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BACTERIAL EXPRESSION, PURIFICATION AND PARTIAL CHARACTERIZATION OF NEW RECOMBINANT CYSTEINE PROTEASE FROM MAIZE LEAVES: POST-TRANSCRIPTIONAL CHANGES UNDER OZONE STRESS

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Abstract

Cysteine proteases are implicated in senescence, defense signaling pathways and cellular responses to biotic and abiotic stresses. In this context, we have cloned a novel cDNA encoding for papain family of cysteine protease from maize leaves. Mature part of papain-like protease was expressed in *Escherichia coli* using T7 promoter system. The recombinant protein was purified from inclusion bodies, refolded, characterized and used to produce corresponding antibodies in order to study post-transcriptional level of this specific protease under ozone stress. The results showed that ozone enhanced significantly papain-like cysteine protease at post-transcriptional level in 12th and 10th leaves of field grown maize plants. Simultaneously, senescence induced a rise in cysteine protease activity in both leaves. All together, these results suggest that ozone stress stimulates senescence processes, such as those related to proteolysis.

Introduction

The cysteine proteases are a group of enzymes that belong to 1 of the 4 major classes of proteolytic enzymes, and are generated by a variety of organisms, including viruses (Allaire *et al*., 1994), bacteria, protozoa, yeasts, plants, insects, and mammals (Berti & Storer, 1995, Mukhtar & Haq, 2012). The cysteine proteases perform a pivotal role in the degradation and turnover of intracellular proteins (Bond & Butler, 1987; Chapman *et al*., 1997). These proteolytic enzymes can be classified into more than 20 different families, including the papain family, the calpains, streptopains, clostripains and cysteine proteinases. The largest of these families is the papain family (Rawlings & Barrett, 1994). In plants, papain-like cysteine proteases (PLCPs) are produced as preproteases containing signal peptide, the pro-domain and mature protein domain (Taylor *et al*., 1995). They are involved in disease resistance and defence against insects and senescence (Feller *et al*., 1977; Pechan *et al*., 2000).

Ozone concentrations in troposphere have increased ~ 38% since industrial times (Menon *et al*., 2007) and considered as atmospheric pollutant with toxic effects on the terrestrial biosphere (Fuhrer, 1997; Tkacz *et al*., 2008; Wittig *et al*., 2009; Lindroth, 2010; Kafiatullah *et al*., 2012). Fowler *et al*., (1999) predicted that ozone concentration will reach upto 60 $nL.L^{-1}$ in 2100, a concentration known to damage plants (Miller *et al*., 1963). Accelerated leaf senescence (Eckardt & Pell, 1996), decrease in growth rate (Wittig *et al*., 2009), closure of stomata (Wilkinson & Davies, 2009, 2010), decrease in root dynamics (Vollsnes *et al*., 2010) and necrotic lesions are typical symptoms of ozone injury. Plants response to these symptoms by an up-regulation of defence and stress related genes and compounds (Kargajärvi *et al*., 1994; Kargajärvi *et al*., 2005; Lindroth, 2010) and down-regulation of photosynthetic components (Bagard *et al*., 2008; Leitao *et al*., 2007; Goumenaki *et al*., 2010; Wahid *et al*., 2011). Among defence and stress related genes and compounds, plant proteases play an essential role against pathogen and pests and are involved in programmed cell death, accumulation and mobilization of non functional proteins (Callis 1995, van der Hoorn, 2008, Kidokoro *et al*., 2009).

In this study, we have cloned a novel cDNA encoding for papain family cysteine protease from maize leaves and denoted as Mor-CP. Mature part of this papain-like protease was expressed in *Escherichia coli* using T7 promoter system. The recombinant protein was purified from inclusion bodies using Bug Buster extraction regent and refolded. It was characterized after partial purification. The purified recombinant protein was used to produce corresponding antibodies in order to study changes at post-transcriptional level of this specific protease under ozone stress.

Materials and methods

Bacterial strains, plasmids, medium and primers: *E. coli* Nova Blue Giga Singles (Novagen, France) strain was employed as a primary host for the transformation and propagation of plasmids. The *E. coli* BL21 (DE3) strain was utilized for the expression of the T7 promoter system, particularly for non-toxic protein expression. *E. coli* BL21(DE3) pLysE harbors the same DE3 lysogen as the BL21(DE3) pLysS which harbors the T7 lysozyme, a T7 RNA polymerase inhibitor, which prevents leaky expression in un induced cells, and also harbors the pLys plasmid with the T7 lysozyme, which is useful for the expression of toxic proteins. The pET30 (Ek/LIC) vector (Novagen, France) was utilized in the cloning of the PCR products. The BL21 (DE3) plasmid harboring the T7 promoter was utilized in the construction of the expression plasmids containing the cysteine proteases genes. *E.coli* cells harboring the plasmid were grown in Luria-Bertani (LB) medium (Sambrook *et al*., 1989) supplemented with 25 µg of kanamicyin/mL.

In order to generate the partial gene fragment of the cysteine protease, the oligonucleotide primers were designed from the conserved sequences flanking the active site histidine, asparagine, and cysteine residues of

the eukaryotic cysteine proteases, which are characteristic of the C1 family of papain like cysteine proteases. The vector pET30 (Ek/LIC) was used for expression of genes (insert), is ligation independent and 5' phosphorylation of the primers is not necessary. Therefore, primer 5'-ends must incorporate the following sequences of vector. Sense primer: 5' GACGACGACAAGATG–insert-specific sequence 3' antisense primer:5' GAGGAGGAAGCC CGGTTA–insert-specific sequence 3'. Mature Mor-CP from cysteine amino acid at 166 positions to last amino acid Alanine at 354 (C^{166} to A^{354}) was produced using sense primer 5'-GACGACGACAAGATGTGTTGCTGG GCCCTCGCT-3 and antisense primer 5'-GAGGAGAAGCCCGGTTATGCGACCGGGTAGGAG GCTT-3'**.**

Amplification of cysteine protease and construction of expression vector: The total RNA was extracted from 100 mg of powdered maize leaves, using "RNeasy Plant Mini Kit" (Qiagen, France) according to the manufacturer's instructions. The reverse transcription reaction was performed to obtain cDNAs using the "Omniscript RT Kit" (Qiagen, France). For the isolation of partial cDNA of Mor-CP maize gene, 50 ng of total RNA from different extracts were used. The cDNA was amplified in a thermal cycler (USA) under the following conditions: 95°C 5 min (1 cycle); 95°C 1 min, 55°C 30 sec, 72°C 1 min (35 cycles); 72°C 10 min (1 cycle). The PCR products were then fractionated on 1.5% agarose gel and the DNA fragments were recovered. The nucleotide sequence amplified by PCR was purified with "QIAquick PCR Purification Kit" (Qiagen, France). The purified PCR product was treated with T4 DNA polymerase (Novagen, France) to generate sharp edges on the insert that was compatible with the vector. For annealing the Vector and Insert, 0.02 pmol of T4 treated insert was mixed with 50ng of pET30 Ek/LIC Vector and incubated at 22°C for 5 min then 25mM EDTA was added and incubated at 22°C for 30 min.

Recombinant protein expression: The recombinant plasmid harboring the mature form of the cysteine protease was transferred into *E. coli* strain BL21 (DE3). The bacterial culture was cultivated and when the cell density achieved an *A*600 value of 0.5, 1 mM IPTG (Isopropyl- β-D-thiogalactoside) was added and incubated for 3h. The bacterial culture was centrifuged at 10,000 xg for 30 min and bacterial pellets were collected.

Purification of recombinant cysteine protease: Recombinant cysteine protease was embedded in inclusion bodies. First of all, the one gram of bacteria pellets were re-suspended in 5mL Bug Buster reagent (Novagen) and mixture was incubated on a shaking platform for 20 min at room temperature. Cell suspension was centrifuged at 16,000 × g for 20 min at 4°. The bacteria pellets contained insoluble (inclusion bodies) proteins while supernatant contained soluble protein. The pellet was re-suspended in the same volume of Bug Buster as mentioned above. rLysozyme solution was added to a final concentration of 1 KU/mL. Inclusion bodies was washed three times with 6 volume of Bug Buster reagent (1:10) and centrifuged at 16,000 × g for 15 min at 4°. The final pellet of inclusion bodies was resuspended in 1X Solubilization Buffer (50 mM CAPS, pH 11.0) of Protein Refolding Kit (Novagen) at a concentration of 20 mg/mL supplemented with detergent 0.3% N-lauroylsarcosine and 1 mM DTT. The mixture was centrifuged at 10,000 x g for 10 min at room temperature. Supernatant containing the solubilized protein was collected. 2-3mL of Ni-NTA agarose resin (Qiagen) was added into solubilised inclusion bodies protein. The mixture remained on ice with agitation for one hour and put it into polypropylene column. Binding buffer 1X (500 mM NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 7.9) was added in column and the column was washed three times with 1X washing buffer (500 mM NaCl, 20 mM Tris-HCl, 60mM imidazole, pH 7.9). The bound proteins were eluted with 1X elution buffer (500 mM NaCl, 20 mM Tris-HCl, 1000mM imidazole, pH 7.9). 4.5M urea (W/V) was added in extracted protein and homogenized for 20 min at room temperature. Dialyze was performed using a bead Spectra / Por Float-A-Lyzer dialysis appartus (G235055, Spectrumlabs.com) for 3 hours at 4°C in 1X Dialysis Buffer. This step was reapeated 3 times and each time new Dialysis Buffer was used. The concentration of extracted protein was measured by Bradford. SDS-PAGE separation of recombinant proteins were carried out using a Phast-system apparatus (Amersham Pharmacia Biotic, France) and 8-25% polyacrylamide gradient gels, according to Laemmli (1970).

Synthesis of polyclonal antibodies: 2 mg extracted recombinant protein (Mor-CP) was sent to Proteogenix (France) for the synthesis of polyclonal antibodies in rabbit. The serum of rabbit was used for immunodetection of protein in control and ozone treated samples.

Ozone fumigation, extraction and determination of *Z. mays* leaf protein: In 2008, maize plants (*Zea mays* L., cv. NK Perform) were grown in a field plot at the INRA (Thiverval Grignon, France, 48° 50' N, 1° 57' E). Seventy-one days after sowing, when the the crop was fully developed, three days before flowering, ozone was applied artificially by means of a linear free air fumigation device for 50 days. The detail procedure of ozone fumigation is mentioned in Ahmad *et al.,* 2012. Maize leaf soluble proteins from control (senescence) and ozone fumigated plants were extracted in extraction buffer (100 mM Hepes pH 7.5, 150 mM NaCl, 0.1% CHAPS, 10% glycerol, 10 mM DTT and 50 mM $CaCl_2$). Leaf homogenates were sedimented at 16 000×*g* for 10 min at 4°C, supernatants represented the soluble extracts were collected. Protein concentration was measured according to Bradford using protein assay kit (BioRad, USA) and bovine serum albumin as a standard.

Western blot analysis: The proteins were separated by SDS-PAGE (SDS - PolyAcrylamide Gel Electrophoresis) in 4% and 15%. Polyacrylamide slab gels, using a Bio-Rad Mini-Protean II (Bio-Rad Laboratories, France). For each sample, 75µg extracted proteins were mixed with 2X Laemmli buffer: 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, 0.125 M Tris HCl. The reaction mixture was incubated at 95°C for 5 minutes (additional protein denaturation). The separation was performed at 60 V per 1.5 mm gel thick. The separated proteins were transferred to a nitrocellulose membrane (Amersham Biosciences UK) at 100 V (250

mA) for two hours. The membrane was rinsed in a blocking solution 1: powdered milk (5% w/v), Tris-HCl pH 6.8 (100 mM), NaCl (0.9% w/v), Tween 20 (0.1% v /v) for 30 min at room temperature with moderate agitation. The powdered milk saturate the surface of the membrane to avoid nonspecific hybridization of primary and secondary antibodies.

Immunodetection of cysteine protease: After rinsing in blocking solution, the membrane was incubated overnight at 4°C under gentle agitation in blocking solution 2 (powdered milk 0,5% w / v, Tris-HCl pH 6.8 (100 mM, NaCl 0.9% w/v, Tween 20 0.1% v/v) with rabbit polyclonal antibodies (Proteogenix, France) raised against the maize cysteine protease, diluted at 1 : 7500. The membrane was washed 10 min twice with blocking solution without milk. Then the membrane was rinsed in blocking solution 2, supplemented with secondary antibodies 1/ 5000 and incubated for 1.5 h. After washing twice the membrane in blocking solution without milk, the revelation of the chemiluminescence signal produced by the secondary antibody in the antigen/antibody reaction was performed using the chemiluminescence kit (Immobilon Western, Millipore, France) The signal was recorded by autoradiography (Amersham Biosciences films, UK) using the KODAK GBX solutions.

Results

Isolation of maize Mor-CP cysteine protease cDNA and analysis of the deduced protein sequences: Searching the public databases for maize papain-like cysteine protease (PLCP) sequences (C1A, family C1, clan CA), using *Arabidopsis thaliana* drought inducible RD21a (accession no. AAM13065) sequence as a query and the (blastp) program (http://blast.ncbi.nlm.nih.gov/Blast.cgi), led to the retrieval of several putative maize CP sequences and for preliminary study, we selected two hypothetical or unknown proteins (Accession numbers NP_001140922 and NP_001130503) having RD21a characteristics. Their alignment showed that they corresponded to papain-like cysteine proteases. Each were used to designed PCR primers for RT-PCR, using cDNA obtained from ozone-treated and control leaf samples (maize cv. NK Perform), as templates. The most ozone-responsive gene was further referred to as Maize Ozone Responsive-CP (Mor-CP). Analysis of the deduced Mor-CP protein sequence (354 amino acid residues in total) identified a N-terminal putative signal peptide terminating after residue A^{31} followed by a 125-amino acid-long propeptide sequence spanning to the L^{137} residue(Fig. 1). The putative mature Mor-CP protein (176 amino acid residues) included the C, H and N residues required for activity in all cysteine proteases (Powers *et al*., 2002).

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Mir1            MRPTRSAVSATALLLLAVALALAATAAARHSYTTTTTRVPAPAERADEEVRRMYEAWKSK 60
RD21            ---MGFLKPTMAILFLAMVAVSSAVDMSIISYDEKHG-VSTTGGRSEAEVMSIYEAWLVK 56
Mor-CP          -MAPYIVVNKTVIAFTAVALTILAVKTMMAEARDLSS--TSTGGYGEEAMKVRHQQWMAE 57
Rcr3            --------MAMKVDLMNILITLFFVISMFNTQTRGRS-------QPKLSVSERHELWMSR 45
CP              ---MAQWSLLIVLFCVTTAAAGFSFHDSNPIRMVSDAEEQLLQVIGESRHAVSFARFANR 57
                                                                            

Mir1            HGRGGSSNDDCDMAPGDDEQEEEDRRLRLEVFRDNLRYIDAHNAEADAGLHTFRLGLTPF 120
RD21            HGKAQSQNS----------LVEKDR--RFEIFKDNLRFVDEHNEKN----LSYRLGLTRF 100
Mor-CP          HGRTYRD-------------EAEKAHRFQVFKANADFVDASNAAGDDK-KSYRMELNEF 102
Rcr3            HGRVYKD-------------EVEKGERFMIFKENMKFIESVNKAGN---LSYKLGMNEF 88
CP              YGKLYDS-------------VDEMKLRFKIFSENLELIRSTNKRR----LSYKLGVNHF 99
                                                                            

Mir1            ADLTLEEYRGRVLGFRARG--RRSGARYGSGYSVR----GGDLPDAIDWRQLGAVTEVKD 174
RD21            ADLTNDEYRSKYLGAKME---KKGERRTSLRYEARV----GDELPESIDWRKKGAVAEVKD 154
Mor-CP          ADMTNDEFMAMYTGLRPVP--AGAKKMAGFKYGNVTLSDADDNQQTVDWRQKGAVTGIKN 160
Rcr3            ADITSQEFLAKFTGLNIPNSYLSPSPMSSTEFKINDLS-DDYMPSNLDWRESGAVTQVKH 147
CP              ADWTWEEFKSHRLGAAQN---------CSATLKGNHKITDANLPDEKDWRKEGIVSEVKD 150
                                                                            

Mir1            QQQCGGCWAFSAVAAIEGVNAIATGNLVSLSEQEIIDCDAQ--DSGCDGGQMENAFRFVI 232
RD21            QGGCGSCWAFSTIGAVEGINQIVTGDLITLSEQELVDCDTSY-NEGCNGGLMDYAFEFII 213
Mor-CP          QGQCGCCWAFAAVAAVEGIHQITTGNLVSLSEQQVLDCDTEG-NNGCNGGYIDNAFQYIA 219
Rcr3            QGRCGCCWAFSAVGSLEGAYKIATGNLMEFSEQELLDCTTN--NYGCNGGLMTNAFDFII 205
CP              QGHCGSCWTFSTTGALESAYAQAFGKNISLSEQQLVDCAGAFNNFGCSGGLPSQAFEYIK 210
                                                                            

Mir1            GNGGIDTEADYPFIGTDGTCDASKEKNEKVATIDGLVEVASNNETALQEAVAIQ-PVSVA 291
RD21            KNGGIDTDKDYPYKGVDGTCDQIR-KNAKVVTIDSYEDVPTYSEESLKKAVAHQ-PISIA 271
Mor-CP          GNGGLATEDAYPYTAAQAMCQSVQ----PVAAISGYQDVPSGDEAALAAAVANQ-PVSVA 274
Rcr3            ENGGISRESDYEYLGEQYTCRSRE--KTAAVQISSYKVVPEG-ETSLLQAVTKQ-PVSIG 261
CP              YNGGLETEETYPYTGSNGLCKFTS--ENVALKVLGSVNITLGSEDELKHAVAFARPVSVA 268
                                                                            

Mir1            IDASGRAFQHYSSGIFNGP-CGT---SLDHGVTAVGYGS-ESGKDYWIVKNSWSASWGEA 346
RD21            IEAGGRAFQLYDSGIFDGS-CGT---QLDHGVVAVGYGT-ENGKDYWIVRNSWGKSWGES 326
Mor-CP          IDAHN--FQLYGGGVMTAASCSTP-PNLNHAVTAVGYGTAEDGTPYWLLKNQWGQNWGEG 331
Rcr3            IAASQD-LQFYAGGTYDGN-CAD---QINHAVTAIGYGTDEEGQKYWLLKNSWGTSWGEN 316
CP              FEVVHD-FRLYKSGVYTSTACGNTPMDVNHAVLAVGYGI-EDGIPYWHIKNSWGGDWGDH 326
                                                                            

Mir1            GYIRMRRNVPRPTGKCGIAMDASYPVKDTYHP---GTG-----------------TATA 385
RD21            GYLRMARNIASSSGKCGIAIEPSYPIKNGENPPNPGPSPPSPIKPPTQCDSYYTCPESNT 386
Mor-CP          GYLRLER----GANACGVAQQASYPVA---------------------------------- 354
Rcr3            GFMKIIRDSGDPSGLCDIAKMSSYPNIA---------------------------------- 344
CP              GYFKMEMG----KNMCGVATCSSYPVVA---------------------------------- 350
                                                                            

Mir1            RAAAMDVIKMVLA------------------------------------------------- 398
RD21            CCCLFEYGKYCFAWGCCPLEAATCCDDNYSCCPHEYPVCDLDQGTCLLSKNSPFSVKALK 446
Mor-CP          ------------------------------------------------------------
Rcr3            ------------------------------------------------------------
CP              ------------------------------------------------------------

Mir1            ----------------
RD21            RKPATPFWSQGRKNIA 462
Mor-CP          ----------------
Rcr3            ----------------
CP              ----------------
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Fig. 1. Alignment of corn cysteine proteases deduced amino acid sequences. The putative maize cysteine protease (our study) Mor-CP (NP_001140922) was compared with other cysteine proteases: Mir1 (*Zea mays*), (AF019145); CP (*Lathyrus sativus*), (ACV72067.1); RD21 (*Arabidopsis*) (D13043) and Rcr3 (*Lycopersicon pimpinellifolium*), (AAM19207). Active site amino acid residues cysteine (C), histidine (H) and asparagine (N), in mature domain, are highlighted.

Purification of recombinant Mor-CP: The mature Mor-CP was expressed in *E. coli* and partially purified. Immunoblot and SDS-PAGE analysis of various preparations are presented in Fig. 2. The concentrated preparation obtained after elution from Ni-NTA contained one molecular specie of approximately 26 kDa.

Quantification of Mor-CP: Pre-mature Mor-CP like signal was detected at approximately 34 kDa in control and ozone stressed leaves (Fig. 3). Accumulation of polypeptide in ozone stressed leaves is more intense than that of the control plants showing the involvement of Mor-CP at post-transcriptional level under ozone stress conditions.

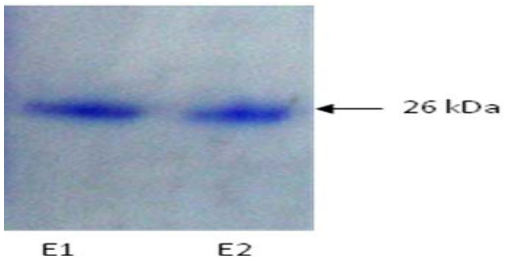

Fig. 2. Purification of recombinant Mor-CP. The recombinant Mor-CP was purified from *E.coli* as described in section 2 (material and methods). Fractions (E1 and E2) containing the recombinant protein (arrows) were concentrated and electrophoresed on a 15% polyacrylamide SDS-PAGE gel.

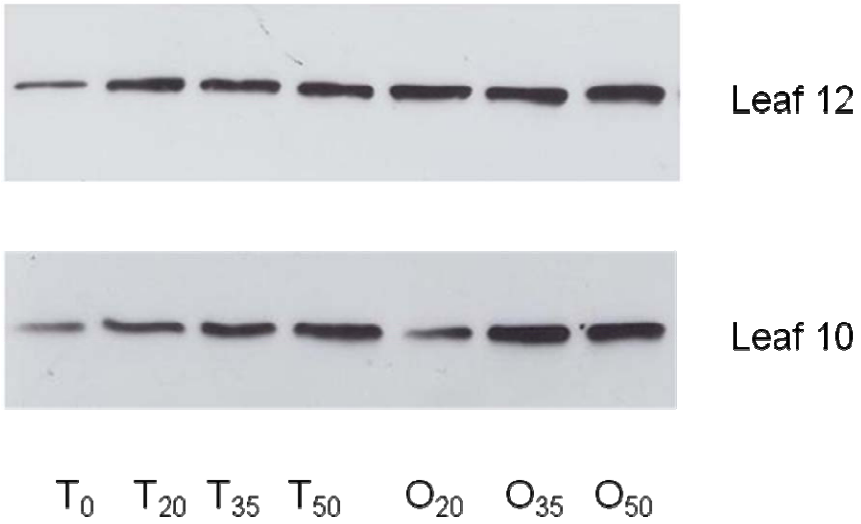

Fig. 3. Effect of controlled ozone stress on the expression of Mor-CP like polypeptides in the senescence (T_0, T_{20}, T_{35}, T_{50}) and ozone stressed leaves (O_{20}, O_{35}, O_{50}). The amount of total soluble proteins loaded in each well was 70 µg. The immunoblot was developed with antiserum raised against the recombinant Mor-CP (1:10000).

Discussion

In this study, a new cysteine protease gene, designated *Mor-CP*, was cloned from *Zea mays leaves*. It seemed to be involved in cellular catabolism under ozone stress in maize leaves at post-transcriptional level. The cysteine protease gene of our study was closely related to drought responsive cysteine protease "RD21" of *Arabidopsis thaliana* and papain from papaya. It has already been demonstrated in literature that cysteine protease degrade the cytoplasmic and/or membrane protein under biotic and abiotic stress conditions (Dramé *et al*., 2007; van der Hoorn, 2008). In humans, they are involved in diseases such as osteoporosis, asthma (Cimerman *et al*., 2001) & cancer (Hirai *et al*., 1999) and in plants, disease resistance signaling, pathogen perception, defence against insects and senescence (Feller *et al*., 1977; Pechan *et al*., 2000; Gilroy *et al*., 2007). Based on these data, we developed an experimental system allowing the manipulation of different O_3 concentration in maize leaves; with the purpose of identifying new papain-like cysteine protease gene involved in ozone stress. Our results showed that cysteine protease (Mor-CP) increased dramatically at post-transcriptional level in response to ozone stress. These results are concurrent with those obtained in previous transcriptomic and activity assays of cysteine protease in senescing plant tissues (Drake *et al*., 1996; Martinez *et al*., 2007), biotic stress (Pechan *et al*., 2000) and abiotic stresses (Matsushima *et al*., 2002, Nakashima *et al*., 2000). This increase of activity of Mor-CP at post-transcriptional level could result in stimulating ozone-induced senescence processes. Moreover we observed for the first time that the Mor-CP increased under senescence and this trend was more in ozone stress leaves.

The experimental approach used here could also prove useful tool to characterize cysteine protease and its regulation at post-transcriptional level to gain more comprehensive understanding of its role under ozone pollution. However, the result presented here are from single year experiment, therefore more experiments should be conducted in order to precise the results of post-transcriptional changes of Mor-CP under ozone stress. Moreover, the studies regarding purification of recombinant Mor-CP for mass production, and the biological activities of the recombinant cysteine protease,

will be required to make the detailed elucidation of the physiological function of the enzyme possible.

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