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Molecular cloning and characterization of novel cystatin gene in leaves *Cakile maritima* halophyte

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Summary

Cakile maritima (Brassicaceae) is a halophyte that thrives on dunes along Mediterranean seashores, with high tolerance to salty and dry environments. We have previously shown that there is great morphological and physiological diversity between ecotypes. We investigated the expression of cysteine protease inhibitor (cystatin) genes in the response to hydric and saline constraints, as cystatins are known to participate in the response to environmental constraints in plants. We isolated, from *C. maritime*, a new cystatin cDNA (CmC) that encodes a 221 amino acid protein with a calculated molecular mass of 25 kDa. It displays a moderate-to-high amino acid sequence similarity with previously reported phytocystatin genes. The predicted protein is hydrophilic, with only one hydrophobic region, just at its N-terminus, and a calculated isoelectric point of 6.7. Sequence analysis revealed a monocystatin structure with one cystatin-like domain. The predicted protein CmC contains the main conserved motifs characteristic of the plant cystatins, and a putative site of phosphorylation by casein kinase II (TPSD). As some cystatins, it contains a C-terminal extension of 106 amino acid residues, with several conserved cystatin motifs. The expression was constitutive in non-stressed plants, with different levels between the ecotypes, and without apparent relation to the climatic area of origin. Augmented expression was observed under severe salinity except in the ecotype from the arid region. Water deficit also increased CmC expression in two ecotypes, with the highest value observed in the ecotype from the humid region. These results indicate that *C. maritima* responds to high salinity and water deficit by

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expressing a cystatin gene that is a known component of defense against abiotic constraints or biotic aggression and survival machinery.

Introduction

The cystatins constitute a superfamily of proteins, which function as reversible inhibitors of papain-like cysteine proteinases (Brown and Dziegielewska, 1997). Cystatins have been identified in vertebrates, invertebrates and plants. In animals, they have been classified into three distinct families on the basis of M_r, the number of disulphide bonds, subcellular localization and primary structure characteristics. Family 1 cystatins have an M_r around 11 kDa and lack a disulphide bond. Cystatins in family 2 contain two disulphide bonds and their M_rs is around 13 kDa. Family 3 contains cystatins with M_r between 50 and 114 kDa (Cornwall and Hsia, 2003). All cystatins contain the signature QxVxG that is generally located in the central region of the protein sequence. Two other amino acids are partially conserved between cystatins: a glycine residue located within the N-terminal region and a tryptophane residue within the C-terminal region (Margis et al., 1998; Nagata et al., 2000). Cystatins from plants referred to as phytocystatins (PhyCys) have been claimed to be an independent family, containing particular consensus motif [LVI]-[AGT]-[RKE]-[FY]-[AS]-[VI]-x-[EDQV]-[HYFQ]-N found in the region corresponding to a predicted N-terminal α-helix (Margis et al., 1998), and they cluster on a distinct branch from other cystatin families on phylogenetic trees. Most PhyCys have a molecular mass in the 12–16 kDa range and no disulphide bond. However, the so-called multicystatins from potato and tomato have M_r of ~85 kDa and contain eight similar cystatin domains. (Waldron et al., 1993; Wu and Haard, 2000), whereas cystatins from soybean, cabbage, sesame and strawberry of ~23 kDa contain a longer C-terminal end (Lim et al., 1996; Misaka et al., 1996; Shyu et al., 2004).

Cystatins are of great interest for researchers because of their regulatory and protective functions in plant tissues (Misaka et al., 1996). These inhibitors might protect the cells from inappropriate endogenous or external proteolysis and/or could be involved in the control mechanism responsible for intracellular or extracellular protein breakdown (Turk and Bode, 1991). They inactivate proteases by trapping them in a tight equimolar complex (Barrett et al., 1998). In animals, cystatins are implicated in various pathologies such as inflammation, Alzheimer's disease, viral diseases and tumor malignancy (Turk and Bode, 1991). Rice oryzacystatin I has been tested for its antiviral action against the HSV-1 virus, and its antiherpetic effect was similar to that of acyclovir (Aoki et al., 1995). Phytocystatins can be considered as candidates in crop protection as well as in human and veterinary medicine (Massonneau et al., 2005).

Plant cystatins, homologous to animal cysteine protease inhibitors (Brown and Dziegielewska, 1997), have been characterized in several monocots and dicots, including rice, maize, soybean, chestnut, potato and tomato (Abe et al., 1987; Pernas et al., 1998). Cystatins show different expression patterns during plant development and responses to abiotic constraints (Felton and Korth, 2000; Belenghi et al., 2003), including heat shock and salinity (Pernas et al., 2000; Gaddour et al., 2001), cold shock (Dopico et al., 1993), water-deficit stress and chilling (Wang et al., 2003) and abscisic acid (Hildmann et al., 1992). Moreover, cystatins may play a role in the regulation of protein turnover during seed development (Pernas et al., 2000; Arai et al., 2002; Corre-Menguy et al., 2002) and plant defense against insect predation and pathogens (Pernas et al., 1998; Oppert et al., 2003). The involvement of cystatins as modulators in plant programmed cell death has been also reported (Solomon et al., 1999).

Sea rocket, *Cakile maritima* (Brassicaceae), is an annual halophyte that naturally spreads on the littoral dunes of the Mediterranean areas (Clausing et al., 2000). This species requires moderate salinities (up to 50–100 mM NaCl) for maximal growth and seed production, but survives even at salt levels close to sea water (500 mM NaCl) (Debez et al., 2004). It is known that exposing plants to salinity leads to modified gene expression profiles, and subsequently to various adaptive responses at the cell and whole plant levels (Hasegawa et al., 2000; Xiong et al., 2001). Various genes and proteins that respond to salinity are also induced by dehydration (Gong et al., 2001; Zhu, 2001). In the present work, the characterization of a novel cDNA coding for a cystatin from *C. maritima* leaf is described. We also compare the variability of cystatin transcript accumulation in four Tunisian