed using a binocular (Stemi SV11; Zeiss, Jena, Germany) or by a differential interference contrast microscope (Leica, Vienna, Austria), using Nikon camera (Analis, Suarlée, Belgium).

Transverse sections of the root

For anatomical sections, GUS-stained samples were fixed overnight in 1% glutaraldehyde and 4% paraformaldehyde in 50 mM phosphate buffer, pH7. Samples were dehydrated and embedded in Technovit 7100 resin (Heraus Kulzer, Bern, Germany) according to the manufacturer's protocol. For proper orientation of the samples, we used a two-step embedding methodology, based on a pre-embedding step to facilitate orientation in 0.5 ml Eppendorf tubes (De Smet et al., 2004). Sections of 5 mm were cut with a microtome (Minot 1212, Leice Microsystems, Wetzlar, Germany) dried on Vectabond-coated object glasses, counterstained for cell walls with 0.05% ruthenium red for 8 minutes (Fluke, Buchs, Switzerland) and rinsed in tap water for 30 s. After drying, the sections were mounted in DePex medium (British Drug House, Poole, UK) and covered with cover slips. Samples were observed using a differential contrast microscope and a Nikon camera (Analis, Suarlée, Belgium).

5.7.3 Y2H mating

Yeast expression vectors for bait (pDEST32, LEU selection marker, DB containing vector) and pey protein (pDEST22, TRP selection marker, AD containing vector) were provided with the Proquest yeast two-hybrid system (Invitrogen, Carlsbad, USA) and were made Gateway compatible. Both vectors contain a C-terminal fusion with the DNA binding domain or activation

domain for expression of the fusion bait or prey protein respectively. Both wild type and mutant versions of the metacaspases were cloned in both vectors by Gateway recombinatorial cloning, resulting in pDEST22-Atmc1-9(CA) and pDEST32-Atmc1-9(CA). For mating purposes, yeast strain pJ69-4A and -4a (MATa *trp1-901 leu2-3,112 ura3-52 his3-200 gal4*" *gal80*" *LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*) were used (James et al., 1996) (reporter genes *HIS3* and *LacZ*). pDEST22-Atmc vectors were transformed in pJ69-4A strain and pDEST32-Atmc vectors in pJ69-4a strain, using the Lithium Acetate (LiAc) method and 0.5 mg-1 mg of the plasmid DNA (Gietz et al., 1992). Selection in a minimal dropout (SD) medium containing all amino acids except Trp (for pDEST22) or Leu (for pDEST32) (Clontech, Palo Alto, CA, USA).

Mating of Atmc2CA and Atmc3CA against the 92 core cell cycle genes was performed using replica-plating. Cell cycle genes were available in pDEST32 (bait) and pDEST22 (prey) vectors, in the Functional Genomics Division Group of the Departement of plant Systems Biology). Cell cycle genes were spotted with Biomek Robot (Beckman Instruments Inc., Fullerton, CA, USA) as bait or prey respectively and grown on selective medium (SD-Leu for bait, and SD-Trp for prey) for at least 20 hours. For Atmc2CA and Atmc3CA, glass beads were used to make lawns of metacaspase-bait and -prey on the appropriate selective medium, and were grown for at least 20 hours. Subsequently, the spotted cell cycle genes are replica-plated to rich YPD medium by using a sterile velvet. Mating occurs by replica-plating the metacaspase as a lawn on a YPD plate and applying the spotted replica-plate on it. One or more successive cleaning steps are needed after mating until the spots can no longer be detected on the YPD plate. The YPD plate is grown overnight to allow mating, and then the mated YPD plate is replica plated to a SD-Leu-Trp plate to allow selection for both plasmids. After at least another 20 hours from this SD-Leu-Trp plate replica plates are made to medium lacking His supplemented with the appropriate AT concentration, here 10 mM AT and 40mM AT. Hereby, three or four cleaning steps are required.

5.8 References

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Chapter 6: Building an interactome of *Arabidopsis* metacaspases via Tandem Affinity Purification strategy

6.1 Abstract

To identify interactors of the Type I metacaspases, a Tandem Affinity Purification (TAP) was perfomed which allowed us to detect interactions of the metacaspases *in vivo*, in *Arabidopsis* cells. Therefore, both N-terminal and C-terminal TAP tagged versions of wild type and mutant metacaspases were overproduced in *Arabidopsis* cell suspensions. The identification of interactors of the metacaspases gave some hints for putative roles of the metacaspases in different biological processes such as the cell death process, cytoskeletal organization, plastid development and energy metabolism. Compared with the yeast two-hybrid approach to detect protein-protein interactions (Chapter 4), no overlap with the yeast two-hybrid data could be established. This indicates that the two technologies provide complementary data, as was already shown for the budding yeast (Gavin et al., 2002).

In addition, a two-dimensional blue native PAGE on cell cultures overproducing metacaspase 1 was also performed and demonstrated that Atmc1 is resided in complexes within the cell, varying from the monomeric metacaspase to complexes of at least 440 kDa.

6.2 Introduction

Recently, Tandem Affinity Purification (TAP) strategies have been established as an efficient approach for purifying protein complexes for many different organisms. The TAP system was first described to purify protein complexes in yeast (Gavin et al., 2002), subsequently the applicability of such a system in many other different organisms was demonstrated by a number of studies. TAP-purified protein complexes have been isolated from bacteria (Gully et al., 2003), mammalian cells (Knuesel et al., 2003), insect cells (Forler et al., 2003), tobacco leaves (Rohila et al., 2004) and rice (Rohila et al., 2006). The method is low-priced allowing large-scale applications, all directly and indirectly interacting components are identified in a single experiment and a low number of false positives occur due to a two-step purification. In addition, the method is characterized by its simplicity, high yield, speed and reliability. Taking into account also the recent improvements in mass spectrometry (MS) techniques, it seems an ideal strategy for high throughput **protein complex component identification** ((Graumann et al., 2004); (Puig et al., 2001)). Moreover, in contrast with methods such as yeast two-hybrid screenings, the ability to establish the interactions *in vivo*, in the plant cell itself, is of big advantage. In addition, the TAP system allows protein isolation under native conditions, therefore allowing functional studies in which the activity or post translational modifications on a protein or a protein complex can be examined (Puig et al., 2001). The success of the TAP approach for the characterization of protein complexes relies on the conditions used for the assembly and retrieval of the complexes. As it was demonstrated from studies in yeast, TAP/MS-based functional proteomics approach may constitute the largest analysis of protein complexes to date (Gavin et al., 2002).

Other large-scale approaches, such as extensive two-hybrid screens have been demonstrated to be complementary to TAP/MS based methodologies, as both methodologies address different aspects of protein interaction. Yeast two-hybrid data reveals binary interactions, whereas the TAP/MS method yields data on complex

composition. Comprehensive two-hybrid approaches do not seem particularly suited for characterization of protein complexes, which supports the view that complex formation is more than the sum of binary interactions. Therefore, it is interesting to combine both complementary approaches in order to build a more complete interaction network. Results obtained from yeast two-hybrid screenings (Chapter 4) and yeast two-hybrid mating with the Type I metacaspases (Chapter 5) enabled to start the building of a network of these metacaspases. However, for further interactome exploration, TAP technology was chosen as an additional approach. Hereby, protein interactions are studied in physiological settings (in contrast with Y2H were artificial conditions are used), both direct and indirect interactions can be detected, and as e.g. for the budding yeast there is a small overlap with Y2H data (Gavin et al., 2002), TAP technology results are used complementary with the Y2H data. In addition, the TAP method has proven to be a very useful tool for the detection of interacting partners of a target protein and for determining protein composition of macromolecular complexes with low levels of false positives and false negatives, due to a two-step purification procedure (Rigaut et al., 1999).

TAP on yeast metacaspase (YCA1) has already been attempted, however the purification was not successful (Krogan et al., 2006). Nevertheless, YCA1 was found as an interacting protein upon TAP purification with ARC1 protein (YGL105w). ARC1 is a protein which binds to tRNA and methionyl- and glutamyl- tRNA synthetases, hereby delivering tRNA to the synthetases and stimulating catalysis (Deinert et al., 2001). Interestingly, the mammalian homologue of ARC1, p43, is a known target of caspase-7 during apoptosis (Behrensdorf et al., 2000). This links progression of apoptosis to the inhibition of protein translation. Similarly, Y2H of yeast caspase also revealed interactors involved in protein synthesis (see Chapter 4, Table 1).

6.3 Tandem Affinity Purification procedure: Principle and characteristics

The TAP method has been designed to allow **fast purification of protein complexes under native conditions**, without the prior knowledge of composition, activity or function of the protein complex and even when the proteins are expressed at their natural level. Biochemical purification of proteins followed by mass spectrometry allows identification of proteins interacting with a given target protein. Currently, approximately 100 fmol of a protein can be detected and identified by MS, allowing rapid characterization of any protein present in a complex mixture, given that the target complex is sufficiently purified in reasonable quantity. Moreover, the availability of complete genomic sequences for several organisms has facilitated the identification of proteins by MS. Therefore, the current limiting step seems to be protein purification rather than protein identification (Puig et al., 2001). A generic purification protocol is therefore developed to allow routine protein complex purification for proteome analysis, making use of fusion of the **TAP tag**, either C- or N-terminally, to the protein of interest. The TAP tag is a fusion cassette encoding a calmodulin-binding peptide (CBP), a TEV cleavage site, and two IgG-binding units of protein A of *Staphylococcus aureus* (ProtA). ProtA allows for efficient binding to the IgG matrix, however it can only be released from matrix-bound IgG under denaturing conditions at low pH. Therefore, a specific TEV protease recognition sequence was inserted (Dougherty et al., 1989), which allows proteolytic release of the bound material under native conditions, upstream of the Prot A tag. The CBP tag allows for efficient selection and specific release from the Calmodulin affinity column under mild conditions.

The TAP method involves fusion of the TAP tag to the target protein and the introduction of the construct in the host cell or organism. The expression of the fusion protein can be maintained at, or close to, its natural level, or alternatively there might be chosen for an overproduction of the fusion protein. On one hand, overexpression of the fusion protein may lead to the formation of nonspecific and/or nonnatural protein interactions with host proteins. On the other hand, the TAP tagged fusion protein should replace the endogenous wild type protein as competition between the corresponding endogenous protein and the introduced TAP tag fusion protein

may interfere with the TAP procedure. Overexpression of the fusion protein may be an approach for competing with the endogenous protein. The fusion protein and associated components are recovered from cell extracts by affinity selection on an IgG matrix. After washing, the TEV protease is added to release the bound material. Subsequently, the eluate is incubated with calmodulin-coated beads in the presence of calcium. This second affinity step is required to remove the TEV protease as well as traces of contaminants remaining after the first affinity selection. After washing, the bound material is released with EGTA (Figure 1). Both purification steps are required for **highly specific purification with very low background.**

A problem which may be encountered with the use of a tag in the method, is the possibility that the tag might affect protein function, protein expression levels, or the tag might not be sufficiently exposed to allow binding of the protein to the affinity beads or to the interacting proteins. However, mostly this can be solved by changing the location of the tag (N- or C- terminus). Another problem that might occur is that the target protein or an associated protein contains a TEV protease cleavage site (glutamic acid–asparagine–leucine–tyrosine–phenylalanine–glutamine–glycine) and is hence cleaved by the TEV protease. In our case, none of the metacaspase proteins harbours such a TEV cleavage site, however whether the associated proteins harbour such a cleavage site can not be known beforehand. Nevertheless, database searches suggest that only a very limited number of cellular proteins are cleaved by the TEV protease ((Dougherty et al., 1989); (Puig et al., 2001)).

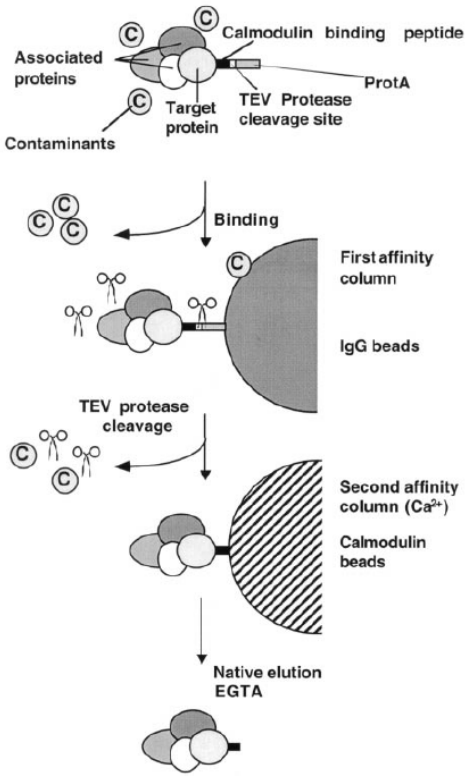


Figure 1: Schematic overview of TAP strategy (Puig et al., 2001). In the first affinity step, binding of the Prot A subunits of the TAP tagged target protein to the IgG affinity column retains its interacting proteins and releases contaminating, non-binding, proteins. Cleavage with the TEV protease releases the target protein complex through the presence of a TEV protease cleavage site in the TAP tag. In the second purification step, the CBP subunit of the TAP tag allows binding to the Calmodulin affinity resin in the presence of calcium. Elution of the target protein and its interacting partners occurs under mild conditions using EGTA.

6.4 Results and discussion

6.4.1 Cloning of *Arabidopsis* Type I metacaspases and transformation of *Arabidopsis* cell culture

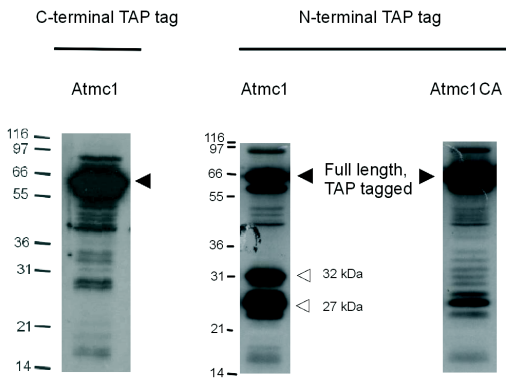
Wild-type and mutant (C/A) versions of the open reading frames (ORF) of the Type I *Arabidopsis* metacaspases Atmc1, 2 and 3 were cloned into both the N-terminal (NTAPi.289.gw.gck) (Rohila et al., 2004) and C-terminal (pK7mGW43Dnew) (Stals et al., in preparation) TAP tag containing vectors. In the resulting vectors, the fusion proteins are under transcriptional control of the Cauliflower Mosaic Virus 35S (CaMv35S) promoter. Subsequently, the constructs were transferred to the *Arabidopsis thaliana* cell suspension cells by *Agrobacterium*-mediated transformation (Table 1). Selection of transgenic calli occurred by selective growth (N-terminal fusions) or GFP-fluorescence (C-terminal fusions). Transgenic calli were mixed (per construct) and brought into suspension.

6.4.2 Molecular analysis of TAP tagged transformed *Arabidopsis* cell suspension

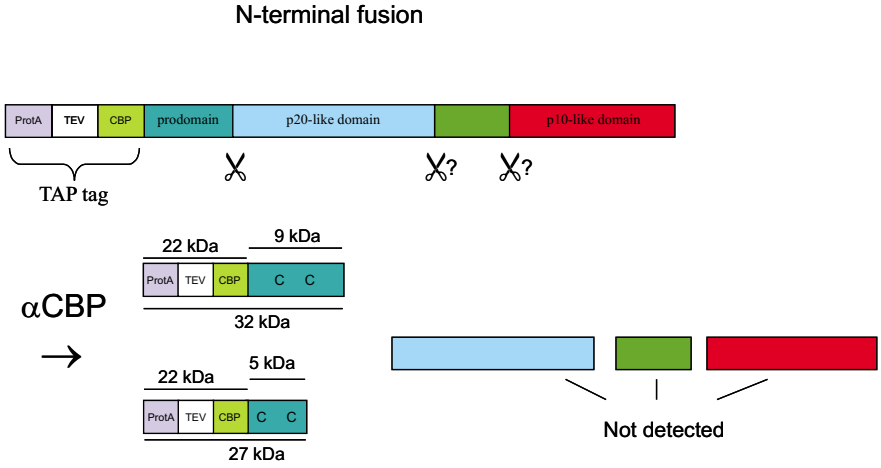
6.4.2.1 Atmc1 and Atmc1CA

Figure 2A clearly demonstrates strong overproduction of the Atmc1 and Atmc1CA TAP tagged fusion proteins in the suspension culture by Western blot. The observed fragments in the Western blot all contain the TAP tag, as detection occurred with the anti CBP antibody, and are hence the full length tagged proteins or N-terminal derived fragments. The full length TAP tagged Atmc1 / Atmc1CA protein migrates around 68 kDa. Interestingly, the **N-terminal** TAP tagged Atmc1 shows autoprocessing into p20- and p10-like subunits, as seen by the presence of the bands at 32 kDa and 27 kDa (Figure 2Ab). The band migrating at 32 kDa most probably represents the TAP tagged

Aa


C-terminal TAP tag
N-terminal TAP tag
Atmc1
Atmc1
Atmc1CA
116
97
66
55
36
31
21
14
Full length,
TAP tagged
32 kDa
27 kDa

Ab


N-terminal fusion
ProtA
TEV
CBP
prodomain
p20-like domain
p10-like domain
TAP tag
αCBP
22 kDa
9 kDa
C C
32 kDa
22 kDa
5 kDa
27 kDa
Not detected

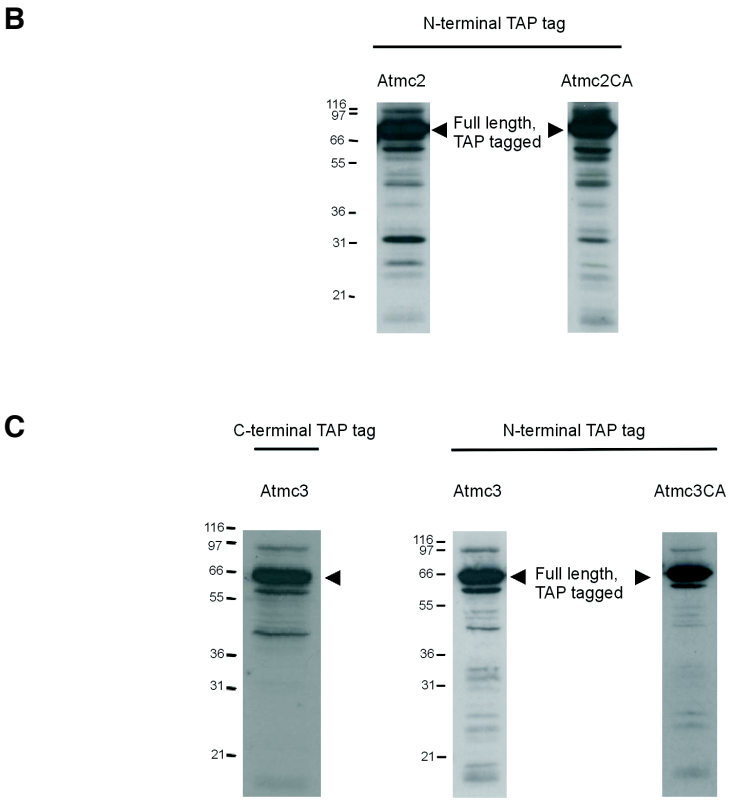


Figure 2: Overexpression of TAP tagged metacaspases Atmc1 (A), Atmc2 (B) and Atmc3(C) in *Arabidopsis* cell suspension cells. Western blot probed with anti-CBP antibody. Full length TAP tagged metacaspases are indicated by black triangles, fragments derived from autoprocessing by open triangles. The TAP tagged fusion proteins of Atmc1, Atmc2 and Atmc3 are clearly overproduced, both in case of N-terminal fusion and C-terminal fusion. For Atmc1, the N-terminal TAP tag fusion protein undergoes autocatalytic processing as demonstrated by the presence of the bands at 32 kDa and 27 kDa (Figure A1and A2).

prodomain. The smaller band at 27 kDa is probably a further degradation product of the TAP tagged prodomain, either aspecifically or by specific cleavage of the prodomain after the Zinc finger cysteines (Figure 2Ab). Indeed, there is an arginine-containing tetrapeptide (alanine-aspartate-proline-arginine) present in the sequence of the prodomain, after the two zinc fingers, which might be a possible candidate for autocleavage. The corresponding size of the zinc finger cysteine-containing prodomain is about 5 kDa, which fits with the apparent size of the observed 27 kDa TAP tagged fragment (22 kDa TAP tag + 5 kDa zinc finger containing prodomain). The p20- and p10-like subunits are not detectable because of loss of the TAP tag after autoprocessing. Most of the Atmc1 TAP tagged protein is present in its autoprocessed form. Autoprocessing is not observed with the mutant metacaspase 1. Remarkably, autoprocessing is not observed in case of **C-terminal** tagging of the Atmc1 protein (Figure 2Aa), which indicates that the position of the tag can influence the functionality of the protein. It can be suggested that positioning of the tag at the N-terminus has a positive effect on autoprocessing. This can be explained by the fact that the TAP tag might lead to an active dimerization of the metacaspases. Indeed, the CBP subunit of the tag might dimerize in presence of endogenous calmodulin (present in the cell) which can possibly lead to an active dimerization of the metacaspases and improve autoprocessing. This will probably be the reason that in this case autoprocessing of a Type I metacaspases is observed, while in plants no autoprocessing could be observed until now (see Chapter 2). Fusion of the tag at the C-terminus of the Atmc1 does not have a positive effect on dimerization as autoprocessing cannot be detected in case of a C-terminal fusion (Figure 2Aa). Indeed, also in mammalian systems dimerization occurs via N-terminal located domains such as dead effector domain (DED) or caspase recruitment domain (CARD) (Earnshaw et al., 1999).

6.4.2.2 Atmc2 and Atmc2CA

A clear overproduction of the Atmc2 and Atmc2CA protein can be detected in the suspension culture, the full length fragments of both proteins have an apparent molecular weight of 73 kDa (Figure 2B). No clear autoprocessing occurs in case of the wild type metacaspase, despite an N-terminal fusion of the TAP tag with the Atmc2. The exact reason why Atmc2 does not show autoprocessing, in contrast with N-terminal fusion of Atmc1, remains elusive. However, concentration-dependency or need for additional (yet unidentified) cofactors might be an important issue. No C-terminal fusions of the wild type or mutant form of the Atmc2 protein were available.

6.4.2.3 Atmc3 and Atmc3CA

Similarly to the Atmc2 protein, the Atmc3 and Atmc3CA proteins were clearly overproduced in the *Arabidopsis* suspension culture. The full length TAP tagged fragments migrate at a molecular weight of 69 kDa and no autoprocessing is detectable of the Atmc2 protein, not in case of an N-terminal neither in case of a C-terminal fusion (Figure 2C).

Conclusively, expression analysis of the *Arabidopsis* cell cultures showed clear overexpression of the TAP tagged Type I metacaspases, both wild type and mutant versions, for all available N- and C-terminal constructs. Hence, cultures containing the TAP tagged metacaspases were grown for upscale for the TAP procedure, as approximately 15 g is required for one TAP.

6.4.3 Blue Native gel electrophoresis (BN PAGE) for the analysis of metacaspase-protein complexes in *Arabidopsis* cell suspension cultures

To analyze if the *Arabidopsis* metacaspases are resided in protein complexes (size, number of complexes) within the cell a blue native electrophoresis (BN PAGE) experiment was done. BN PAGE can be used for a high-resolution separation of intact protein complexes by their molecular mass using a standard polyacrylamide gel electrophoresis system in which in a first dimension the ionic detergent SDS is replaced by mild detergents, for the solubilization of proteins, and the dye Coomassie blue, for adding negative charges to the proteins. Hereby, native and enzymatically active protein complexes are obtained. When followed by an SDS PAGE in the second dimension, a complete denaturation of the protein complexes leads to subsequent separation of the protein complexes in their subunits. The working principle of the BN PAGE and BN / SDS PAGE is depicted in Figure 3. BN offers a broader proteomics approach as it does not only provide data on protein complex composition, but also on the composition of many small and large protein complexes and therefore the interaction patterns of all their protein subunits in a relatively complex sample which can be determined in a single experiment (Nijtmans et al., 2002). In contrast with more popular proteomics techniques such as Y2H, TAP and others, which require that the organism of choice is subjected to genetic engeneering, the BN PAGE does not involve such modifications. Moreover, these techniques mostly focus on a single protein and determine its binding partners, while BN PAGE can be an ideal tool to study new protein complexes in plants (Eubel et al., 2005).

In our case, BN / SDS PAGE could be used to gain information in which complexes the metacaspases reside in the cell. Moreover, by performing MS analysis on the separated subunits of the protein complexes more information can be gained about the interaction partners within the protein complex, which offers an additional, complementary way to analyze protein interactions *in vivo*. A two-dimensional approach, combining the BN PAGE with SDS PAGE, was performed on both total

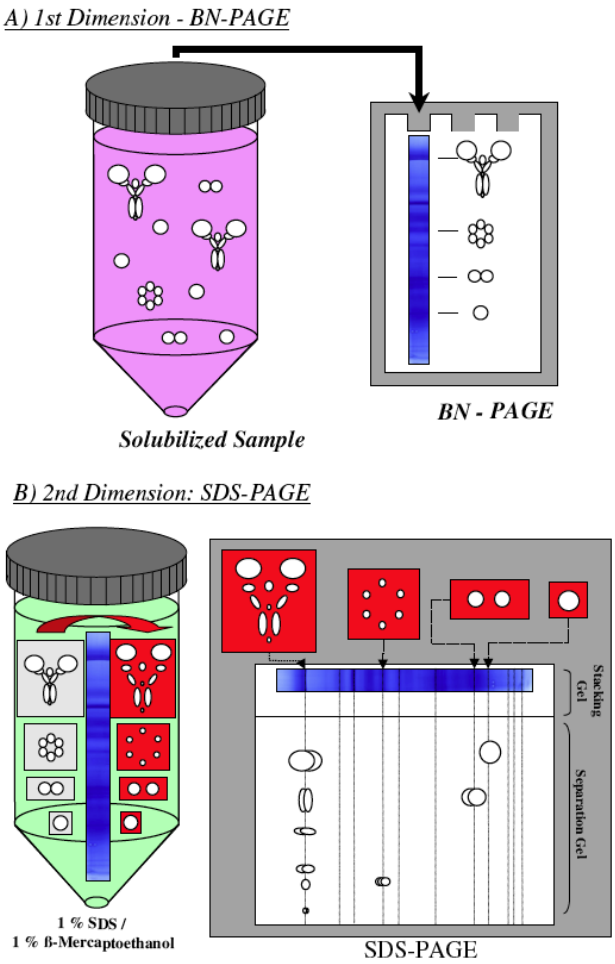


Figure 3: Principle of BN and BN / SDS PAGE (Eubel et al., 2005) A) Separation of mixture of different native protein complexes in the first dimension according to their molecular weight. For the solubilization of the samples mild detergents (such as digitonin) are used. The dye Coommassie blue G250 is responsible for separation in the native gel by binding and negatively charging the solubilized protein complexes. **B)** Following the first dimension gel run, the lane of the gel is excised, subjected to a denaturing solution (containing SDS and beta-mercaptoethanol) and run perpendicular in the second dimension of an SDS PAGE. This leads to denaturation and subsequent dissociation of the native protein complexes (underlayed in grey colour) in their constituent subunits (underplayed in red colour). SDS is used in the denaturing step and negatively charges the subunits, separating them according to their molecular weight upon electrophoresis. Hence, subunits of a protein complex are detected on a vertical row on the second dimension gel.

extract and TAP eluate of *Arabidopsis* suspension cultures overexproducing N-terminal TAP tagged Atmc1 protein. In that way additional information on the efficiency of the TAP procedure could also be obtained. The first dimension was run on a 5 to 16% polyacrylamide BN gel, and as second dimension a 12% polyacrylamide gel. The 2D approach on **total protein extract** shows that the metacaspase 1 is present in a series of complexes, varying from the monomeric metacaspase to complexes of at least approximately 440 kDa (detection of higher complexes is more difficult due to low abundancy) (Figure 4A). It is an interesting finding that the metacaspases are resided in complexes of varying sizes within the cell. BN / SDS PAGE on **TAP eluate** shows only the monomeric form of the metacaspase 1 (around 46 kDa), and most probably a processed form of the Atmc1 protein (approximately 35 kDa) (Figure 4B). This processed form could represent the TAP tagged proform followed by the p20-subunit (after TEV cleavage). However, as the Western blot could not be probed with anti-CBP antibody and MS analysis failed to identify this band, this is not completely confident. However, because this band is recognized by the anti Atmc1-antibody and Atmc1 was found to be processed, it is most likely that this band is derived from autoprocessing. Unfortunately, MS analysis also failed to identify the interacting partners of Atmc1 within the Atmc1-protein complexes. Strikingly, the 2D analysis demonstrated that upon TAP purification only the monomeric complexes are retained, and the bigger complexes are apparently lost during TAP purification. This might explain the relatively low number of interacting proteins purified with the different metacaspase baits using the TAP technology. Moreover, the fact that many of the complexes are lost during the TAP procedure, will lead to a further optimalization of the different steps during the purification. This loss can be due to the long duration of the procedure (more than 12 hours), or a TEV cleavage step which was performed at 17°C. Hereby, larger complexes are more sensitive to dissociation than the smaller complexes. For example, the effect of an overnight TEV cleavage step at 4°C will be tested.

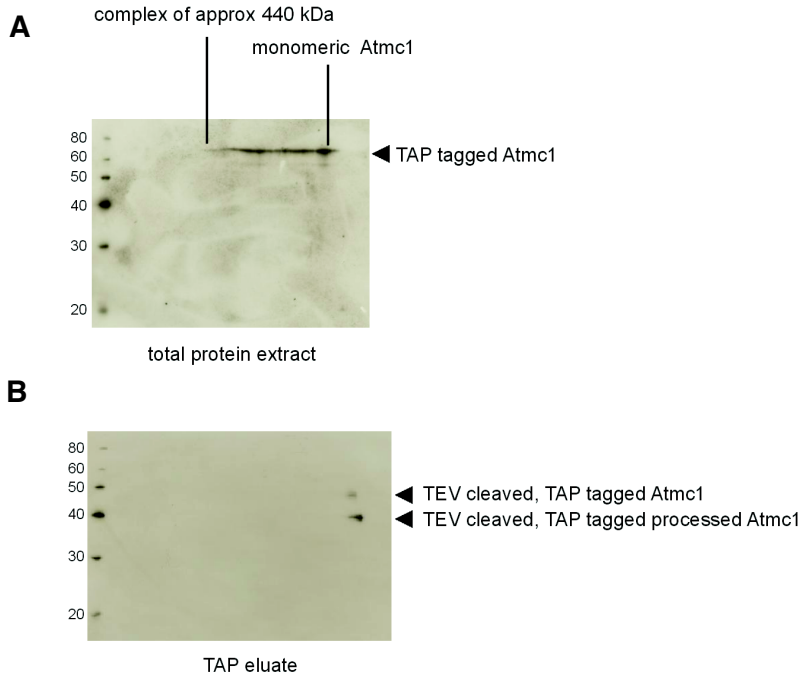


Figure 4: BN on total protein extract (A) and TAP eluate (B) of *Arabidopsis* suspension culture overexpressing N-terminal TAP tagged Atmc1. Immunoblotting with antibody generated specifically against Atmc1 (PS729-Atmc1). In total protein extract the full length Atmc1 tagged protein (68 kDa) resides in a series of complexes, varying from the monomeric metacaspase to complexes of approximately 440 kDa. Upon TAP purification, only the monomeric metacaspases are retained. The TEV cleaved, TAP tagged proform of Atmc1 can be detected around 46 kDa, the other lower molecular weight fragment is probably a processed form of the Atmc1 protein. This processed form could represent the TAP tagged proform followed by the p20-subunit (after TEV cleavage), this fragment has an apparent molecular weight of 35 kDa.

6.4.4 Tandem affinity purification of the Type I metacaspases

TAP purification was performed on two-days old *Arabidopsis* cell suspension overproducing the fusion proteins. TAP purification was performed on all available constructs (Table 1), except on the C-terminal fusion of Atmc3. The different steps in

	cloning	transgenic calli	TAP
N-terminal TAP tag vector			
N-Atmc1	+	+	+
N-Atmc1CA	+	+	+
N-Atmc2	+	+	+
N-Atmc2CA	+	+	+
N-Atmc3	+	+	+
N-Atmc3CA	+	+	+
C-terminal TAP tag vector			
C-Atmc1	+	+	+
C-Atmc1CA	+	-	/
C-Atmc2	-	-	/
C-Atmc2CA	-	-	/
C-Atmc3	+	+	/
C-Atmc3CA	+	-	/

Table 1: Overview of the TAP tagged metacaspase constructs and transgenic calli. Cloning in the C-terminal TAP tag vector did not succeed for Atmc2 and Atmc2ca, and transformation of C-terminal tagged AtMC1CA and AtMC3CA did not result in transgenic calli. TAP was performed for all constructs indicated with +.

the TAP procedure are depicted in Figure 5, with TAP on the N-terminal fusion of Atmc3CA as a representative. Starting material of the *Arabidopsis* suspension cells, shows clear overexpression of the TAP tagged fusion protein of Atmc3CA (Figure 5, lane 1). The flow through fraction of the IgG beads shows there is a considerable fraction of full length TAP tagged protein which does not bind to the IgG column (Figure 3, lane 2). This can be due to an inaccessibility of the TAP tag in some fusion proteins which results in an inability to bind to the IgG affinity resin. Cleavage of the TAP tagged proteins by TEV protease is detected by a shift in molecular weight of approximately 10-15 kDa, due to cleaving off the IgG subunits of protein A (ProtA). Remarkably, TEV protease cleavage did not occur optimally as upon cleavage the full length TAP tagged protein can still be observed (Figure 5, lane 4). It is possible that this is due to the fact that the TEV cleavage site of the TAP tag is somehow not optimal accessible to the protease, which could be attributed to binding of other proteins to the TAP tagged protein, such as for instance oligomerization of the TAP

tagged metacaspases. There was indeed a substantial fraction of full length TAP tagged fusion proteins which was not released by TEV cleavage as seen upon boiling of the IgG beads after TEV treatment (Figure 5, lane 3). In the flow through fraction of the Calmodulin affinity resin particularly full length fusion protein is detected (Figure 5, lane 5). Most probably these full length proteins can bind less easy to the affinity column due to decreased accessibility of the CBP unit of the TAP tag, as in the not cleaved TAP tag the CBP unit is not exposed to the outside. After these two affinity purification steps, the final elution fractions from the Calmodulin affinity resin were collected separately (Figure 5, lanes 6-12). Finally, the TAP purified Atmc3CA protein is mostly observed in the first two elution fractions, although it has to be remarked that in all fractions there is an excess of non TEV cleaved Atmc3CA protein due to non-optimal cleavage by the TEV protease.

The TEV cleavage and elution step for the different N-terminal TAP tagged Type I metacaspases are depicted in Figure 6. TEV cleavage results in fragments of about 55 kDa for Atmc1 and Atmc3 (both wild type and mutant form) and of 62 kDa for Atmc2 (also both forms). It can be deduced that TEV protease cleavage is not optimal for any of the metacaspases, wild type nor mutant form. In addition, for the Atmc1 fusion protein TEV cleavage resulted in an additional lower MW-fragment probably due to aspecific degradation of the TEV cleaved protein by contaminating proteases. Interestingly, for most metacaspases (except for Atmc3CA) it is predominantly the TEV cleaved protein, and not the full length (non TEV cleaved) protein, which is eluted at the end. Probably, as shown in Figure 5, the second purification on the Calmodulin affinity resin is responsible for loss of a substantial fraction of the full length non-cleaved protein as binding to this affinity column through its CBP subunit is less efficient. Remarkably, Atmc1 showed autoprocessing in the suspension culture (Figure 2A), however these autoprocessed fragments could not be retained during the TAP procedure as these fragments were not detected in the elution fraction. Apparently, the TAP tagged prodomain (and TAP-tagged zinc-finger containing prodomain) were lost during the TAP procedure, the reason why this occurred remains elusive.

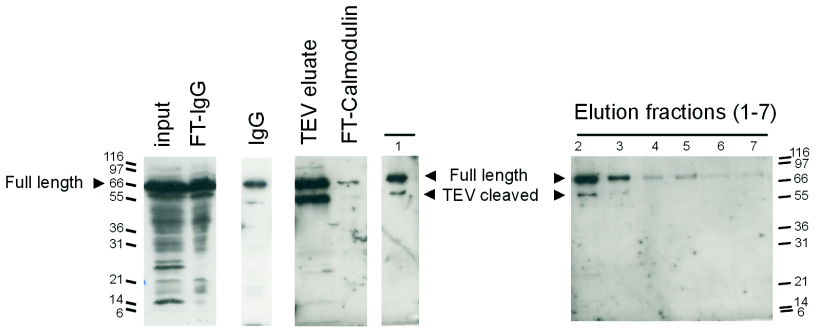


Figure 5: Western analysis using anti-CBP antibody shows the different steps through TAP procedure. As a representative TAP purification of the N-terminal fusion protein of Atmc3CA is depicted. The TAP tagged Atmc3CA protein which is overproduced in the suspension culture is purified and subsequently eluted, predominantly in the first two elution fractions. First lane, *Arabidopsis* cell culture overproducing N-terminal TAP tagged Atmc3CA (around 68 kDa), used as starting material for the TAP procedure. Second lane, Flow through (FT) of IgG beads; Third lane, boiled IgG beads; fourth lane, TEV eluate; fift lane, FT of Calmodulin beads. Last lanes, elution fractions 1 to 7.

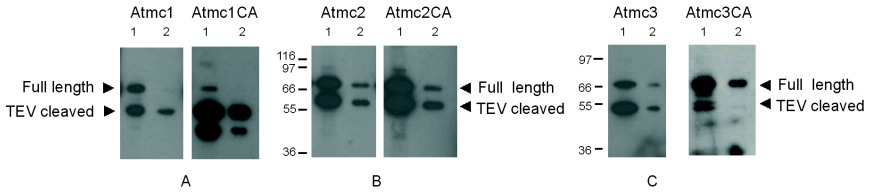


Figure 6: TEV cleavage (lanes 1) and elution step (lanes 2) of the N-terminal TAP tagged metacaspases during TAP procedure. For all metacaspases, TEV cleavage did not occur optimal; however for most metacaspases it is predominantly the TEV cleaved tagged protein which is eluted. The TEV cleaved protein of Atmc1 and Atmc3 (both wild type and mutant) migrates around 55 kDa, that of Atmc2 (also both forms) around 62 kDa. For Atmc1CA, upon TEV cleavage an additional lower molecular weight fragment can be observed (around 45 kDa) which might be due to aspecific degradation of the TEV cleaved product.

6.4.5 Expansion of the protein interaction map of the Type I metacaspases: detection of putative novel interacting partners by TAP / MS based approach

Elution fractions from the Calmodulin affinity resin in which the TAP tagged purified protein was detected, were pooled and TCA precipitated. For all performed TAP purifications (see Table 1), one-dimensional 4 to 12% % gradient gels were run, subsequently Coomassie Blue G stained and the excised bands were subjected to MS analysis. In addition, for one TAP, the TAP of N-terminal target protein of Atmc3CA, a two-dimensional gel was run, with separation according to the protein's isoelectric point (pI) in the first dimension, so called isoelectric focusing (IEF). As a second dimension 4 to 12 % gradient SDS gels were run.

The **1D-pattern** obtained after TAP procedure is quite different amongst the different metacaspases, also between wild type and mutant form (Figure 7). Despite the detection of some similar bands, the overall pattern with the distribution of low and high molecular fragments is quite different which suggests there are some different interacting partners in the interaction maps of the different metacaspases. The TAP tagged metacaspases (= **bait** proteins) are often detected multiple times, both in lower and higher molecular weight fragments than the TEV cleaved proteins. The TEV cleaved Atmc-proteins (55 kDa for Atmc1 and Atmc3, 62 kDa for Atmc2) are clearly detectable by the presence of a more intense band. All bait fragments are indicated in the figure (black triangles). The fact that the bait metacaspases are also observed in higher molecular weight fragments, can be due to an insufficient reduction of the sample before putting on gel which could allow reformation of disulfide bridges. In addition, an effect of position of the tag could be revealed as the pattern obtained with the C-terminal fusion protein was considerably different from the N-terminal fusion protein. This was already observed in Figure 2 where processing could be observed with the N-terminal tagged Atmc1, in contrast with the C-terminal tagged protein. This confirms the assumption that the position of the tag has an effect on the functionality

of the protein and/or folding and hence also on the proteins interacting with the bait protein. Remarkably, the bait proteins are not always detected by MS analysis, as is the case for the TAP with Atmc2 and Atmc2CA, which can be due to the fact that the bait protein is present under the detection limit or that it is present in a fragment underneath a high concentration of another protein. On the other hand, the bait proteins Atmc2 and Atmc2CA were detected by Western blot in the elution fractions (Figure 6). The results of the **interacting** proteins identified via mass spectrometric analysis are represented in Table 2.

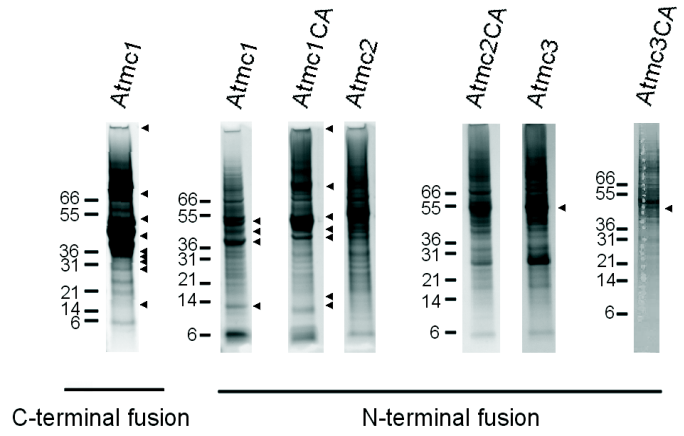


Figure 7: One-dimensional gels on TAP purified metacaspases. Pictures were taken after Coomassie G staining, and prior to subjection to MS analysis (picture from Atmc3CA was taken after picking with the robot). Bands in which the TAP tagged, bait metacaspases were identified are indicated with a black triangle. In the TAP with Atmc2 and Atmc2CA the bait metacaspases could not be identified by mass spectrometric analysis.

	1D/2D	AGI code	protein name
N-terminal tagged Atmc1			
	1D	At2g43040	Hypothetical protein At2g43040
	1D	At1g02170	latex-abundant family protein (AMC1) / caspase family protein
	1D	At1g47710	serpin, putative / serine protease inhibitor, putative
N-terminal tagged Atmc1CA			
	1D	At1g02170	latex-abundant family protein (AMC1) / caspase family protein
C-terminal tagged Atmc1			
	1D	At3g02560	40S ribosomal protein S7 (RPS7B)
	1D	At5g16130	40S ribosomal protein S7 (RPS7C)
	1D	At3g23990	chaperonin (CPN60) (HSP60)
	1D	O23612 (At4g17720)	Hypothetical protein dl4895c (RNA recognition motif (RRM)-containing protein)
	1D	At1g02170	latex-abundant family protein (AMC1) / caspase family protein
	1D	At2g43080	oxidoreductase, 2OG-Fe(II) oxygenase family protein
	1D	At4g04570	protein kinase family protein; receptor-like protein kinase
	1D	At1g47710	serpin, putative / serine protease inhibitor, putative
N-terminal tagged Atmc2			
	1D	At2g39460 or At3g55280	60S ribosomal protein L23A (RPL23aA or RPL23aB)
	1D	At3g04120 or At1g13440	glyceraldehyde 3-phosphate dehydrogenase, cytosolic, putative / NAD-d
	1D	At2g44060	late embryogenesis abundant family protein / LEA family protein
	1D	At5g09810	actin 7 (ACT7) / actin 2
N-terminal tagged Atmc2CA			
	1D	At3g23990	chaperonin (CPN60) (HSP60)
	1D	At3g04120 or At1g13440	glyceraldehyde-3-phosphate dehydrogenase, cytosolic (GAPC) / NAD-depe
	1D	At2g44060	late embryogenesis abundant family protein / LEA family protein
N-terminal tagged Atmc3			
	1D	At5g45390	ATP-dependent Clp protease proteolytic subunit (ClpP4)
N-terminal tagged Atmc3CA			
	1D	At5g49530	SIN-like family protein low similarity to Sex-lethal interactor
	1D	At4g39650	gamma-glutamyltranspeptidase family protein
	1D	At3g17530	F-box family protein contains Pfam profile: PF00646
	1D	At5g64240	metacaspase 3 / latex-abundant family protein (AMC3) / caspase family protein
	1D	At1g06260	cysteine proteinase, putative contains similarity to thiol-protease
	1D	At1g45976	expressed protein
	1D	At1g56190	phosphoglycerate kinase, putative
	1D	*	60S ribosomal protein L6 (RPL6)
	1D	At5g18110	novel cap-binding protein (nCBP)
	1D	*	40S ribosomal protein S7
	1D	*	60S ribosomal protein L18
	1D	*	60S ribosomal protein L23A
	1D	At1g42700	hypothetical protein
	1D + 2D	At3g48870	ATP-dependent Clp protease ATP-binding subunit (ClpC)
	1D + 2D	*	actin
	1D + 2D	At2g41800	expressed protein contains Pfam profile PF04862
	1D + 2D	At3g04120	glyceraldehyde-3-phosphate dehydrogenase, cytosolic (GAPC)
	1D + 2D	At2g44060	late embryogenesis abundant family protein / LEA family protein
	2D	At2g25350 phox	(PX) domain-containing protein
	2D	At2g39730	ribulose bisphosphate carboxylase/oxygenase activase / RuBisCO activase
	2D	At3g13920	eukaryotic translation initiation factor 4A-1 / eIF-4A-1

Table 2: Interacting proteins identified via MS analysis upon TAP purification with the different metacaspase baits. Proteins which were identified via MS analysis, however also detected in control TAPs (using GFP/GUS TAP tagged bait proteins) are not withdrawn in the table, as they have a high chance of being false positives. The table indicates whether the proteins were identified by 1D or 2D. If MS analysis was not able to identify a single gene, this is indicated with *.

6.4.5.1 There is no overlap with datasets obtained by Y2H

Comparing the TAP purified proteins identified with the different metacaspase-baits, it can be remarked that the common interactors are predominantly the ones that are also found back in control TAPs (ribosomal proteins, heat shock proteins, tubulin a/b chain and elongation factor 1-alpha) and as such have a high probability of being false positives. Remarkably, interactors that have been identified with the wild type metacaspase-bait are not always found back in the mutant form. There might be several reasons for that. First of all, as shown in case of Atmc2, the bait protein could not be identified by mass spectrometric analysis with the wild type nor mutant form. However, the bait proteins could be identified in the TAP elution fractions of Atmc2 and Atmc2CA on a Western blot using the CBP antibody, which demonstrates that the MS platform can be limited in its ability to identify proteins. Second, it might be possible that mutation of the metacaspase has an effect on binding capacity or binding partners. Especially for Atmc1 an effect of the mutation can be expected, as for Atmc1 autoprocessing occurs this can be reflected in different or extra binding partners which bind specifically to the TAP tagged prodomain. Although, during the purification, these processed forms could not be detected. On the other hand, there are some interactors that are found back in both the wild type and mutant form and that seemed to be specific for a certain metacaspase. Atserpin1 showed to be an interactor of both Atmc1 and Atmc1CA, although it was demonstrated that the interaction is not confined by Atmc1 alone, as shown by a serpin-Atmc9 interaction (discussed below). For Atmc2 and Atmc2CA both the glyceraldehyde 3-phosphate dehydrogenase and late embryogenesis abundant family protein seemed to be specific interactors. Clp proteases are only identified with Atmc3 and Atmc3CA (ClpP4 for Atmc3 and ClpC for Atmc3CA). To what extent these interactors are specifc, or rather general metacaspase-interactors is not clear at present. Elucidating this, it will also be important to implement this information to obtain more insight into the specific function of each metacaspase and the metacaspase family in general. In addition, as is the case for any protein interaction method, the biological relevance and functionality

of the interaction also has to be determined.

If we compare the dataset obtained by TAP purification with the one obtained by **Y2H-screening**, there is no overlap between the different datasets. Using Atmc2CA and Atmc3CA as bait proteins in both a Y2H screening and a TAP purification, no common interactors were obtained. However when comparing the datasets in general (not taking into account which metacaspase was used as a bait) it can be remarked that there are only some ribosomal, RNA-associated or heat shock proteins found in common, and these are not even exactly the same proteins but other members of the family. There is one protein, the serpin protein which was found in common with both techniques, however with different bait proteins (in Y2H with Atmc9CA (Tine Beunens, submitted), and in TAP with Atmc1/CA). However, this small overlap between Y2H data and TAP data has also been seen in *Saccharomyces cerevisiae* (Gavin et al., 2002). Only 7% of the protein interactions was found to be similar. This is due to the fact that both methods address different aspects of protein interactions. Two-hybrid analysis is of exceptional value for the detection of pairwise and transient interactions, while TAP/MS based methodologies provides information of protein complex composition. As such, both methods can be used in a complementary way.

Interestingly, there was an overlap found with proteins which were identified as a substrate of the metacaspases (Type II metacaspases Atmc4 and Atmc9) in a parallel assay. This suggests that these interactors identified via TAP, have a higher likelihood to be substrates of the Type I metacaspases as well. The fact that these substrates would then be identified for both Type I and Type II metacaspases, could suggest that these substrates might be general substrates for the metacaspase family. Moreover, this indicates that TAP was able to identify protease-substrate interactions in addition to protease-regulator (e.g. inhibitor) interactions.

Conclusively, every technique has its own limitations and advantages, and a combination of different protein interaction methods is ideal to get a more complete overview of an interactome of a certain protein of interest. Indeed, the different techniques often yield datasets which are complementary to one another.

6.4.5.2 Some interacting partners give lead to a specific function of the metacaspases in cytoskeletal organization, apoptosis, plastid development and energy metabolism.

One of the interacting proteins is **actin**, which was detected upon TAP purification with Atmc2 and Atmc3CA (Table 2). Actin is an ubiquitous component of the plant cytoskeleton and participates in a number of important subcellular processes ((Hussey et al., 2006); (Staiger and Lloyd, 1991). The microfilament cytoskeleton is involved in cell division plane localization, cytokinesis, cell elongation and cell shape determination, cytoplasmic streaming, maintenance of cell architecture, cell wall deposition, transport and organelle movement (Kobayashi et al., 1987; Lloyd, 1991; Cleary *et al.*, 1992; Palevitz *et al.*, 1987; Seagull, 1989; Staiger and Schliwa, 1987). Also the response of plants to external stimuli, such as the gravitrophism of root growth and light on chloroplast streaming and orientation, may also require the actin-based cytoskeleton (White and Sack, 1990; Ytow *et al.*, 1992). Plant actin is encoded by a multigene family in most multicellular eukaryotes. Quantitative evolutionary studies suggest that the different actin subclasses diverged very early in vascular plant evolution (Hightower and Meagher, 1986). It has been proposed that such ancient and divergent gene family members have been maintained in the plant genome because they have distinct patterns of regulation and/or encode proteins with distinct functions. Indeed, data from diverse species suggest that plant actin genes are expressed differentially (Meagher and Williamson, 1994). The *Arabidopsis thaliana* actin family consists of ten genes (ACT1, 2, 3, 4, 5, 7, 8, 9, 11, 12) (McDowell *et al.*, 1996). Eight of them are highly transcribed at some time in development, while two might be pseudogenes. This gene family can be subdivided into two ancient classes of genes that diverged early in land plant evolution and may have separated vegetative from reproductive actins. Subsequent divergence produced a total of six distinct subclasses of actin, and five showed a distinct pattern of tissue specific expression (McDowell *et al.*, 1996; Meagher *et al.*, 1999).

A role for the metacaspases in actin cytoskeletal organization or disruption of

the cytoskeletal organization during apoptosis can be suggested. A function in the latter was also proposed for mammalian caspases. In mammalian systems alpha-actin (Communal et al., 2002), beta-actin (Mashima et al., 1997) and many other cytoskeletal and structural proteins (Fischer et al., 2003) are identified as substrates of the caspases. Disruption of such cytoskeletal proteins may lead to some of the morphological changes associated with cell death such as changes in cell shape. In addition, in yeast a role for actin in yeast ageing and apoptosis has been suggested (Gourlay and Ayscough, 2005). It was proven that reduced actin dynamics led to increased levels of ROS and correlated decreased cell viability. Therefore, it was proposed that the actin cytoskeleton could function as a regulator of ROS release from the mitochondria and as a key regulator of apoptosis.

A protein involved in protein synthesis was identified as a candidate Atmc3CA-binding protein, namely **eukaryotic translation initiation factor eIF4A-1**. Initiation factors were also identified as mammalian caspase substrates. Induction of apoptosis results in inhibition of the rate of overall protein synthesis. Cleavage of these proteins results in inactivation of the protein, leading to loss of translation ((Satoh et al., 1999); (Marissen et al., 2000); (Bushell et al., 2000); (Tee and Proud, 2002)). Possibly, the eIF4A-1 protein is also a substrate of the metacaspase(s) during apoptosis. This link of inhibition of protein translation with progression of apoptosis was also revealed in TAP with ARC1, a tRNA-binding protein, where yeast metacaspase was detected as an interacting potein (Krogan et al., 2006).

TAP purification of Atmc3 and Atmc3CA resulted in the characterization of a **Clp protease**. TAP purification with Atmc3 resulted in identification of ClpP4 and with Atmc3CA of ClpC2. Clp proteases are a class of ATP-dependent, serine proteases that are present in eubacteria, plants and mammals (Adam and Clarke, 2002). The proteases are composed of a serine-type proteolytic subunit ClpP and a regulatory ATPase subunit ClpC (molecular chaperone), as such two essential functions are separated in two different polypeptides. The Clp proteases have a barrel-like structure

housing a catalytic triad characteristic of serine-type proteases. In *E. coli*, the Clp protease has a central core comprised of two face-to-face heptameric rings of ClpP flanked at one or both sides by a single hexameric ring of ClpA or -X (molecular chaperones) (Grimaud et al., 1998). In an ATP-dependent fashion, the regulatory subunits selectively bind and unfold the polypeptide targeted for degradation and then translocate it into the central cavity of the barrel-like ClpP complex (Wang et al., 1997). Within this proteolytic chamber, ClpP rapidly degrades the polypeptide into smaller fragments that then diffuse out. The incorporation of the activity of molecular chaperones results in targeting specific polypeptide substrates and avoid accidental degradation of others. ClpC is a molecular chaperone of the Hsp100 family, and in higher plants there are two chloroplast (stroma)-localized paralogs (ClpC1 and ClpC2). *Arabidopsis clpC1* T-DNA insertion mutants display a retared-growth phenotype, leaf chlorosis, lower photosynthetic activity, and a specific reduction in photosystem content (Sjogren et al., 2004). Hence, it is suggested that ClpC plays a vital role in chloroplast function and leaf development and is likely involved in photosystem biogenesis. In *Arabidopsis*, six ClpP genes are identified and antisense lines for Clp3-6 and all show chlorotic appearance on the leaves (Clarke et al., 2005). The interaction with the Clp proteases suggests a role for the metacaspases in photosynthesis. However, as not much is known about the functionality of these Clp proteins, it is difficult to predict how exactly the metacaspases would be involved in the photosynthetic process. It is possible that the Clp proteases would be a substrate of the metacaspases. However, the effect, function or relevance of cleavage of ClpC by the metacaspase(s) remains elusive.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was found to be a candidate binding protein of Atmc2 (both wild type and mutant) and Atmc3CA. GAPDH is known for its pivotal role in glycolysis and subsequent energy production. GAPDH catalyzes the oxidation and phosphorylation of D-glyceraldehyde-3-phosphate to 1,3 -diphosphoglycerate with the conversion of NAD^+ to NADH. Several more recent studies suggest that the mammalian glycolytic enzyme displays a number of activities

unrelated to glucose metabolism (Sirover, 1999). These include its role in membrane fusion, microtubule bundling, phosphotransferase activity, nuclear RNA export, DNA replication and DNA repair. Interestingly, a function for GADPH in tubulin polymerization/bundling into microtubules provides additional evidence for a putative role for the metacaspases in cytoskeletal organization (cfr interaction of the metacaspases with actin). These new activities may be related to the subcellular localization and oligomeric structure of GAPDH *in vivo* (Sirover, 2005). Furthermore, other investigations suggest that GAPDH is involved in apoptosis (Berry and Boulton, 2000). For the apoptotis-related function its nuclear translocation appeared to be essential and GAPDH is assumed to function as an intracellular sensor for oxidative stress during early apoptosis (Chuang et al., 2005). In plants, GAPDH has found to act in a GAPDH/CP12/PRK complex, this complex is identified in many photosynthetic organisms and may have an important role in the regulation of CO2 assimilation (Graciet et al., 2004). In addition, a role for GADPH in ROS signalling in plants has also been suggested (Hancock et al., 2005). Concerning the interaction with GAPDH multiple roles for the metacaspases can be proposed, in energy metabolism, apoptosis or cytoskeletal organization. If GAPDH could be a substrate of the metacaspase(s), altering for example the function, activity or conformation of the GAPDH protein upon its cleavage remains to be resolved.

Another glycolytic enzyme identified with Atmc3CA-TAP purification is **phosphoglycerate kinase (PGK)**. PGK is an ubiquitous monomeric enzyme that has been isolated from diverse species ranging from humans and plants to bacteria (Watson and Littlechild, 1990). PGK is an ATP-generating glycolytic enzyme that forms part of the glycolytic, gluconeogenic and photosynthetic pathways (Banks et al., 1979). In higher plants it occurs in chloroplasts, cytosol and nuclei (Anderson et al., 2004).

Another photosynthetic-related enzyme identified as a putative Atmc3CA binding protein is **Rubisco activase**. Ribulose 1,5-bisphosphate carboxylase / oxygenase (Rubisco) activase is a chloroplast protein that has an ability to activate and relieve the inhibition on the Rubisco protein by removing various inhibitory sugar phosphates

that either block substrate binding or prevent carbamoylation (Wang and Portis, 1991). Photosynthetic organisms produce ATP and NAD(P)H as chemical energy using sunlight, and fix CO2 into an organic compound using this chemical energy in the Calvin cycle. Rubisco, key enzyme in this photosynthetic pathway, catalyzes the carboxylase reaction that fixes CO2 in the substrate ribulose-1,5-bisphosphate (RuBP). The carboxylase reaction is the starting reaction in the Calvin cycle and fixed CO2 is utilized as the carbon source for growth of photosynthetic organisms (Ashida et al., 2005). Plants lacking activase or having a very low level of activase cannot survive at atmospheric CO2 levels (Sommerville *et al.*, 1982; (von Caemmerer et al., 2005)) and those with reduced levels showed reduced rates of photosynthesis and growth ((Mate et al., 1993); (Hammond et al., 1998)).

Gamma-glutamyltranspeptidase (GGT) was identified as a possible Atmc3CA-binding partner. GGT is a heterodimeric glycoprotein that catalyzes the transpeptidation and hydrolysis of the gamma-glutamyl group of gluthatione and related compounds and plays a role in the metabolism of glutathione.

Identification of ClpC protease, GAPDH, PGK, GGT and Rubisco activase as putative interactors of the metacaspases, may suggest a putative role for the metacaspases in photosynthesis and/or energy metabolism. How the metacaspases would be involved, and if these proteases could be substrates of the metacaspases is not known to date and remains to be studied.

Upon TAP purification with Atmc2 (WT and CA) and Atmc3CA, a **late embryogenesis abundant (LEA)** protein was identified. LEA proteins are expressed late in embryo maturation and during the developmentally regulated period of dehydration at the end of seed development (Hong-Bo et al., 2005). They are thought to play an important role in the maturation process, and to be involved in protecting higher plants from damage caused by environmental stresses, especially drought (dehydration). Hereby, a putative role for metacaspases in seed development and response to stress can be suggested.

Identified in TAP purification with Atmc3CA is a **SIN (Sex-lethal interactor)-like**

family protein. SIIN is a Drosophila protein that associates with the RNA binding protein Sex-lethal (Dong and Bell, 1999). Sex-lethal encodes an RNA binding protein that regulates gene expression in Drosophila sex determination and dosage compensation and is best known as a regulator of alternative RNA splicing (Bell et al., 1988). An **F-box family protein**, specificity factors for a family of ubiquitin protein ligases, has also been identified in a TAP with Atmc3CA.

Another interactor identified from the TAP purification with Atmc1 (both N- and C-terminal) is **serpin**. Serpins (**ser**ine **p**roteinase **in**hibitors) are a superfamily of protease inhibitors that are widespread among organisms, being the only proteinase inhibitor found in eukarya, bacteria, archaea and viruses (Rawlings et al., 2004). In animals, the inhibitory mechanism is well understood (van Gent et al., 2003). The serpins fold into a well-conserved, metastable structure, consisting of three b-sheets (A, B and C), nine a-helices and a reactive centre loop (RCL), which is necessary to employ a unique suicide substrate-like inhibitory mechanism. The RCL, which is recognized by the target protease is a key feature of the serine protease inhibitors. Upon RCL-cleavage by the protease, the serpin undergoes a strong conformational change (Figure 8). During this rearrangement, the RCL moves from an exposed position to one in which it forms an extra strand in the centre of the b-sheet A. Hereby the catalytic site of the bound protease is distorted and the serpins irreversibly neutralize their targets by forming a covalent acyl-enzyme intermediate ((Gettins, 2000); (Huntington et al., 2000)). Most serpins inhibit serine proteases of the chymotrypsin, trypsin or elastase families. However, *cross-class inhibitors* have been identified. The viral serpin CrmA (and to a lesser extent SERPINB9), inhibit caspase-1 (Komiyama et al., 1994), and SERPINB3 neutralizes the potent papain-like cysteine proteinases, cathepsins K, L, S, and V. In animals, the serpins are known to be involved in a number of biological processes such as blood coagulation, complement activation, cell migration, tumor suppression, and others ((Patston, 2000); (van Gent

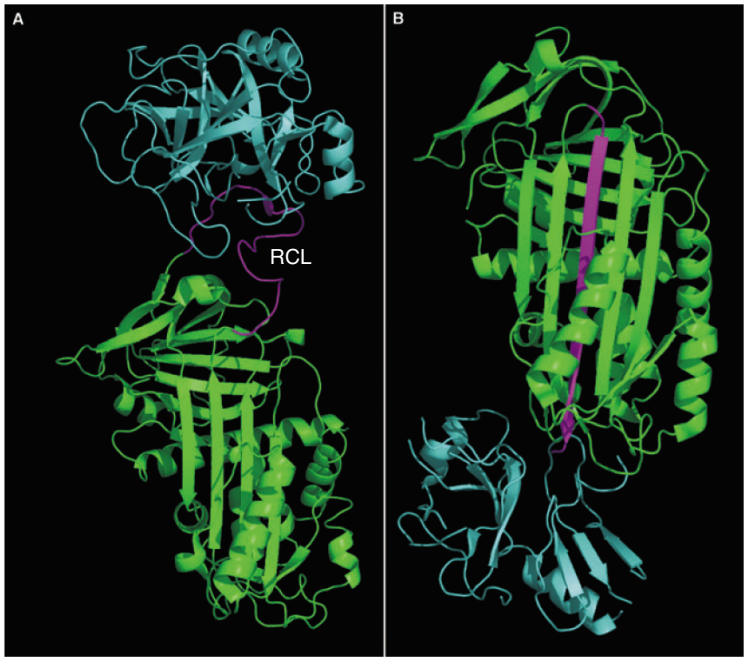


Figure 8: Irreversible suicide substrate mechanism of the serpin (Whisstock et al., 2005). A) Complex formation of a serpin (green colour) with trypsin (blue colour). The reactive centre loop of the serpin is coloured in purple. **B)** Conformational change of the serpin upon RCL-cleavage to its most stable inhibitory form. S to R transition is associated with insertion of the RCL in the centre of the A β-sheet. Hereby, the active site of the trypsin protease is distorted and the protease remains attached to the serpin RCL via an acyl bond.

et al., 2003)).

In plants, the high abundance of serpins in cereal seeds has led to the hypothesis that they could be involved in defense against insects feeding on the storage tissues of seeds, as the serpins can inhibit digestive serine proteases of the insects (Hejgaard and Hauge, 2002). Biochemical studies with plant serpins from cereals have demonstrated their inhibitory action against different animal proteases (such as trypsin, chymotrypsin, cathepsin G and elastase) ((Ostergaard et al., 2000); (Hejgaard and Hauge, 2002); (Hejgaard, 2001)). Nevertheless, no plant target protease of plant

serpins had been identified until now.

TAP purification identified **Atmc1 as a potential target protease for *Arabidopsis* serpin**, Atserpin1. In the *Arabidopsis* genome, ten genes exist which putatively encode for proteins showing homology to Atserpin1 and possessing a RCL. Based on the number of existing cDNAs and ESTs, Atserpin1 is by far the most abundant, while several of the other gene homologues are probably pseudogenes. Recently, Atserpin1 was identified as a suicide-substrate for **Atmc9** (Vercammen *et al.*, submitted), hereby characterizing the plant metacaspases as target proteases for a plant serpin. In this study, Atserpin1 was found to inhibit Atmc9 in a mechanism similar to that of other serpin-protease systems. Atserpin was shown to interact with Atmc9 in a yeast two-hybrid (Vercammen *et al.*, submitted) and was cleaved by Atmc9 in the RCL. Similarly, cleavage of the serpin was also detected with Atmc1. Western blot with anti-serpin antibody on the TAP eluates of the type I metacaspases could identify serpin as a substrate / interactor of Atmc1 / Atmc1CA (Figure 9). Remarkably, the serpin was not detected by mass spectrometric analysis upon TAP with Atmc1CA, which proved that the MS platform is limited in its ability to identify proteins. As already discussed for the failure of identification of bait proteins Atmc2 / Atmc2CA, this can be due to the fact that the protein did not reach the detection limit or that it is present in a fragment underneath a high concentration of another protein which disrupts the identification.The full length Atserpin1 was detected upon TAP with Atmc1CA, which migrates around 45 kDa. Interestingly, with Atmc1 (both N- and C-terminal TAP tagged) a lower molecular weight fragment was detected, around 40 kDa. It can be assumed that this fragment is the cleaved form of Atserpin1, upon cleavage of the RCL by the metacaspase. The N-terminal tagged bait protein Atmc1 was found to be processed before the start of the TAP, however this was not shown for the C-terminal TAP tagged Atmc1. Despite this discrepancy the cleaved form of the serpin was detected in both cases (for C-terminal tagged version this was shown by MS analysis, data not shown), which means the metacaspases were active before hand or became active during the purification. It was demonstrated for N-terminal tagged Atmc1 that processing occurred (Figure 2A), however not for the C-terminal version. It is suggested that for

type I metacaspases active dimerization would be sufficient to have an active metacaspase, and processing simply leads to a stabilization (see Chapter 2). Active dimerization of the metacaspases is very likely due to the presence of the tag, especially upon binding on the affinity column. In case of the C-terminal fusion this might not lead to processing, as it is a C-terminal fusion (see § 5.4.2.1.). The mutant metacaspase is catalytically inactive and hence unable to cleave the serpin, therefore in this case the full length is observed. Indeed, this is concomitant with the results obtained with Atmc9 (Vercammen *et al.*, submitted): incubation of the recombinant serpin with active recombinant Atmc9 resulted in a shift of 5 kDa, which is in agreement with the predicted cleavage site (P1-Arg) of the Atmc9 near the C-terminus in the

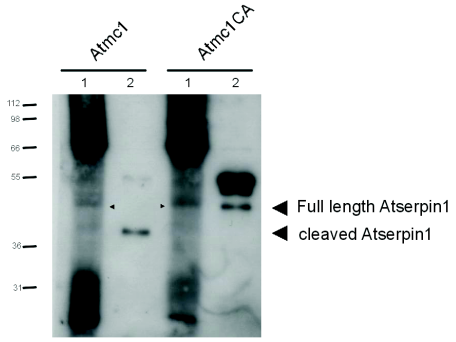


Figure 9: Atserpin1 is an interactor of metacaspase 1: the full length serpin interacts with the mutant metacaspase and the cleaved form of the serpin with the wild type metacaspase. Western blot analysis was performed with antibody generated specifically against Atserpin1, on input material (lanes 1) and final TAP eluates (lanes 2) of N-terminal TAP tagged bait proteins Atmc1 and Atmc1CA. The full length serpin is detected with an apparent mass around 45 kDa upon purification with Atmc1, and the cleaved, probably the most stable inhibitory form of the serpin is observed around 40 kDa. The full length serpin is already detected in the input material however, is clearly enriched upon the purification. For Atmc1, in addition the cleaved form is also visible at the start before purification.

RCL. The identity of the fragment around 55 kDa is currently unknown.

Remarkably, western blot with anti-serpin on TAP eluates of the other Type I metacaspases could not identify serpin as an interactor of Atmc2 or Atmc3 (wild type nor mutant form) (data not shown). However, this does not exclude that the serpin is not interacting with the other metacaspases. First, it is possible that the TAP purification of Atmc2 and 3 did not succeed as well as for Atmc1 and as such the serpin is purified in lower quantities, just underneath the detection limit. Second, due to the low abundance of the serpin, competition of the endogenous metacaspases might titrate out the serpins and as such too little complexes with the TAP tagged metacaspases, so prone to TAP purification, can be formed. As the serpin is identified with both Atmc1 and Atmc9, representatives of Type I and Type II metacaspases respectively, this suggests that the serpin is a general metacaspase inhibitor, which might involve a conserved region of the metacaspase family. Inhibition studies on other recombinant Type I / Type II metacaspases will help to establish how specific the serpin-Atmc1 / serpin-Atmc9 interaction is. Elucidating the precise function of this serpin and in which processes the inhibition of the metacaspases by the serpin would play a role will inevitably be linked to those of the metacaspases, and vice versa. Therefore, it would be interesting to use uncleavable serpins mutated at the RCL-cleavage site and *Arabidopsis* plants with perturbed serpin expression levels.

In conclusion, TAP enabled the identification of some novel interactors of the Type I metacaspases, which was useful to suggest putative (novel) functions of the metacaspases in biological processes. Some interacting proteins were also identified in other methods as a substrate of the (Type II) metacaspases. However, it is necessary to unravel the biological relevance of the interaction. In addition, for the two proteins to interact, it is also a prerequisite that the two proteins colocalize, at least at the moment of interaction. Whether or not the interactors are substrates of the metacaspases should also be investigated. Therefore it would be interesting to produce uncleavable mutant substrates or express the cleavage product to gain further insight.

Furthermore, if the interactors are specific to one metacaspase or more general to the metacaspase family is also of interest. Nevertheless, it must be taken into account that the identification of novel interactors is only a first step and it is important to validate the interactions by other means, confirm that the interaction occurs *in vivo* and elucidate the biological relevance and effect of cleavage / interaction, as it is for any protein interaction method.

6.5 Experimental procedures

6.5.1 Cloning of Type I metacaspases in N- and C-terminal TAP tagged vectors

Vectors used for N-or C-terminal fusion of the target protein to the TAP tag are NTAPi.289.gw.gck ((Rohila et al., 2004) and pK7mGW43Dnew (Stals et al., in preparation). Cloning of the metacaspases in these vectors was obtained by Gateway recombinatorial cloning. For the C-terminal fusion, a multisite Gateway reaction was performed, using three entry vectors: one harbouring the CaMv35S promotor, one with the ORF of the metacaspase (without stop codon), and one with the TAP tag. All entry vectors were used in equimolar quantities and at a concentration of approximately 50 ng/ml. The destination vector pK7mGW43Dnew was used at a concentration of approximately 150 ng/ml.

Both destination vectors for N- and C-terminal fusion contain Spectinomycin for selection in *E. coli*. To allow selection in plants, the N-terminal TAP tag vector contains the BASTA resistance gene and the C-terminal vector the Kanamycine resistance gene. In addition, the C-terminal vector harbours a GFP expression cassette, which allows for easy selection of *Arabidopsis* calli. All constructs were checked by restriction digest. Binary constructs were transformed into *Agrobacterium tumefaciens* strain C58C1Rif^{R} [pMP90] via electroporation, and *Agrobacterium* colonies were checked via PCR for the presence of the metacaspase fusion protein.

6.5.2 Transformation of *Arabidopsis* cell suspension with *Agrobacterium tumefaciens*

Agrobacterium strains containing the constructs were grown at 28°C and resuspended in MSMO medium (containing 3% sucrose, 0.05% NAA and 0.005% kinetin, pH5.7). Subsequently, the Agrobacteria (OD 1) were incubated with two-days old *Arabidopsis* cell suspension cells, in the presence of 200 mM acetosyringone during two days. Cells were washed three times with MSMO medium and plated out on 0.8% plant agar in MSMO supplemented with the antibiotics Carbenicilline (500 mg/ml), Vancomycin (500mg/ml) and Kanamycine (50 mg/ml)

or BASTA (5 mg/ml). After approximately one week, transformed calli can be expected. A mixture of these calli were brought in suspension in liquid MSMO medium supplemented with Kanamycine or BASTA. To have enough yield for one TAP procedure, cell cultures were scaled up to two liters, which yields around 15 g upon harvesting in liquid nitrogen.

6.5.3 TAP procedure

The TAP purification protocol ((Puig et al., 2001); (Graumann et al., 2004)) was used with some modifications. After grinding in liquid nitrogen, the plant material was extracted in extraction buffer (25mM Tris pH 7.6, 15mM MgCl2, 5 mM EGTA, 150 mM natriumchloride (NaCl), 15 mM para-nitrophenylphosphate, 60 mM beta-glycerophosphate, 0.1% Nonidet P-40 (NP40), 0.1 mM natriumvanadate (Na3VO4), 1 mM natriumfluoride (NaF), 1 mM dithiothreitol (DTT), 1mM phenylmethylsufonyl fluoride (PMSF),10mg/ml leupeptin,10 mg/ml aprotinin, 5 mg/ml antipain, 5 mg/ml chymostatin, 5 mg/ml pepstatin, 10 mg/ml SBTI, 0.1 mM benzamidine) by mixing using the ultraTurax T25 (Janke & Kunkel IKA-Labortechnik, Staufen, Germany). After clearing by 2 centrifugation steps the supernatant was mixed with 500 ml equilibrated IgG Sepharose 6 Fast Flow resin (Amersham Biosciences, Piscataway, NY, USA) and incubated at 4°C for two hours with gentle rotation. This was followed by six subsequent washing steps with IgG Sepharose wash buffer (10 mM Tris pH 8, 150 mM NaCl, 0.1 % NP-40) and one washing step with TEV buffer (10 mM Tris pH 8, 150 mM NaCl, 0.1 % NP-40, 0.5 mM EDTA, 1 mM DTT). Elution from the IgG beads was performed by incubating the washed beads with 100 units of TEV protease (Invitrogen, Carlsbad, USA) in TEV cleavage buffer for two times one hour at 17°C with gentle rotation. Eluates were recovered after centrifugation and pooled. IgG beads were loaded on a Miacol Column (MoBiTec GmbH, Goettingen, Germany) and the column was washed two times with Calmodulin binding buffer (10 mM Tris pH 8, 150 mM NaCl, 0.1% NP-40, 10 mM beta-mercaptoethanol, 1 mM imidazol, 2 mM CaCl2, 1mM MgAc). The pooled TEV eluates and wash of the IgG-column were added to the equilibrated Calmodulin affinity resin in the presence of 1 mM CaCl2, and incubated on a rotating wheel for one hour at 4°C. After six washing steps with the calmodulin binding buffer, beads were transferred to a Miacol column and elution from the beads was performed with calmodulin elution buffer (10 mM Tris pH8, 150 mM NaCl, 0.1% NP-40, 10 mM beta-mercaptoethanol, 1 mM imidazol, 2 mM EGTA). Elution fractions were collected separately. Before concentration of the elution fractions via TCA / HCl precipitation, aliquots were taken

for Western blot analysis and silver staining.

6.5.4 Separation of TAP purified bands on SDS polyacrylamide gel electrophoresis (PAGE) and subsequent mass spectrometry (MS) analysis

For the two-dimensional gels, after TCA precipitation the pellet is dissolved in resolubilisation buffer (2 M thiourea, 7 M ureum, 4 % CHAPS, 1%DTT, 0.5% Pharmalyte pH 3-10 (Amersham Biosciences, Buckingham, England). Immobilized pH gradient (IPG) Immobiline dry strips (Amersham Biosciences, Buckingham, England) were rehydrated with equilibration buffer (50 mM Tris pH 8.8, 6 M urea, 30% glycerol, 2% SDS, bromophenol blue). The strip holder was put onto the cooling plate's electrode contact area of the IPGphor (Amersham Biosciences, Buckingham, England) and the equilibration buffer was evenly pippeted in the strip holder channel, 1-2 cm from the electrodes. The IPG strip was lowered, gel-side-down, onto the equilibration buffer (containing the sample) without trapping air bubbles, and overlayered with silicone oil before application of the plastic cover. Once the safety lid was closed, IEF was carried out automatically, overnight, according to the programmed settings. IEF is started with low voltage (200 V), followed by 500 V and 1000 V and to finish at 3000V for five hours. Prior to the second dimension, the IPG strip was equilibrated for two times 15 minutes, once in equilibration buffer supplemented with 1 % DTT (w/v), and once with buffer containing 4 % iodoacetamide (w/v). After equilibration of the strip, the strip was applied to vertical SDS gel. As a second dimension, Criterion 4 to 12 % bisacrylamide Bis-Tris gradient gels (Bio-Rad, Hercules, CA, USA) were run in 1 x Mes running buffer. Electrophoresis was performed for 15 minutes at 10 mA and for approximately 90 minutes at 20 mA, until the bromophenol blue dye had migrated off the strip by 3-4mm.

For separation of TAP purified proteins on one-dimensional SDS-PAGE, the TCA precipitated pellets were first dissolved in NuPAGE SDS sample buffer (with reducing agent). NuPAGE Novex Bis-Tris gels 4-12% were used for gel electrophoresis (Invitrogen, Carlsbad, CA, USA). The upper buffer chamber of the gel system was filled with in 1 x NuPAGE SDS running buffer supplemented with NuPAGE antioxidant, the lower buffer chamber was filled with 1 x NuPAGE SDS running buffer. Gel was run at 200 V for approximately 35 minutes.

Both one- and two-dimensional gels were Coomassie brilliant blue G stained, suited for subsequent mass spectrometry analysis. For colloidal Coomassie staining, gel was first fixed (at least two hours to) overnight in 50 % ethanol and 2 % H_3PO_4, subsequently washed three times with water. Afterwards, to stain the gel the water was replaced by stain solution concentrate

(34 % methanol, 17 % ammoniumsulphate, 3 % H_3PO_4), whereto Coomassie brilliant blue G powder (1g/L) is added.

TAP purified and stained protein bands were excised from SDS-PAGE gel and subjected to mass spectrometry analysis, in collaboration with E. Witters, CEPROMA (Centre for Proteome analysis and Mass spectrometry).

6.5.5 Blue Native (BN) gel electrophoresis (as described by Remmerie *et al.*, in preparation)

BN PAGE

For sample preparation of *total protein extract*, grinded material from two-days old *Arabidopsis thaliana* cell suspension cells was extracted in ice-cold extraction buffer (25mM Tris pH 7.6, 15mM $MgCl_2$, 5 mM EGTA, 150 mM natriumchloride (NaCl), 15 mM para-nitrophenylphosphate, 60 mM beta-glycerophosphate, 0.1% Nonidet P-40 (NP40), 0.1 mM natriumorthoorthovanadate (Na_3VO_4), 1 mM natriumfluoride (NaF), 1 mM dithiothreitol (DTT), 1mM phenylmethylsufonyl fluoride (PMSF), 10 mg/ml aprotinin, 10 mg/ml antipain, 10 mg/ml chymostatin, 10 mg/ml pepstatin, 10 mg/ml SBTI, 0.1 mM benzamidine). Cell lysates were centrifuged at 20000g at 4°C for 45 minutes. The protein-containing supernatant was then subjected to buffer exchange at 4°C using Ultra4 Amicon centrifugal device (Millipore, USA) against BN sample buffer (30 mM HEPES pH 7.0, 150 mM potassium acetate, 1% (v/v) protease inhibitor cocktail, 10% (v/v) glycerol). Protein content was determined by Bradford assay (Biorad, Hercules, CA, USA). For sample preparation of *TAP eluate*, a centrifugation step of 30 minutes at 14000 rpm (4°C) was performed prior to buffer exchange. Per sample 400 mg of protein was used and digitonin was added in a final concentration of 8.5 g/g protein. Samples were incubated on ice for 30 minutes, and prior to loading on gel, unsoluble material was removed by centrifugation at 20000 g for 45 minutes at 4°C.

BN 5-16% gradient gels were cast on the SE600 Ruby system (GE Healthcare, Fairfield, CT, USA). Samples were loaded, and gels were run overnight at 4°C, at the following conditions: 45 minutes at 100 V/ 7 mA and 10-15 hours at 500 V (max)/ 15 mA. The entire gel run was performed with the blue cathode buffer (50 mM Tricine, 15 mM BisTris, 0.2 % Coomassie 250 G pH7) and the anode buffer (50 mM BisTris, pH 7), i.e. the cathode buffer was not exchanged with a colorless cathode buffer during the run. For immunoblotting of BN gels, TE 77PWR Semi-dry Transfer Unit was used (GE healthcare, Fairfield, CT, USA). Gels were first equilibrated for 15 minutes in transfer buffer (12 mM Tris, 96 mM glycine, 20 % methanol,

0.05 % SDS). Two short preblots are necessary to remove the excess of Coomassie (2 x 10 minutes at 30 V). Afterwards blot was performed during one hour at 30 V. After blotting, the membranes were put in blocking buffer (1% skimmilk in PBS containing 0.05% Tween 20). Rest of the detection protocol is performed using standard procedures. Anti-CBP antibody is provided by Upstate (Charlottesville, VA, USA).

SDS PAGE

For further separation in a second dimension 12 % SDS PAGE, the lanes from first-dimension were cut out and incubated for at least one hour in BN denaturing buffer (50 mM Tris pH 6.8, 66 mM Na_2CO_3, 10 % (w/v) glycerol, 2 % (w/v) SDS and 2% B-mercaptoethanol). First-dimension strips were then placed onto the stacking gel (4%) and overlayed with 0.5 % agarose. For running the second dimension, the Ettan Dalt Six system (GE healthcare, Fairfield, CT, USA) was used and running conditions of 1h minutes at 600 V / 400 mA / 2.5 W for one gel and several hours at 100W. Second dimension run and immunoblotting were performed according to standard protocols. In case gels were stained, a Ruthenium II tris (bathophenantroline disulfonate) (RuBP) staining (Lamanda et al., 2004) was performed including overnight fixation of the gels in 30 % ethanol and 10 % acetic acid, three rinsing steps in 20 % ethanol, incubation of the gels in 1 mM RuBP and equilibration in water. To destain, the gels were incubated in 40 % ethanol / 10% acetic acid for 15 minutes, equilibrated in water and hence scanned using Typhoon 9400 (GE healthcare, Fairfield, CT, USA). After scanning, fixation of the gels in 1.3 % para-phosphoric acid / 20 % methanol for colloidal Coomassie Brilliant Blue staining.

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Summary

Caspases are cysteine dependent proteases, which are known for their key regulator role during apoptosis in mammalian systems. In plants, only recently a distant family of caspases was identified and designated as metacaspases (Uren et al., 2000). These metacaspases have been biochemically characterized and shown to be cysteine proteases as well, however with an arginine / lysine specificity unlike their mammalian counterparts (Vercammen et al., 2004). However, hitherto little functional data are available that can assign a putative function to these metacaspases.

Chapter 1 gives an overview on the current knowledge of the cysteine proteases in plants. It provides information on the general characteristics of these proteases, regulation of their activity, identity of their substrates and the multifunctional role that they play in plant growth and development. Moreover, the cysteine proteases can be classified in different clans and families according to their evolutionary conservation, each clan and family with different characteristics. In *Arabidopsis*, at least 149 cysteine proteases can be identified and a phylogenetic tree could be constructed according to their evolutionary conservation. Recently, in plants various caspase-like activities have been detected and mainly associated with plant cell death. Despite the initial assumption that metacaspases would be the first candidates to perform these activities, other proteases have been characterized which could fulfill this role. In addition, the general features, processing, activity and putative role of the metacaspase family in programmed cell death are discussed and a comparison with their mammalian counterparts is punctuated.

To help decipher the putative roles of the metacaspases in plants, transgenic *Arabidopsis thaliana* plants were produced which overexpress the *Arabidopsis* metacaspases. In **Chapter 2** a molecular analysis was performed which demonstrated that mere overproduction of the Type II metacaspases was sufficient to induce selfcatalytic processing into its p20- and p10-like subunits. Furthermore, for at least metacaspase 4 and metacaspase 9 this processing seemed to be correlated with

metacaspase activity in the transgenic lines, as demonstrated by both fluorometric and in-gel assays. This was in contrast with overexpression of the Type I metacaspases, which did not show detectable autoprocessing upon their overproduction in the tested conditions. This suggests that Type I and Type II metacaspases use different mechanisms of activation, which might be reflected in the functional divergence between type I and type II metacaspases. This is analogy with the mammalian initiator and executioner caspases.

The transgenic plants did not show an obvious phenotype under normal conditions. **Chapter 3** describes the phenotypical analysis which was performed on the transgenic plants. Only transgenics overproducing metacaspase 4 showed a minor phenotypical change in root morphology. In addition, no enhanced cell death and no obvious effect on DNA ploidy levels could be observed. Furthermore, different biotic and abiotic stress stimuli were applied on the overproducing plants. However, no altered response towards any of the tested stress stimuli was observed when compared to the wild type plants.

Building a protein interactome by identification of protein-protein interactions is extremely useful to assign a function to a protein with an unknown function. In **Chapter 4**, a yeast two-hybrid screening was performed on Type I metacaspases metacaspase 2 and metacaspase 3, which resulted in the identification of various potential substrates and regulators of the metacaspases. Interestingly, many of the identified metacaspase-interactors showed a homology with known interactors of mammalian caspases. Based on these interactors, hints towards possible roles for *Arabidopsis* metacaspases in different processes such as apoptosis and cell cycle were proposed. Putative interactions between Type I and Type II metacaspases were also investigated by a yeast two-hybrid assay, to examine a possible existence of a metacaspase cascade, in analogy to the mammalian caspases. However, yeast two-hybrid did not suggest the existence of a metacaspase cascade.

One interaction which resulted from the yeast two-hybrid screening, namely the interaction of metacaspase 3 with KRP2 was analyzed in detail in **Chapter 5**. KRP2 is an important cell cycle regulator which exhibits its function by inhibiting the CDK /

cyclin complex, which results in cell cycle arrest. Using different deletion mutants in a yeast two-hybrid assay to identify the domain of interaction, it was demonstrated that the full length KRP2 protein was necessary for interaction. As a representative for metacaspase activity, recombinant metacaspase 9 was used and able to cleave both *in vitro* translated KRP2 and KRP2 extracted from plant tissues. A cleavage assay with the deletion mutants demonstrated that the cleavage site(s) was resided in the N-terminal domain. Moreover, an *in silico* search for potential metacaspase 9-cleavage sites indicated that the N-terminal domain contains the cleavage sites with the highest cleavage probability core. In addition, metacaspase 3 was found to be colocalized with KRP2 in phloem pole-specific pericycle cells of root tissues. Together these data suggest that KRP2 is a likely *in vivo* substrate of metacaspase 3 and indicate a regulatory role for the metacaspases in cell cycle progression by regulation of KRP2 abundance and as such its inhibitory activity.

Another method, namely the Tandem Affinity Purifcation (TAP), which allowed identification of protein-protein interactions *in vivo* was employed and results are described in **Chapter 6**. Almost no overlap with the yeast two-hybrid results were detected and as both protein interaction methods address different aspects of protein interactions they can be used in a complementary way. TAP resulted also in the identification of various interactors which suggest putative roles of the metacaspases in different biological processes such as cell death, cytoskeletal organization, plastid development and energy metabolism. In addition, a two-dimensional blue native PAGE on cell cultures overproducing metacaspase 1 demonstrated that metacaspase 1 is resided in complexes within the cell, varying from the monomeric metacaspase to complexes of at least 440 kDa.

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Samenvatting

Caspases zijn cysteine afhankelijke proteasen, gekend voor hun belangrijke regulatorische functie bij zoogdiersystemen tijdens apoptose. In planten werd echter pas recent een ver gerelateerde familie van caspases geïdentificeerd, de zogenaamde metacaspases (Uren et al., 2000). Deze metacaspases werden reeds biochemisch gekarakteriseerd en het werd aangetoond dat deze eveneens cysteine afhankelijke proteasen zijn, echter in tegenstelling tot hun dierlijke tegenhangers met een arginine / lysine specificiteit (Vercammen et al., 2004). Tot op heden zijn er weinig functionele data beschikbaar die een mogelijke functie aan de metacaspases kunnen toekennen.

In **Hoofdstuk 1** wordt een overzicht gegeven over de huidige kennnis van de cysteine proteases in planten. Er wordt informatie verstrekt over de algemene eigenschappen, regulatie van hun activiteit, identiteit van hun substraten en de multifunctionele rol die deze cysteine proteasen spelen in de groei en ontwikkeling van de plant. De cysteine proteases kunnen geclassificeerd worden in verschillende clans en families op basis van hun evolutionaire conservatie, waarbij elke clan en familie bepaalde kenmerkende karakteristieken vertonen. In *Arabidopsis* konden er minstens 149 cysteine proteases geïdentificeerd worden en op basis van hun evolutionaire conservatie kon een phylogenetische stamboom opgesteld worden. Onlangs werden in planten caspase-achtige activiteiten gedetecteerd die voornamelijk konden geassocieerd worden met plantenceldood. Ondanks de initiele veronderstelling dat de metacaspases de beste kandidaten zouden zijn om deze activiteiten uit te voeren, werden er andere proteases gekarakteriseerd die mogelijks deze rol zouden kunnen vervullen. Daarenboven werden in dit hoofdstuk ook de algemene eigenschappen, “processing”, activiteit en mogelijke rol van deze metacaspase familie besproken. Hierbij werd telkens aandacht besteed aan de analogie met dierlijke systemen.

Om de mogelijke functies van de metacaspases in planten trachten op te helderen werden transgene *Arabidopsis thaliana* planten aangemaakt met verhoogde metacaspase niveaus. In **Hoofdstuk 2** werden deze planten grondig moleculair

geanalyseerd. Hierbij werd aangetoond dat overexpressie van de Type II *Arabidopsis* metacaspases voldoende was om "processing" in zijn p20- en p10-achtige subeenheden te veroorzaken. Bovendien kon door zowel fluorometrische als "in-gel" studies aangetoond worden dat dit, ten minste voor metacaspase 4 en metacaspse 9, gepaard ging met metacaspase activiteit in de planten. Dit in tegenstelling met overproductie van Type I metacaspases die geen aanleiding gaven tot detecteerbare "processing" in de geteste condities. Dit suggereert dat Type I en Type II metacaspases andere activeringsmechanismen gebruiken, wat mogelijks gereflecteerd wordt in de functionele divergentie tussen beide Types. Dit is in analogie met zoogdier initiator- en executiecaspases.

De transgene planten vertoonden geen duidelijk fenotype bij normale omstandigheden. **Hoofdstuk 3** beschrijft de fenotypische analyse die op de planten werd uitgevoerd. Enkel transgene planten die metacaspase 4 tot overproductie brachten vertoonden een subtiel fenotype inzake wortelmorfologie. Bovendien werd geen verhoogde celdood noch een effect op de DNA ploidyniveaus vastgesteld. Daarenboven werden deze transgene planten getest voor hun respons op verscheidene biotische en abiotische stress stimuli. Er werd echter geen gewijzigde sensitiviteit waargenomen van de transgene planten ten opzichte van de wild type planten.

De opbouw van een eiwitinteractoom door identificatie van eiwit-eiwit interacties is bijzonder nuttig om een functie toe te kennen aan ongekende eiwitten. In **Hoofdstuk 4** werd een "yeast two-hybrid" uitgevoerd met de Type I metacaspases metacaspase 2 en metacaspase 3. Dit resulteerde in de identificatie van verscheidene potentiele substraten en regulatormoleculen van de metacaspases. Het bleek interessant dat veel van deze metacaspase-interactoren een zekere homolgie vertoonden met reeds gekende interactoren van de dierlijke caspases. Dit leidde tot hints tot mogelijke functies van deze *Arabidopsis* metacaspases in verscheidene processen zoals apoptose en celcyclus. Een mogelijke interactie tussen Type I en Type II metacaspases werd eveneens onderzocht door middel van "yeast two-hybrid" om het mogelijks bestaan van een metacaspase cascade te onderzoeken, naar analogie met de dierlijke

caspases. “Yeast two-hybrid” gaf echter geen aanleiding om een metacaspase cascade te vermoeden.

Eén interactie die resulteerde uit de “two-hybrid screening” werd verder uitgediept en beschreven in **Hoofstuk 5**, namelijk de interactie van metacaspase 3 met KRP2. KRP2 is een belangrijke regulator van de celcyclus die zijn functie uitoefent door inhibitie van CDK / cycline complexen, wat uiteindelijk leidt tot inhibitie van de celcyclus. Om het interactiedomein te bepalen werden in een “two-hybrid” test verschillende deletiemutanten van KRP2 gebruikt en hierbij werd aangetoond dat “full length” KRP2 nodig is voor interactie. Recombinant metacaspase 9 werd als representatief gebruikt voor metacaspase activiteit en kon zowel *in vitro* vertaald KRP2 als KRP2 afkomstig van plantenextract verknippen. Een verknippingstest met de verschillende deletiemutanten van KRP2 bewees dat de verknippingsplaats(en) gelegen was in het N-terminale domein. Bovendien kon een *in silico* onderzoek voor mogelijke metacaspase 9-verknippingsplaatsen aantonen dat de verknippingsplaatsen die de hoogste verknippingskans vertoonden effectief in het N-terminale gebied gelegen waren. Daarenboven was metacaspase 3 gecolocalizeerd met KRP2 in pericycluscellen specifiek aan de phloem pool. Al deze data suggereren dat KRP2 wellicht een *in vivo* substraat is van metacaspase 3 en indiceren een regulerende rol voor de metacaspases in celcyclusprogressie door regulatie van KRP2 abundantie en aldus zijn inhibitorische activiteit.

Een andere methode, een Tandem Affiniteitszuivering, werd aangewend voor de detectie van eiwit-eiwitinteracties *in vivo*. De resultaten hiervan worden besproken in **Hoofdstuk 6**. Er werd bijna geen overlap gevonden met de resultaten van de “yeast two-hybrid”, en aangezien beide methodes verschillende aspecten van eiwitinteracties behandelen kunnen deze methodes aangewend worden op een complementaire manier. De Tandem Affiniteitszuivering resulteerde in de identificatie van verscheidene interactoren, wat een potentiële rol voor de metacaspases suggereerde in verschillende biologische processen zoals celdood, cytoskelet organizatie, ontwikkeling van de plastiden en energie metabolisme. Daarenboven werd een twee-dimensionele “blue native” gelelectroforese op metacaspase 1-

overproducerende celculturen uitgevoerd. Die toonde aan dat metacaspase 1 zich in de cel bevindt in complexen dewelke variëren in grootte, gaande van het monomerische metacaspase tot complexen van ten minste 440 kDa.

Referenties

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