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One of the three proteinase inhibitor genes newly identified in the *Brassica napus* genome codes for an inhibitor of glutamyl endopeptidase

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Abstract Three proteinase inhibitor genes have been identified in the rapeseed (*Brassica napus*) genome. They are highly homologous to other genes of the mustard inhibitor (MSI) family of proteinase inhibitors characteristic of *Cruciferae*.

In germinating seeds, only the transcript of one gene, coding for a trypsin inhibitor, is detectable by Northern analysis. The other two genes are transcribed at basal levels detectable only by reverse transcription PCR. One of the other two genes (*rti-2*) encodes a polypeptide with a glutamic residue in the P1 position, characteristic of glutamyl proteinase inhibitors. The recombinant RTI-2 protein strongly inhibits (K_i = 44 nM) a glutamyl proteinase from *Streptomyces griseus*.

Keywords: Rapeseed; Proteinase inhibitor gene; Germination; Glutamyl proteinase inhibitor

1. Introduction

Hundreds of proteinase inhibitors (PIs) have been isolated and identified from a wide range of plant species. They constitute a defence strategy that plants can activate against attacks of herbivorous insects [1]. Indeed, inhibition of larval gut proteases can cause relevant delays in larval development and eventually their death.

PIs are usually catalogued according to the class of inhibited proteases, and subsequently on the basis of their primary structure. Inhibitors of serine proteases are classified in about 10 different families. From *Cruciferae* seeds, two homologous trypsin inhibitors have been isolated: mustard trypsin inhibitor-2 (MTI-2) and rapeseed trypsin inhibitor (RTI) [2,3] and their biological activity and primary sequence determined. Interestingly, their primary structures differ from those of PIs classified among the already known families of plant PIs. Tentatively these inhibitors have been countered as the first members of a novel PI family, named mustard inhibitor (MSI) [4]. The transfer of the *mti-2* gene to crop and model plants gave interesting results: when expressed in tobacco, arabidopsis and rapeseed, MTI-2 increased plant defences against lepidopteran insects such as *Plutella xylostella*, *Spodoptera littoralis* and *Mamestra brassicae* [5,6].

The *mti-2* gene and several mutants obtained by site directed mutagenesis have been expressed in *Pichia pastoris* and the specificity of the MTI-2 reactive site (R20-I21) demonstrated [7,8]. Other mutants with different affinities against trypsin and chymotrypsin have also been produced by the screening of a phage display library. Among those, a mutant specifically obtained by selection against chymotrypsin proved significantly effective in reducing the life span and vitality of the aphid species *Acyrthosiphon pisum* [9].

Before the identification of RTI, three other PIs had been identified in rapeseed seeds. They were separated by gel filtration chromatography and were indicated as inhibitors I, II and III [10]. Inhibitor III showed stability to heat denaturation; it corresponds to the RTI inhibitor purified and sequenced by Ceciliani [3].

Inhibitors I and II were sensitive to heat denaturation, and were not further characterized. In this paper the name RTI-3 will be used for the RTI inhibitor, according to the nomenclature of the previous literature [11–14].

This paper reports the identification in the rapeseed genome of three distinct genes highly homologous to each other and to the *mti-2* gene. The first gene, *rti-1* codes for a trypsin inhibitor highly homologous to RTI-3. The second gene, *rti-2*, codes for a novel inhibitor having a Glu residue in the P1 position of the reactive site, which makes it a potential inhibitor of glutamyl endopeptidases [15]. The third gene, *rti-3*, corresponds to the RTI-3 protein.

The potential inhibitor of glutamyl endopeptidase has been expressed as recombinant protein using *Pichia pastoris* cells and its activity tested against a specific glutamyl endopeptidase from *Streptomyces griseus* (SGPE).

2. Materials and methods

2.1. Materials

A rapeseed (*Brassica napus*) library constructed in lambda EMBL3 from a partial *Mbo*I digest of genomic DNA was obtained from Clontech (Palo Alto, USA). RNA and protein extracts were isolated from leaves, seeds and flowers of the same species.

Azocasein, bovine β-trypsin and α-chymotrypsin, *Staphylococcus aureus* endoproteinase Glu-C (GluV8), *Bacillus licheniformis* subtilisin A, Protease from *S. griseus* and Protease from *Aspergillus oryzae* were from Sigma. *Tritirachium album* Proteinase K was from Roche. Substrates benzyloxycarbonyl-Arg-Arg-*p*-nitroanilide (Z-RRpNA), *N*-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide (S-AAPLpNA) and *N*-succinyl-Ala-Ala-Pro-Glu-*p*-nitroanilide (S-AAPEpNA) were from Bachem.

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2.2. Plant treatments

Seeds were collected at different days after pollination (DAP). Dry seeds were imbibed on wet paper in sterile Petri dishes in a growth chamber. Cotyledons, flowers, inflorescence and leaves were collected from plants grown under greenhouse conditions. Plants were wounded or treated with methyl-jasmonate (MJ) as already described [16]. Wounded leaves, and distal leaves, were collected at 18 and 48 h after wounding. Induction of proteinase inhibitors by salicylic acid (SA) was tested using plants (12–15 days after planting) dipped in a solution of 0.2 and 5 mM SA for 24 h. Control plants were dipped in water. To test induction of PIs by insect attack, *M. brassicae* larvae were fed for 24 h with rapeseed leaves from adult plants. All seeds and leaves were frozen in liquid nitrogen immediately after collection.

2.3. Detection of proteinase inhibitors

Seeds and leaves were ground in 100 mM Tris–HCl, pH 8.0 at 4 °C. The homogenate was centrifuged at 4 °C for 5 min at $17000 \times g$. The soluble proteins were recovered in the supernatant and the protein concentration determined. Protein seed extracts were subjected to 15% (w/v) SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose Hybond™ C membrane (Amersham). After the transfer, the membrane was blocked for 1 h at room temperature with a solution of 5% (w/v) low fat milk powder in TBS, 0.05% Tween 20. Polyclonal antiserum raised in rabbits against MTI-2 were used as the primary antiserum, and horseradish peroxidase-conjugated anti-rabbit IgC was used as a secondary antibody. Peroxidase activity was detected by chemiluminescence using an ECL™ kit (Amersham) according to the instructions of the manufacturer. Trypsin inhibition in seed and leaf extracts was monitored using gelatin/PAGE [5].

2.4. Procedures for nucleic acids manipulation

The screening of the genomic library, Southern blot analysis, DNA cloning and sequencing, PCR and RNA manipulation were carried out according to standard procedures [17].

RNA isolation, electrophoresis and gel-blot analyses were performed as described [16]. Reverse transcription PCR (RT-PCR) was carried out using Superscript RT (Gibco, Gaithersburg, MD). Fragments amplified by RT-PCR were cloned using the pGEM-T easy vector system (Promega, Madison, WI). Gel blots were quantified using ChemiDoc (Biorad) and Phosphorus Imaging Software (Pharmacia Biotech). Gel blots were hybridized with a 18S rRNA probe for loading control. All experiments were repeated at least twice.

RACE analysis was performed using the Gene Racer Kit (Invitrogen), according to the instructions of the supplier.

2.5. Oligonucleotides

The following primers, designed on the *mti-2* sequence (GenBank Accession No. X84208) or on the specific sequence of each rapeseed gene, were used for RT-PCR and PCR amplifications:
LES: 5′-CAg gAT AgC gAg TgC CTg-3′
LED: 5′-ggC ACA TgC Agg AAC TCA AAT gCC-3′
RTI-RANDOM: 5′-gT(AgCT) CA(Ag) AgA TgC CgT gC(AgCT) g-3′
GSP2RTI-5′RACE: 5′-TTT TCC ACT TAg AgC TCC CTT gTC CgC-3′
rticDNA-5′end: 5′-gAA AgT gAA ACA AAg AAA gTT gAg-3′
rticDNA-3′end: 5′-CgC CTC TAC CAC gAT ACA AAC C-3′
RTI-RACE: 5′-gAA TCT gAC CAg gTT CgT ggC g-3′
rti1-5′: 5′-CgTTAATACACTATggCTTgCAAACgC-3′
rti1-3′: ACCAAACTTATTTTATTACACATAg-3′
rti2-5′: ggTgATTCTgATgTTAAATgC-3′
rti2-3′: CAggCTTTTTATTTCACATATTCgC-3′
rticDNA-5′: 5′-gTAATgTgTATgAAAAATAAAACCgC-3′
rti1cDNA-F: 5′-ATggCgATAAAACCACTTTCC-3′
rti1cDNA-R: 5′-gggTTgATCgTTgCAgTAg-3′
rti2cDNA-F: 5′-CATTCgCAACgAAATCAgTTTC-3′
rti2cDNA-R: 5′-AggTATgTCgCTgCAgTAgTC-3′.

Positions of LES and LED primers in the *mti-2* gene were as already described [18].

2.6. Expression and activity of recombinant RTI-2

The *rti-2* amplified region corresponding to a putative inhibitor of glutamyl endopeptidase was used to produce the recombinant polypeptide (rRTI-2) using *P. pastoris* as expression system essentially as already described [7]. rRTI-2 was purified from cell culture supernatant by gel filtration through a Sephadex G-50 column equilibrated with 50 mM Na-phosphate buffer, pH 7.2 [7].

RTI-2 inhibitor activity assays were performed against either single proteinases (trypsin, chymotrypsin, glutamyl endopeptidases GluV8 and SGPE, in the presence of their specific substrates Z-RRpNA, S-AAPLpNA and S-AAPEpNA, respectively) or cocktail of proteases (Protease from *S. griseus* and Protease from *A. oryzae*, using azocasein as substrate). Assays were carried out as previously described [19].

Inhibitor activity of rRTI-2 towards SGPE was determined by following the hydrolysis of the substrate S-AAPEpNA at 405 nM. Inhibitor and protease were incubated at room temperature for 30 min in 50 mM glycine/NaOH, 2.5 mM $CaCl_2$, pH 10 buffer, before addition of S-AAPEpNA.

K_i determination for the RTI-2/SGPE complex was carried out by the equilibrium method of Green and Work [20] as described by Beekwilder et al. [21]. Data were analyzed by non-linear regression with SIGMAPLOT program (Sigma).

2.7. Expression and activity of recombinant SGPE

Recombinant plasmid pEB-E and *Bacillus subtilis* DB104 strain were gifts of Dr. Thor J. Borgford (Simon Fraser University, Burnaby, Canada). pEB-E is an *Escherichia coli – B. subtilis* shuttle vector containing the gene coding for the pro-mature form of the *S. griseus* glutamyl endopeptidase (SGPE) [22]. Transformation of *B. subtilis* protoplasts with pEB-E and purification of rSGPE were according to reported procedures [22].

Activity of rSGPE was determined incubating a 50 nM solution with 0.25 mM of the SAAPEpNA substrate in 50 mM glycine/NaOH, 2.5 mM $CaCl_2$, pH 10 buffer. Substrate breakdown was monitored at 405 nm.

Proteolytic activity of rSGPE on RTI-2 was assayed incubating 250 ng of protease with 150 ng of inhibitor in 20 μL of 50 mM HEPES, 1 mM EDTA, pH 8.2 buffer at 37 °C for 30′. After incubation, proteins were denaturated and loaded on Tricine SDS-16.5% polyacrylamide gel. 300 ng of bovine serum albumine (BSA) was also incubated with SGPE as control of proteolysis.

2.8. Phylogenetic analysis

Sequences of MSI complete genes or coding sequences were aligned by the CLUSTAL_W program [23] available at European Bioinformatic Institute (EBI, http://www.ebi.ac.uk), using standard alignment parameters.

Phylogenetic analysis of MSI coding sequences was performed by sequentially applying the SeqBoot, DNADist, Neighbor-Joining and Consense programs of the PHYLIP package [24]. 1000 resampling of data were used in the bootstrap procedure. The Jukes and Cantor model for nucleotide substitutions was adopted to calculate the DNA distance matrix [25]. Phylogenetic tree was drawn using the Njplot program [26] available at Bioinformatic Pole of Lyon (http://pbil.univ-lyon1.fr/).

3. Results and discussion

3.1. Detection of serine PIs in seeds and leaves of rapeseed

The detection of rapeseed PIs active against trypsin was carried out by activity gels. Seeds were analyzed at different developmental stages (Fig. 1A). Activity bands at the same mobility of MTI-2 are detectable in immature seeds starting from 50 DAP, dry seeds and imbibed seeds. MTI-2 like inhibitors could not be detected in proteins extracted from wounded or chemically stressed leaves (not shown).

Inhibitors homologous to MTI-2 were identified by Western blot hybridization experiments using a polyclonal antibody against MTI-2 (Fig. 1B). The expression of PIs homologous to MTI-2 could only be detected in seeds at late stages of maturation and in the first hours after germination. These results

were further confirmed by RACE, Northern blot and RT-PCR analysis (see below).

3.2. Detection of mti-2 homologous genes in rapeseed genome

A portion of the *mti-2* gene corresponding to the mature protein was used for the screening of a rapeseed genomic library. The probe was obtained by PCR amplification carried out on the plasmid vector containing the complete gene, using primers LES and LED [18].

The screening of the rapeseed library revealed several positive clones, among which the clone 17.C2.B was further investigated. The complete sequencing of a *RsaI* subfragment of about 1100 bp allowed the identification of a putative gene homologous to *mti-2*, indicated as *rti-1*. The sequence has been deposited at the EBI nucleic acid database with the Accession No. AM162666. By comparison with *mti-2*, the *rti-1* gene shows an intron of 103 bp with the putative mature protein coded entirely in the second exon. The similarity between the putative RTI-1 polypeptide and the MTI-2 precursor protein [18] is around 76%.

Sequence analysis of a second clone, 19.A1.A, led to the identification of a *Hin*dIII subfragment of about 3000 bp containing a different gene indicated as *rti-2*. Accession number of the sequence in the EBI nucleic acid database is AM162667.

The similarity between the putative RTI-2 polypeptide and the MTI-2 precursor is around 76% (Fig. 2). An interesting feature of RTI-2 is its reactive site, since in the P1 position the canonical Arg for a trypsin inhibitor [27] is substituted by a Glu residue. Sequencing analysis of other clones identified

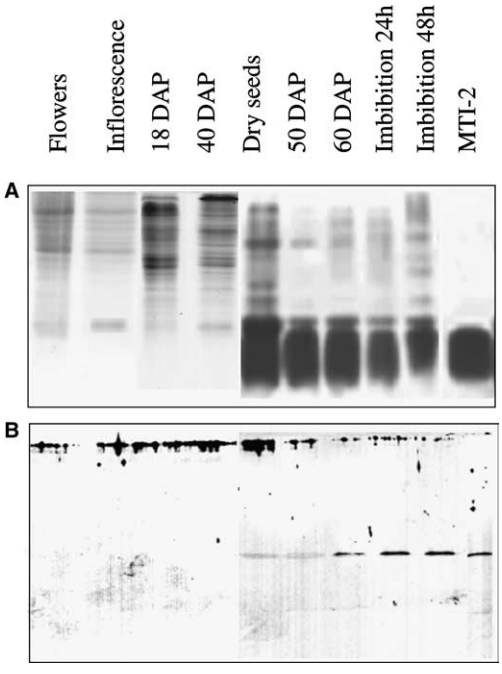

Fig. 1. Expression of trypsin inhibitors in different rapeseed organs. Panel A: 10 μg of extracts and 50 ng of the MTI-2 recombinant protein were separated on acrylamide gels containing gelatin. Gelatin was subsequently degraded by incubation of the gel in a trypsin containing solution. Panel B: Heated extracts were electrophoretically separated and immunologically localized by MTI-2 antibodies. Panel A,B: The proteins were extracted from seeds at different days after pollination (18, 40, 50, 60); from dry seeds; from seeds imbibed for 24 and 48 h and from flowers and inflorescence.

by screening the rapeseed genomic library did not allow to identify further genes than *rti-1* and *rti-2*.

The identification of the gene coding for the RTI-3 protein was achieved using a different strategy. 3′RACE analysis on total mRNA purified from 24 h imbibed seeds was carried out using the degenerated primer RTI-RANDOM, corresponding to aminoacids 27–37 of RTI-3 polypeptide [3]. Sequencing of the clones obtained by 3′RACE led to the identification of the nucleotide sequence corresponding to the final 30 amino-acids of RTI-III. By 5′RACE, using a primer complementary to the cDNA identified by 3′RACE (GSP2RTI-5′RACE) it was possible to identify the nucleotides corresponding to the first 30 amino acids. Finally, RT-PCR carried out using suitable primers (rticDNA-5′end/rti-cDNA-3′end) synthesized on the basis of the results of 3′ and 5′RACE analysis allowed the identification of the full length cDNA corresponding to the *rti-3* gene.

The complete *rti-3* gene was obtained by amplification of genomic DNA using specific primers (rticDNA-5′end/RTI-RACE) located at the 3′ and 5′ termini of the cDNA. The gene shows an intron of 155 nucleotides, with the mature protein entirely encoded in the second exon. The sequence of the amplified fragment has been deposited in the EBI nucleic acid database (Accession No. AM162668). The similarity between the complete RTI-3 polypeptide and the MTI-2 precursor [18] is around 69% (Fig. 2).

The *rti-1*, *rti-2* and *rti-3* genes show a high similarity with the six isogenes identified in the *Arabidopsis thaliana* genome [28] and to the four genes recently described in *Arabidopsis lyrata* [29]. A rapeseed EST sequence corresponding to a MSI gene different from *rti-1*, *rti-2* and *rti-3* has been recently identified (EBI Accession No. cd823866). Its gene will be hereafter indicated as *rti-4*. Interestingly the corresponding inhibitor has a Leu in the P1 position and should therefore correspond to a chymotrypsin inhibitor. It seems therefore that the MSI family in rapeseed is constituted by at least four genes. Table 1 reports a complete list of all the MSI genes identified so far. A multi-alignment of the fourteen complete gene sequences is available in Supplementary Material, together with a similarity table. Fig. 3 shows a phylogenetic tree obtained considering the fifteen available coding sequences for MSI members. Even if supported in some cases by bootstrap values only near to 50%, the four rapeseed genes are clustered together the *atti-1* and *alti-1* genes. The other genes from *Arabidopsis* species form highly supported separated branches.

3.3. Transcriptional analysis of rti genes

In order to study the expression of the three genes described in the previous section, both Northern blot and RT-PCR experiments were carried out.

For Northern blot analysis three specific probes were synthesized by PCR amplification, using primers corresponding to the regions flanking the genes at the 3′ side. The pairs of primers rti1-5′/rti1-3′, rti2-3′/rti2-5′, rticDNA-5′/rticDNA-3′end were designed on the *rti-1*, *rti-2* and *rti-3* genes, respectively. These regions were chosen because they show a very low similarity to each other. Preliminarily, in order to check their specificity, the three probes were tested by cross hybridization which revealed that each probe hybridized only with itself (not shown). RNA was isolated from immature seeds at 50 DAP and from imbibed seeds within 8 and 64 h after the beginning of imbibition. After blotting, RNAs were hybridized with the

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                    .         .         .         .        50
MTI2        MAMAKKSVSSFTLIFILVLVIFEVPEIKAQDSECLKEYGGDVGFPFCAPR
RTI3        .D..T.....LAAFL..F.....M...E..............G....E.
RTI2        ..F.T......AI.............E...D............S....E

                    .         .         .         .
MTI2        IFPTICYTRCRENKGAKGGRCIWGEGTNVKCLCDYCNDSPFDQILRGGI
RTI3        .Y.SF.VQ...AD...LS.K...GQ.G......NF.RHE.-G...-SGI
RTI2        ...SF...N..KD...L..T....-DSD........SDI.NGK......
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Fig. 2. Multialignment of polypeptides deduced from *rti* genes. Polypeptides deduced from *rti-2* and *rti-3* genes have been aligned with the complete MTI-2 protein. MTI-2 and RTI-3 mature forms [2,3] and the RTI-2 portion expressed as recombinant protein are reported in bold. The P1 residues of the reactive sites are boxed.

Table 1
Genes coding for protease inhibitors of the MSI family identified in *Cruciferae* genomes

		Accession number
A. lyrata	alti-1	AJ632267
	alti-2	AJ632267
	alti-4	AJ632267
	alti-6	AJ632267
A. thaliana	atti-1	AJ632250
	atti-2	AJ632250
	atti-3	AJ632250
	atti-4	AJ632250
	atti-5	AJ632253
	atti-6	AJ632250
B. napus	rti-1	This paper
	rti-2	This paper
	rti-3	This paper
	rti-4[a]	cd823866
S. alba	mti-2	X84208

alti, *Arabidopsis lyrata* trypsin inhibitor; atti, *Arabidopsis thaliana* trypsin inhibitor; rti, rapeseed trypsin inhibitor; mti, mustard trypsin inhibitor.
[a]mRNA.

three specific probes. No signals appeared using probes corresponding to *rti-1* or *rti-2* genes on RNA from either immature or imbibed seeds (data not shown). On the contrary, the *rti-3* probe hybridized in both cases to transcripts of about 650 nt. In Fig. 4 the hybridization of RNAs from imbibed seeds is shown. Densitometric analysis of hybridization signals (carried out taking into account the relative intensities of RNA loaded in single lanes) indicated that *rti-3* transcripts reach a maximum value after about 24 h imbibition (not shown).

In order to reveal transcripts not detectable by Northern blot experiments, the transcription of *rti-1* and *rti-2* genes were further investigated using RT-PCR amplifications. Total RNA isolated from rapeseed immature seeds at 50 DAP and from seeds imbibed for 24 h was used as template and amplified with specific couple of primers (rti1cDNA-F/rti1cDNA-R and rti2cDNA-F/rti2cDNA-R). The amplification products were cloned and their sequencing revealed primary structures in full agreement with the described gene sequences. In particular, locations and lengths of hypothesized introns were confirmed. The detection of *rti-1* and *rti-2* transcripts by RT-PCR, and not by Northern blot, can be explained assuming that these genes are transcribed but only at a basal level.

BLAST analysis of plant EST sequences, performed by using *mti-2* cDNA as query sequence, allowed to identify several *Arabidopsis* sequences (about 20 entries from immature seeds, siliques, inflorescence, leaves, culture cells) corresponding only to the *atti-1* gene. Evidence for the transcription of *atti-2*, *atti-3*,

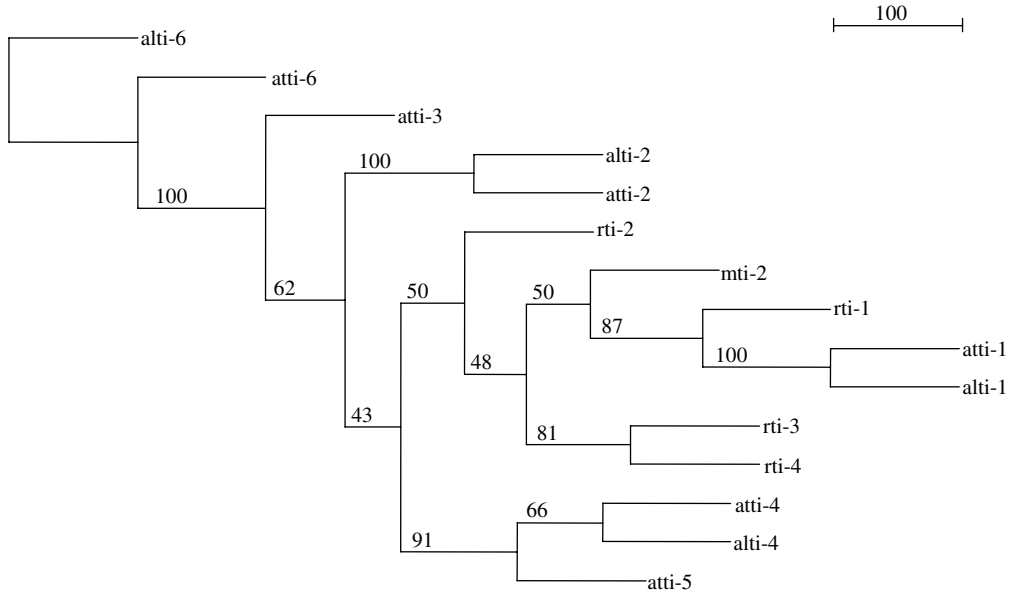

Fig. 3. Phylogenetic tree of MSI members from *Cruciferae*. Neighbor-joining un-rooted tree of coding sequences from MSI genes. Names of genes are as in Table 1. alti-6 sequence was used as outgroup. Bootstrap values are shown above the branches.

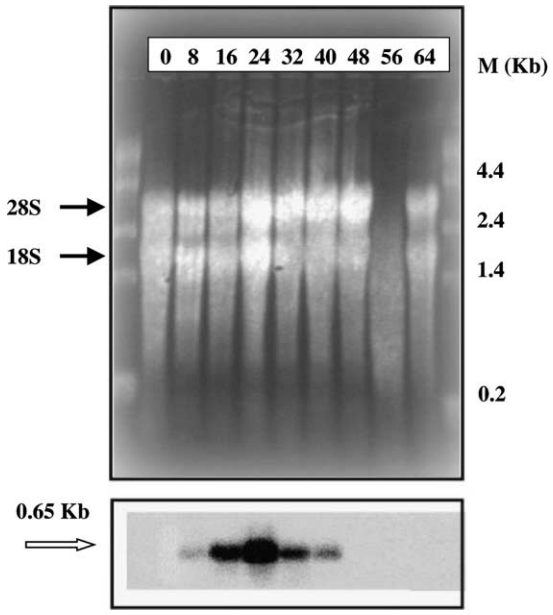

Fig. 4. Northern blot analysis of the *rti-3* transcript. Upper panel: RNA extracted from dry and imbibed seeds at different times (from 0 to 64 h) fractionated on agarose gel. Arrows indicate the bands corresponding to 18S and 28S rRNAs. Lower panel: Northern hybridization using a probe corresponding to the *rti-3* gene. Signals indicated by an open arrow correspond to a fragment of 0.650 Kb.

atti-4 and *atti-6* have been reported by Clauss and Olds only by RT-PCR on leaf RNA [29]. Transcription of the *atti-5* gene (a gene coding for a potential glutamyl proteinase inhibitor) could not be detected [29]. The same BLAST analysis also identified several rapeseed EST sequences corresponding to *rti-3* (more than 20 entries from seeds, germinating seeds and embryos) and *rti-4* (three entries from immature and mature seeds) genes. It appears therefore to be a common feature for either *Arabidpsis* sp. and rapeseed genomes the presence of MSI genes which, in the several conditions assayed, are expressed at very low levels detectable only by RT-PCR.

3.4. Expression of recombinant RTI-2 and activity analysis

Among the polypeptides encoded by the three genes detected in the rapeseed genome, we decided to study the activity of recombinant RTI-2 (rRTI-2) owing to the unusual Glu residue present in the P1 position. The P1 residue of canonical PIs is the main determinant in establishing the specificity of the inhibited proteinase [27]. The presence of a glutamic residue in the P1 position suggests that the protein can act as an inhibitor of glutamyl proteinases [15].

Serine glutamyl proteinases have not been described so far among insects but are present in the bacterial kingdom [15,30]. In plants two glutamyl endopeptidases have been identified [31,32], but their characteristics do not allow a certain classification among serine proteinases. Our attempts to identify glutamyl endopeptidase activity in *B. napus* seeds at different developmental stages (immature seeds and imbibed seeds), leaves and wounded leaves gave no results.

Recombinant RTI-2 was produced in the yeast *P. pastoris* and purified according to previously reported procedures [7].

To study the activity of the recombinant polypeptide, inhibition assays were performed on the following proteases/substrate combinations: bovine β-trypsin/Z-RRpNA, bovine α-chymotrypsin/S-AAPLpNA, *S. aureus* endoproteinase Glu-C (GluV8)/S-AAPEpNA, *B. licheniformis* subtilisin A/S-AAPLpNA, *Tritirachium album* Proteinase K/S-AAPLpNA. Azocasein was used as generic substrate in the case of the Protease cocktails from *S. griseus* and *A. oryzae*. With the exception of Protease from *S. griseus*, which was inhibited of about 20% (data not shown), rRTI-2 resulted ineffective in inhibiting activity of all the used enzymes. To verify whether the inhibition of *S. griseus* Protease was due to the inhibition of SGPE, present in the Protease cocktail, the expression of recombinant SGPE (rSGPE) was set up. A *B. subtilis* expression vector containing the SGPE gene was used to produce the recombinant protease. Expression and purification of rSGPE from *B. subtilis* culture media were carried out according to the procedure already described [22].

Before to study the activity of RTI-2 on SGPE, the two enzymes were incubated together at 37 °C for 30 min in order to exclude a possible proteolytic activity of SGPE on RTI-2. After incubation, proteins were immediately analysed by silver staining PAGE (Fig. 5A). The results reported in the figure demonstrate that RTI-2 is resistant to the proteolytic activity of the proteinase.

Activity of SGPE in the presence of RTI-2 was tested using BSA as substrate. RTI-2, at a concentration in the same order of magnitude of SGPE, was clearly active in inhibiting the protease (Fig. 5A).

To better characterize the inhibitor activity of RTI-2 on SGPE, inhibition was followed using the specific SGPE substrate S-AAPEpNA, at different concentrations of the inhibitor. Double reciprocal blots (Fig. 5B) indicate that rSGPE is indeed inhibited by RTI-2 by a typical competitive mechanism. The apparent equilibrium dissociation constant for rRTI-2 toward SGPE (K_i) was determined from the active site titration curve by the method of Green and Work [20] as already reported for other inhibitors of the MSI family [7,8] (a titration curve is supplied in the Supplementary Material). By this method both the K_i value and the concentration of active inhibitor can be established. K_i from three separate experiments gave a result of 44 ± 16 nM.

K_i was also calculated from the double reciprocal plot using the formula $K_i = K_M/(K'_M - K_M) \cdot [I_C]$ (K_M is the Michaelis constant and K' is the apparent K_M, as deducible by the double reciprocal plot; I_C is the concentration of active inhibitor). It results 32.3 nM (for 71 nMrRTI-2) and 30.93 nM (for 142 nM rRTI-2), in good accordance with the value from the titration curve.

Up to now only few inhibitors of glutamyl endopeptidases, or related genes, belonging to the class of serine protease inhibitors have been described in plants. In most cases they belong to the Potato-I family of plant PIs [4]. This family is not restricted to potato and *Solanaceae*, and members of this family can be found in other plants, like *Arabidopsis*, barley, broad bean and maize. Potato-I inhibitors retain a specificity towards chymotrypsin-like and elastase-like proteases, even if in some cases, they are reactive against trypsins. Accordingly, the P1 residues of the inhibitors are often Leu, Met, Ala and Lys. In tomato [33] a gene coding for a putative Potato-I inhibitor with a Glu residue in the P1 position has been described. However, the corresponding protein has never been isolated. In tobacco [34] a Potato-I inhibitor has been identified which did not result active against bovine chymotrypsin, but was active

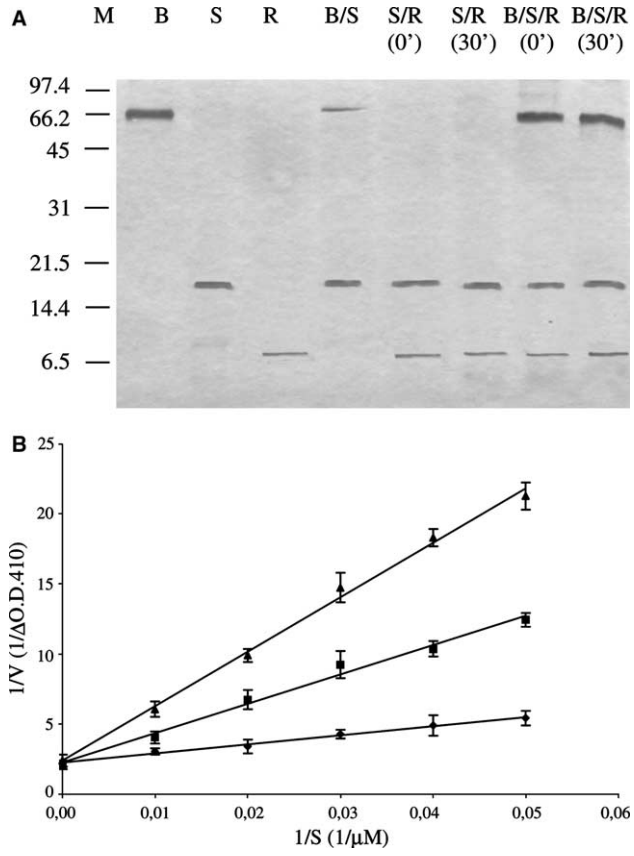

Fig. 5. Activity analysis of rRTI-2. (A) PAGE analysis of SGPE proteolytic activity. M, protein molecular weight marker; B, BSA; S, SGPE; R, RTI-2; B/S, BSA incubated with SGPE for 30 min; S/R, SGPE incubated with RTI-2 for 0 and 30 min; B/S/R, BSA incubated with SGPE and RTI-2 for 0 and 30 min. (B) Double reciprocal plots of the inhibition of SGPE by RTI-2. Substrate (S) was S-AAPEpNA at concentrations of 0.01, 0.02, 0.03, 0.04 and 0.05 mM. 50 nM rSGPE was incubated without (solid circle), or with 71 nM (solid square), or 142 nM (solid triangle) of active rRTI-2. Each point is the mean of three assays.

in inhibiting bacterial (subtilisin from *B. subtilis* and proteinases from *S. griseus*) and fungal (proteinase K from *T. album* and alkaline proteinase from *A. oryzae*) enzymes. Another inhibitor of the Potato-I family, identified in bitter gourd [35], resulted active against a specific glutamyl endopeptidase from *S. griseus*. The K_i of the inhibitor against the bacterial enzyme was about 70 nM. However, the inhibitor did not result active against two other bacterial (from *B. subtilis* and *S. aureus*) glutamyl endopeptidases. To further complicate this scenario, the reactive site of the inhibitor has an Ala residue in the P1 position, and not the expected Glu. Other two genes coding for Potato-I inhibitors with P1-Glu have been described in tobacco tumor cells [36] and in tobacco leaves infected by tobacco mosaic virus [37]. The arabidopsis *atti-5* gene of the MSI family (see Table 1) codes for a potential inhibitor of glutamyl endopeptidases, but its transcript has by no means ever been detected [29].

The rapeseed glutamyl endopeptidase inhibitor described in this paper is the first to have been produced as recombinant protein among all the plant inhibitors with such specificity. It will give the possibility of further studies about their function in plants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.01.022.

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