Identification of a protease inhibitor (BnD22) involved in leaf senescence process of oilseed rape (*Brassica napus* L.)

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Abstract

Oilseed rape (*Brassica napus* L.) is an important crop plant which presents a low Nitrogen Use Efficiency (NUE) leading to a large restitution of this element in the soil. The mobilization of N from leaves is generally considered to be very weak by agronomists. This low leaf N mobilization is essentially related to an incomplete degradation of foliar proteins (low protease activities or inhibition of proteolytic process) during senescence. The aim of this work was to identify the leaf proteomic changes which appeared in N mobilization during developmental leaf senescence (control) or induced foliar senescence (provoked by deprivation of nitrate in soil or foliar pulverization of methyl jasmonate (MeJA)). In the young leaves, deprivation of nitrate delayed senescence initiation and induced trypsin inhibitor (TI) activity at 19 kDa. The degree of TI activity induction tented to decline with the leaf age indicating that TI activity disappeared after the senescence is started. Protein inhibitor activity levels are greatly induced by MeJA allowing detection of TI activity on 2-DE gels. Image analysis of 2-DE gel using silver staining allowed to detect two 19 kDa protein spots (p*i* 5.1 and 5.2) and ESI-LC/MSMS analysis, demonstrated that these proteins perfectly matched with BnD22, a protein previously identified in oilseed rape leaf submitted to water stress. It was the first study which reported that BnD22 possessed a protease inhibitor activity. Our results suggest that BnD22, a Kunitz proteinase inhibitor, may be involved in the regulation of leaf senescence.

Introduction

In oilseed rape (*Brassica napus* L.), the grain yield is mainly related to the plant capacity to recycle efficiently biogenic elements such as nitrogen (N) from the source organs. To meet the N requirements of seed growth, a large amount of endogenous N (protein and amino acids) was mobilized from leaves by foliar senescence. In oilseed rape, the mobilization of leaf N is generally considered to be very weak by agronomists. This low valorisation of endogenous N would not be due to a limitation of the amino transport systems from leaf to phloem (Tilsner et al., 2004) but would be primarily related to an incomplete hydrolysis of foliar proteins (leading of low recycling of endogenous foliar N). This led to an important return of organic N on the soil, not directly available for plant without preliminary mineralisation, with important consequences at economical and environmental levels. The effective recycling of the N compounds from source leaves to sink growing tissues or storage organs requires a fine coordination of the proteolysis processes during the foliar senescence (Hörtensteiner and Feller 2002; Zimmerman and Zentgraf, 2005). Foliar senescence depends on the leaf age but can also be modulated by different biotic (Noh and Amasino 1999; Pourteau et al. 2004) or abiotic factors, such as drought conditions or mineral N limitation (Gombert et al. 2006) which led to accelerate the initiation of leaf senescence.

The cysteine and serine proteases were the proteolytic systems the more frequently implied in N mobilization process associated to senescence (Buchanan-Wollaston and Ainsworth 1997). Many genes encoding cysteine proteases were induced during leaf senescence such as *SAG2*, *SAG12* and *RD21* in *Arabidopsis thaliana* (Yamada et al. 2001) or *BnSAG12-1* and *BnSAG12-2* in oilseed rape (Noh and Amasino, 1999). Moreover, few studies have suggested the involvement of protease inhibitors in the control of the senescence process in leaves (Sugawara et al. 2002). In oiseed rape submitted to water deficit conditions, Reviron et al. (1992) have reported that BnD22, a protein presenting high homology with trypsin inhibitors, was accumulated in young leaves. These authors suggested that this accumulation of BnD22 may contribute to protect the young leaves by delaying their senescence.

The objective of this study is to identify the protease inhibitors potentially involved in the control of N mobilization occurring during the leaf senescence of oilseed rape. This work was conducted in leaf senescence-induced plants (by deprivation of mineral N or MeJA treatment). In order to follow the senescence status of each leaf rank, we used an accurate molecular indicator (changes of *SAG12* and *Cab* gene expression) recently developed by Gombert et al. (2006).

Materials and methods

Plant material

After 60 days of growth under greenhouse conditions (16h/20°C-day and 8h/18°C-night, 200 µmoles photons m^{-2} s^{-1}, nutrient solution supplied every 2 d), *Brassica napus* L. (cv Capitol) plants were submitted to two treatments. Firstly, plants were supplied every 2 d with nutrient solution without KNO_3 (N-deprived plants) or with 2 mM of KNO_3 (control plants) during 21 d. Control and N-deprived plants were sampled in triplicate at Day 0 and after 2, 4, 7, 14, 17 and 21 d of treatment. Secondly, plants grown with nutrient solution containing 2 mM of KNO_3 were subjected to pulverization with MeJA solution

(100 μM, 12.5 mL. plant^{-1}. d^{-1}) or not (control plants) during 7 d. Control and MeJA treated plants were sampled in triplicate at Day 0 and after 1, 2, 3 and 7 d.

Extraction of soluble proteins

Frozen leaf samples (200 mg fresh weight) were ground in a mortar with liquid nitrogen and extracted in citrate Na-phosphate buffer (20 mM citrate and 160 mM Na$_2$HPO$_4$, pH 6.8) in presence of 150 mg PVPP (Polyvinylpolypyrrolidone). After centrifugation (12000 g, 4°C for 1h), the resulting supernatant was used for determination of soluble protein concentration by protein-dye staining (Bradford, 1976).

Protein separation by 2-DE gels

The purification of soluble proteins was performed according to Wang et al. (2003). The pellet (100 μg of proteins) was resuspended and rehydrated in R2D2 buffer (Mechin et al., 2003). IEF was carried out using 18 cm long, pH 4-7 Immobiline DryStrips (IPG strips, Amersham Biosciences) and a Bio-Rad PROTEAN IEF unit. After equilibration of IPG strips, second dimension was carried out on 14% polyacrylamide gels (17 cm × 20 cm) using a BioRad system (300 V, 100 mA). Gels were stained using the silver staining procedure described by Blum et al. (1987).

Detection of trypsin inhibitor (TI) activity by SDS-PAGE and 2-DE

SDS-PAGE was performed using a 5.5% polyacrylamide (w/v) stacking gel and a 18% polyacrylamide (w/v) resolving gel. One volume of each triplicate was mixed together and prepared in 2× lysis buffer without β-mercaptoethanol. Seventy five micrograms of soluble proteins were loaded per lane and separated by SDS-PAGE. For 2-DE gel, 340 μg of soluble proteins were purified using a ReadyPrep 2-D cleanup kit (BioRad). After rehydratation in R2D2 buffer, proteins were separated by 2-DE gels as described above. For detection of trypsin inhibitor activity, gels were stained using a procedure adapted from Yeh et al. (1997). Gels were scanned with the ProXPRESS 2D proteomic Imaging System and TI activity was estimated with the Millipore BioImage computerized image analysis system by measurement of integrated intensity.

Protein Identification by ESI-LCMS

Selected polypeptide spots were manually excised from gels and destained as described by Gharahdaghi et al. (1999). After digestion with trypsin, the peptide was resuspended in 20 μL of solution acid acetic (0.1%). Samples (5μL) were injected directly onto C18 column before the ESI-MS/MS system. The fasta *Brassica napus* database was generated from the file br.seq.all downloaded from http://bioinfo.hku.hk/db/unigene/UNIGENE/.

Results and Discussion

Induction of leaf senescence by mineral N deprivation

Kinetic of leaf senescence progression

According to Gombert et al. (2006), the concomitant up regulation of *SAG12* gene and down regulation of *Cab* gene were used to characterize the theoretical leaf rank in source/sink transition for N (initiation of senescence) at each date for control or N-deprived plants (Fig. 1).
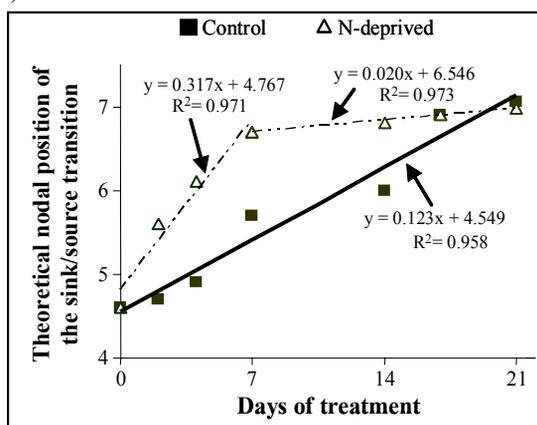
Figure 1- Kinetic of the progression of leaf senescence along the axis of oilseed rape supplied (Control: ■) or not (N-deprived: △) with nitrate.

In control plants, slope of the straight line indicated that temporal senescence progression was theoretically 0.123 leaf rank per d during all the experiment. In N-deprived plants, this progression was accelerated during the 7 first d of treatment to reach 0.317 leaf rank per d. At this date around 6.5 leaf ranks was theoretically engaged in senescence process *versus* 5.5 in control plants. In contrast, in the more youngest leaves (theoretical leaf rank ≥7), the temporal progression of leaf senescence in N-deprived plants observed after 7 d was highly reduced to 0.02 leaf ranks per d (Fig. 1) as compared to control plants.

Detection of anti-protease activities

The anti-trypsin activity (TI) was tested in overall leaf ranks of control or N-deprived plants (Fig. 2A). SDS-PAGE under

non denaturing conditions allowed detecting a polypeptide harbouring TI activity with a molecular weight of 19 kDa. In mature leaf (leaf#6), the TI activity rapidly decreased after the senescence was started (Fig. 2C).
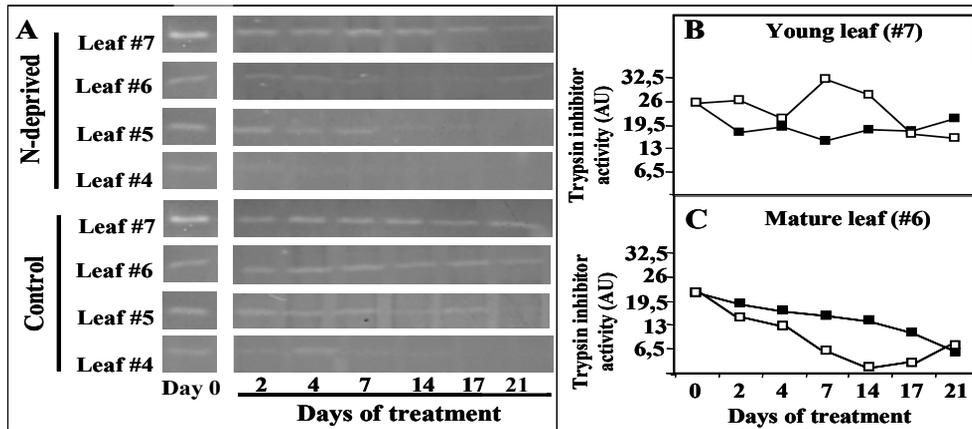
Figure 2- Kinetic evolution of the 18 kDa anti-trypsin activities in the different leaves from Control and N-deprived plants (**A**) and estimation of this evolution by image analysis in leaf #7 (**B**) and leaf #6 (**C**) of oilseed rape supplied (Control: □) or not (N-deprived: ■) with nitrate during 21 d.

These zymograms clearly showed that the TI activities were higher in young leaves than in older ones. The TI activity tented to decline with leaf age, suggesting the presence of a gradient of TI activities which decreased from the youngest to the oldest leaves.

Leaf senescence progression as compared to TI activity changes

As expected, the mineral N deprivation accelerated the initiation of leaf senescence in mature leaves (Fig. 1). Compared to control plants, TI activity was not detected in the older leaf (leaf #4) of N-deprived plants whereas in the same leaf the activity was present up to day 4 in control plants (Fig. 2A). Additionally, TI activity of leaf #5 disappeared after 17 d of treatment in control plants and after 7 d in N-deprived plants. Contrary to mature leaves, mineral N deprivation provoked a delay of the senescence initiation in young leaf as compared to control plants (Fig. 1). Interestingly, the level of TI activity in young leaf (leaf #7) in the N-deprived plants was equal or superior to control plants during 17 d of treatment (Fig. 2B). This high accumulation of TI in young leaves is accordant with previous studies reported by Cipollini and Bergelson (2000). These authors have shown that a TI activity was detected in the first true leaves of 10-day-old *Brassica napus* seedlings. Overall results confirm that leaves possess a TI activity since the beginning of their life, and suggest that the expression of TI in young leaf could be constitutive. This result also supposes an important physiological function of this TI.

Induction of senescence by MeJA and identification of protease inhibitor

Many plant hormones play an important role in the regulation of leaf senescence. Foliar pulverization of MeJA was currently used to induce leaf senescence and also to mimic pathogen attacks. In plants treated with MeJA, chlorophyll and proteins levels decreased more rapidly than in control (data not shown). In the young leaf (leaf #7), after 7 d of MeJA treatment, TI activity was 7 times higher than control (Fig. 3A). This protein sample was used to attempt detecting TI activity after 2-DE gel.
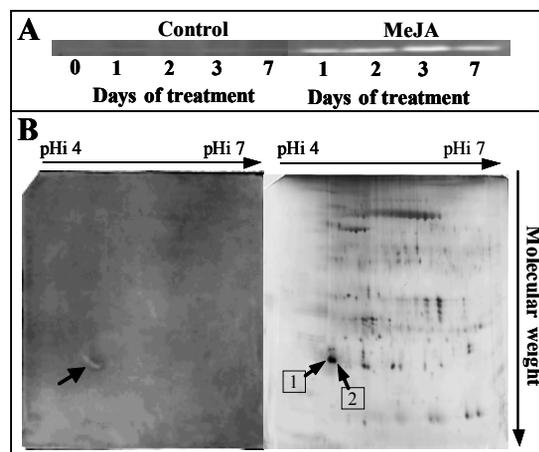
Figure 3- A: Changes of the 18 kDa TI activities in leaf #7 of plants treated (MeJA) or not (Control) with MeJA during 7d. **B:** 2-DE gels of leaf #7 proteins from plants treated with MeJA after TI staining (left) or silver staining (right). Arrows indicate spots (1 and 2) corresponding with the TI activity.

Table 1- Identification of protein spots corresponding with TI activity by ESI/LCMS/MS. (AN : Accession Number)

N° spot	Protein/ AN/ organism	Mr	pI	Sequenced peptides	Max Sp Score
1	BnD22/ X65637.1/ *Brassica napus*	19 kDa	5.1	KEPAIIIGGESTAPNSLK	1019 (20/34)
				(K)LWAVDVSSSAAK	905 (15/22)
2	BnD22/ X65637.1/ *Brassica napus*	19 kDa	5.2	(K)EPAIIIGGESTAPNSLFK	821 (17/34)
				(K)LWAVDVSSSAAK	832 (15/22)
				(K)TLFVK	366 (6/8)

To our knowledge, our study shows for the first time that a TI activity could be detected after 2-DE gel separation (Fig. 3B). In the aim to characterize the TI that appeared in the young leaves, proteins extracts were analyzed by 2-DE gels and stained either with TI or silver staining (Figure 3B). Two polypeptides spots that were common in both gels were identified at a molecular mass of 19 kDa and p*i* of 5.1 and 5.2, respectively. Analysis with ESI-MSMS revealed that both spots were homologous to BnD22 (a *Brassica napus* drought-induced protein; Table 1). BnD22 was firstly characterized in a study focused on the adaptation of *Brassica napus* to osmotic stress (Reviron et al., 1992). This putative inhibitor of proteases represented approximately 1% of the leaf soluble protein contents in plants adapted to drought and disappeared after rehydration (Downing et al. 1992; Ilami et al. 1997). These authors have also reported that BnD22 amino acid sequence presented high degree of homologies with the Kunitz protease inhibitor family although the functional significance remained unclear. Using *in vitro* assay with purified BnD22 protein, Ilami et al. (1997) have showed that BnD22 (i) did not inhibit the endogenous leaf proteases, (ii) was able to slightly inhibit chymotrypsin, and (iii) has no significant effects on others serine proteases tested such as trypsin. In our study, 2-DE gels clearly revealed that BnD22 harboured *in vitro* protease inhibitor activity which was coherent with its homology with the Kunitz-type proteinase inhibitor family.

Such a role of protease inhibitors in the control of senescing process have been reported in previous works (Shatters et al. 2004, Sin and Chye, 2004). For instance, in *Citrus paradisi*, *lkiL-l* and *lkiL-2* (two genes encoding inhibitor of serine proteases of Kunitz type) were up regulated in the young leaves whereas they were strongly down regulated in the mature leaves (Shatters et al. 2004). In *Dianthus caryophyllus* L., when senescence of the petals occurs, the protein degradation appears with the induction of the expression of the cysteine-protease (*DC-CP 1*) and the concomitant disappearance of protease inhibitor *DC-CPIn (*Sugawara et al., 2002*).* Interestingly, BnD22 accumulation was concomitant with a lower proteolytic activity in the leaves of oilseed rape submitted to water stress (Reviron et al., 1992). Consequently, protease inhibitor may contribute to regulate or delay senescence process. Because BnD22 is specifically accumulated after treatment with MeJA, a phytohormone involved in senescense induction and in response of plant to pathogens; it could be assumed that the high abundance of BnD22 in young leaves have several physiological functions. For instance, BnD22 accumulation could be a mean to prevent the young leaf of (i) premature protein–N degradation (especially induced by a low mineral N availability) or (ii) pathogen attacks.

Acknowledgments

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