Proteasome comprising a β1 inducible subunit acts as a negative regulator of NADPH oxidase during elicitation of plant defense reactions

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Abstract Elicitation of defense reactions in tobacco by cryptogein, triggered a production of active oxygen species (AOS) via the NADPH oxidase, NtrbohD, and an accumulation of β1din, a defense induced β-type subunit of 20S proteasome. The proteasome inhibitor, MG132, stimulated this AOS production. Tobacco cells transformed with sense constructs of *β1din* showed an inhibition of the AOS production following elicitin treatment, whereas the antisense transformed cells showed a strongly enhanced AOS production. In cells transformed with sense construct of *β1din*, the NtrbohD transcripts failed to be induced by cryptogein as observed in control and antisense transformed cells. Conversely, in tobacco cells transformed with antisense constructs for *NtrbohD*, β1din transcripts remained at a low level after elicitation. These results constitute the first demonstration of proteasome comprising β1din acting as a negative regulator of NtrbohD and contributes to the regulation of AOS generation during plant defense reactions.

Keywords: Proteasome; NADPH oxidase; AOS production; Cryptogein; Tobacco BY-2 cells; Plant defense

1. Introduction

Plants are exposed to a great number of pathogenic microorganisms, although only a relatively small proportion of them are able to cause diseases. Indeed, plants defend themselves against pathogens by triggering a wide range of mechanisms including the hypersensitive response (HR), which leads to cell death lesions at the infection sites, thus limiting pathogen growth to a restricted area of the plant [1,2]. HR plays a central role in acquisition of the systemic resistance that protects plants against attacks by virulent pathogens, since pathogens that do not trigger HR can establish compatible interactions with the host, resulting in plant disease [3–5]. The development of this HR involves the recognition by the plant of a signal which then triggers a great amount of cellular responses [6]. This detection of pathogens, which constitutes a critical step for activation of plant defense reactions, can be mediated by elicitors, including oligosaccharides, lipids or proteins, secreted or generated by various pathogens [7]. For example, elicitins, a family of proteinaceous elicitors synthesized by *Phytophthora* species [8] induce a hypersensitive-like response and a systemic acquired resistance (SAR) in tobacco [9], (for review see [10]), after binding of the elicitor to a high affinity site on the plasma membrane [11]. Although most of the responses induced by such elicitors have been extensively described in various models of plant–pathogen interaction, their molecular basis and fine regulation is often still under investigation. Moreover, cross-talk between these different cellular events are poorly understood and the need exists for new signal transduction elements to help to put together the pieces of the puzzle.

The HR, rapidly induced after recognition of the microorganism, is characterized by intense and rapid production of active oxygen species (AOS). Because the generation of these AOS is believed to contribute to several disease resistance strategies (direct antimicrobial activity, cross-linking of cell wall proteins, induction of defence related genes, cell death, etc.), the mechanism and the regulation of their biosynthesis have been extensively studied (for reviews see [12–15]). We recently demonstrated that NtrbohD an enzyme similar to $gp91^{phox}$, the NADPH oxidase of mammalian neutrophils, was responsible for the production of AOS in tobacco cells treated with the fungal elicitin cryptogein [16]. However, if some characteristic features are conserved between these plant and animal oxidases, the similarity of their regulation remains questionable and very little is actually known about the precise mechanisms of regulation of this plant oxidase. Indeed AOS production in plants undergoing an incompatible interaction has been proven to depend on very early and general phenomena such as calcium influx or protein phosphorylations [17], but numerous molecular elements of the signal transduction pathway leading from the crucial step of pathogen recognition to AOS production are still missing.

We previously showed a transcriptional activation of various genes, rapidly following elicitation of defense reactions in tobacco by elicitins [18,19]. Amongst these activated genes, we studied particularly *β1din*, a gene encoding a β subunit of proteasome [20,21]. In eukaryotes, the 26S proteasome is a multicatalytic complex comprising the 20S core particle and 19S regulatory particles, that recognize proteins targeted for

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Abbreviations: AOS, active oxygen species; din, defense induced; BY-2, Bright Yellow line 2; HR, hypersensitive response; SAR, systemic acquired resistance; S, sense; AS, antisense; NF, nuclear factor

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degradation, especially polyubiquitin targeted proteins [22,23]. The 20S proteasome is a barrel-shaped structure of two outer rings formed by seven α subunits (α1–α7) and two inner rings of seven β subunits (β1–β7) enclosing cavities with the active sites for processive protein degradation [23]. In mammals, proteasomes were shown to be not only involved in degradation of misfolded proteins [24], in cell cycle progression, cell death and development [25] but also in specific cleavage of propeptides to activate the peptides (for example transcription factors), or allowing the antigene presentation during immune reactions (for a review, see [26]).

In plants, 20S proteasome has been shown to be involved in cell cycle progression [27], in differentiation of tracheary elements [28], in senescence, in early stages of seedling development [29] and in elicitation of defense responses [30,31].

We also previously showed that only one β subunit, β1 (tcI7) and two α subunits (α3 and α6) were upregulated by cryptogein in tobacco cells and plants [32]. Expression of these induced subunits, renamed β1din, α3din and α6din (din for *d*efense *in*duced), probably included in newly reassembled proteasomes called "plant defense proteasomes " was also shown to be tightly correlated with the establishment of SAR in various experimental models [33]. We also observed that inhibition of the oxidative burst, strongly and rapidly induced in the hypersensitive reaction, is correlated with the inhibition of induction of *β1din*, *α3din* and *α6din* [33]. The aim of this work was to investigate the link between induction of the 20S proteasome subunit β1din and the "oxidative burst" occuring in the first steps of elicitation of defense reactions leading to the SAR and thus to the plant protection.

2. Materials and methods

2.1. Materials and treatments

Tobacco BY-2 cells (*Nicotiana tabacum* cv Bright Yellow-2) were grown and maintained as previously described [16]. Gp3, an antisense line for NtrbohD unable to produce AOS after treatment with cryptogein [16] was also used. BY-2 wild-type or transformed cells were harvested at various times after treatment, quickly frozen in liquid nitrogen and stored at −80 °C. Three separate experiments were carried out.

Cryptogein was purified from *Phytophthora cryptogea* as previously described [34]. The proteasome inhibitor MG132 (Z-Leu-Leu-Leu-al) was dissolved in DMSO and stored at −20 °C.

2.2. Antisense gene construction and cell transformation

An *Xba*I/*Sst*I PCR fragment of 700 bp from the β1tcI7 cDNA (β1din) was inserted in sense, into pBI 121(Clontech) downstream from a 35S CAMV promoter deleted from the GUS sequence by *Xba*I and *Sst*I. An *Xba*I/*Sst*I fragment truncated in the 5′-coding region was inserted in antisense. The resulting plasmids, introduced by triparental mating [35] into a disarmed strain of *Agrobacterium tumefasciens*, C58C1 (pMP90) [36] were used to transform BY-2 tobacco cells. A 3-day-old BY-2 culture (2 ml) was co-cultivated with 50 µl of each *Agrobacterium* culture (A_{600} 0.3) on Petri dishes in the dark for 2–3 days at 26 °C. Cells were washed and plated onto agar-MS medium containing 100 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxime. Transformed microcalli were propagated during 4–5 subcultures with the selection agent and then diluted weekly without.

2.3. RNA analyses

RNA extractions were performed using the "Plant RNeasy minikit" (Qiagen, France). Northern blots were carried out according to standard protocols using 15 µg of total RNA per lane. Hybridizations were carried out with ^{32}P labeled probes (*redi*prime, Amersham, France): *NtPB1* (*β1din*) (Accession No.: Y09505) corresponding to the *β1tcI7* 20S proteasome subunit [21], *NtPB2*(*β2*) (Accession No.: AJ291736), and *NtPB5* (*β5*) (Accession No.: AJ291741) [32]. A fragment of 0.86 kb corresponding to a part of the 3′ encoding and non-translated region of *NtrbohD* (Accession No.: AJ309008) [16] was also used as a probe.

Three independent hybridizations were carried out at 42 °C. Filters were washed under stringent conditions and analyzed with a PhosphorImager (Molecular Dynamics, France), using Imagequant for quantification.

2.4. AOS production

Experiments were performed as described previously [16].

3. Results

3.1. Effect of the proteasome inhibitor, MG132, on AOS production in BY-2 wild-type tobacco cells

Inhibitors of proteasome are useful tools for elucidating the role of proteasome in various molecular mechanisms. MG132 was known as an efficient proteasome inhibitor in animal cells [24], in yeasts [37] and in plants [27]. Wild type BY-2 cells were treated with cryptogein (50 nM) or with the proteasome inhibitor MG132 (20 µM) or with cryptogein (50 nM) and MG132 (20 µM) together. MG132 alone had no effect on AOS production whereas in the presence of 50 nM cryptogein, MG132 induced a stimulation of the AOS production triggered by the elicitor (Fig. 1). Insert in Fig. 1 shows that this stimulation of AOS production (summed during 2 h) in wild-type BY-2 cells treated with cryptogein (50 nM) was dose-dependent, with a maximum of stimulation reached at 25 µM of MG132. The NADPH oxidase NtrbohD being responsible for the AOS production observed following cryptogein treatment [16], the increase of AOS production triggered by cryptogein in presence of MG132 suggests the possible involvement of proteasome in the down regulation of the NADPH oxidase NtrbohD. As cryptogein or hydrogen peroxide have been previously shown to induce the expression of *β1din*, a β1 subunit of proteasome [21], it was interesting to further investigate the involvement of β1din comprising proteasomes in the regulation of NtrbohD, by using tobacco cells transformed with sense and antisense constructs for *β1din*.

3.2. Expression of β1din subunit of proteasome in sense and antisense transformed tobacco cells

Expression of *β1din* in BY-2 tobacco cell lines transformed with either the antisense construct of *β1din* cDNA (AS cells), the sense construct (S cells), or the empty vector (pBI cells) was investigated by Northern-blot (Fig. 2A). The analysis of the transformed cell lines showed that the expression of the *β1din* 20S proteasome subunit was significantly decreased in all the antisense lines and increased in all the sense lines compared to wild-type cells and cells transformed with the empty vector (Fig. 2B). All the selected lines were further used but AS15(8) and S(8)6 which were the most affected in *β1din* expression, were considered as the most representative of antisense and sense transformation, respectively. RT-PCR analysis of the lines confirmed these results (not shown). The expression of two other β subunits (β2 and β5) was also monitored as a control. Fig. 2 shows that the level of expression of β2 and β5 remained unchanged in all the selected lines. As an additional control, three peptidasic activities associated with the 20S proteasome, chymotrypsin-like, trypsin-like and peptidylglutamyl-

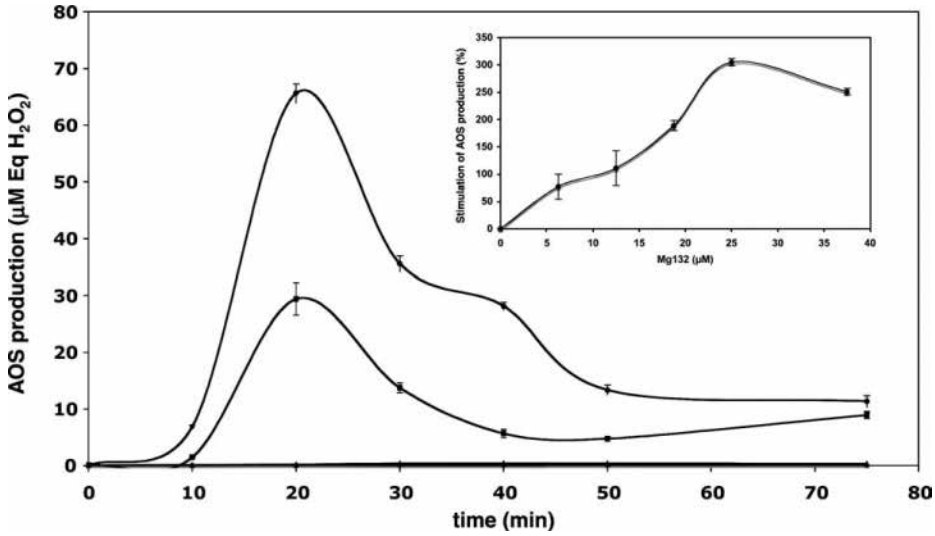

Fig. 1. Effect of the proteasome inhibitor MG132 on AOS production from wild-type BY-2 tobacco cells. AOS production was measured in tobacco cells without treatment (control) ♦, treated with cryptogein (50 nM) (dotted line) ■, with MG132 (20 µM) ▲, or with cryptogein and MG132 together ●. The insert shows the dose-dependent stimulation of AOS production by MG132: AOS production was measured every 10 min and values are summed to give the total AOS production during 2 h. Results are expressed in % of stimulation of AOS production obtained from tobacco cells treated with cryptogein and MG132 related to AOS production in tobacco cells treated only with cryptogein. Experiments were performed in triplicate and results are expressed as mean values.

peptide hydrolase (PGPH) were measured in the selected transgenic tobacco lines (not shown). Chymotrypsin-like activity was unchanged in all cell lines but trypsin-like activity was significantly decreased in antisense cells and increased in sense cells while PGPH activity increased in the antisense transgenic lines and decreased in the sense line, the antisense line AS15(8) and the sense line S8(6) being also the most representative. These results show that proteasomes comprising β1din instead the constitutive β1 subunit have modified peptidasic activities and consequently could have specific target(s).

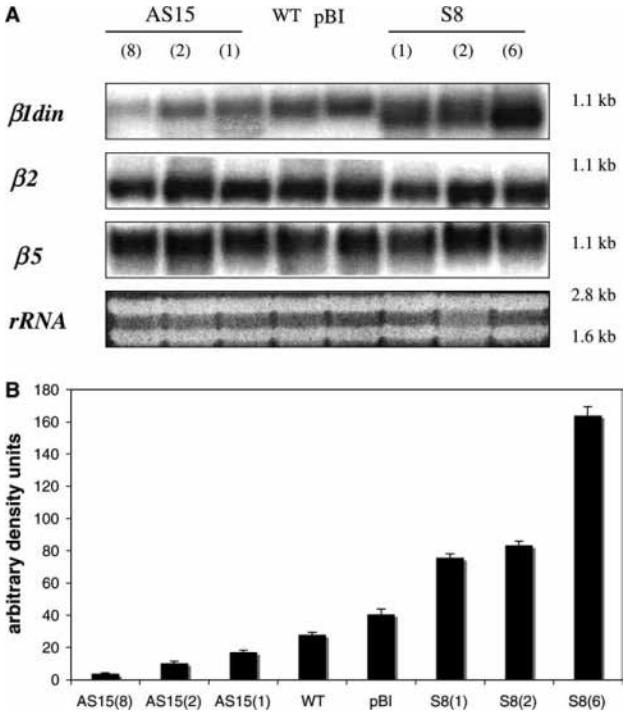

Fig. 2. Characterization of *β1din* sense and antisense cell lines. RNA expression of *β1din*, *β2* and *β5* 20S proteasome subunits in tobacco cells transformed with the sense (S) and antisense (AS) constructs of *β1din*. PBI was the cell line transformed with the empty binary vector and WT the wild-type BY-2 cells. Fifteen micrograms of total RNA extracted from the transgenic tobacco cells were subjected to RNA analysis. (A) Autoradiograms were obtained using ^{32}P labeled *β1 din*, *β2* and *β5* cDNA probes with rRNA as standards. (B) *β1din* expression was quantified using Imagequant (Phosphorimager) and expressed as arbitrary density units.

3.3. AOS production in the β1din transgenic cell lines treated with cryptogein, a proteinaceous elicitor of plant defense reactions

To further address whether β1din induction is associated with the regulation of AOS production, the production of H_2O_2 following cryptogein treatment was investigated in the sense and antisense transgenic cell lines. Upon elicitation with 50 nM cryptogein, BY-2 type cells and pBI (empty vector) transformed cells used as controls exhibited a transitory production of H_2O_2 consistent with previous results whereas this AOS production was very strongly decreased in all sense transformed lines (Fig. 3A). On the opposite, AOS production in antisense transformed cell lines was always higher than in wild-type and pBI cells (Fig. 3B). In particular, no hydrogen peroxide was detected in the sense S8(6) line while the highest production of hydrogen peroxide was observed in the antisense line AS15(8). These results, in addition to those obtained with MG132 on wild-type cells (Fig. 1), suggest that NtrbohD, responsible for AOS production following elicitation with cryptogein, could be downregulated by β1din comprising proteasome, since the level of AOS production is inversely correlated to the level of *β1din* mRNA in wild-type and transformed tobacco cells.

3.4. Expression of β1din and NtrbohD genes in elicited tobacco cells transformed with sense and antisense constructs of β1din

According to the results obtained above, wild-type, *β1din* sense S8(6) and *β1din* antisense AS15(8) tobacco cell lines

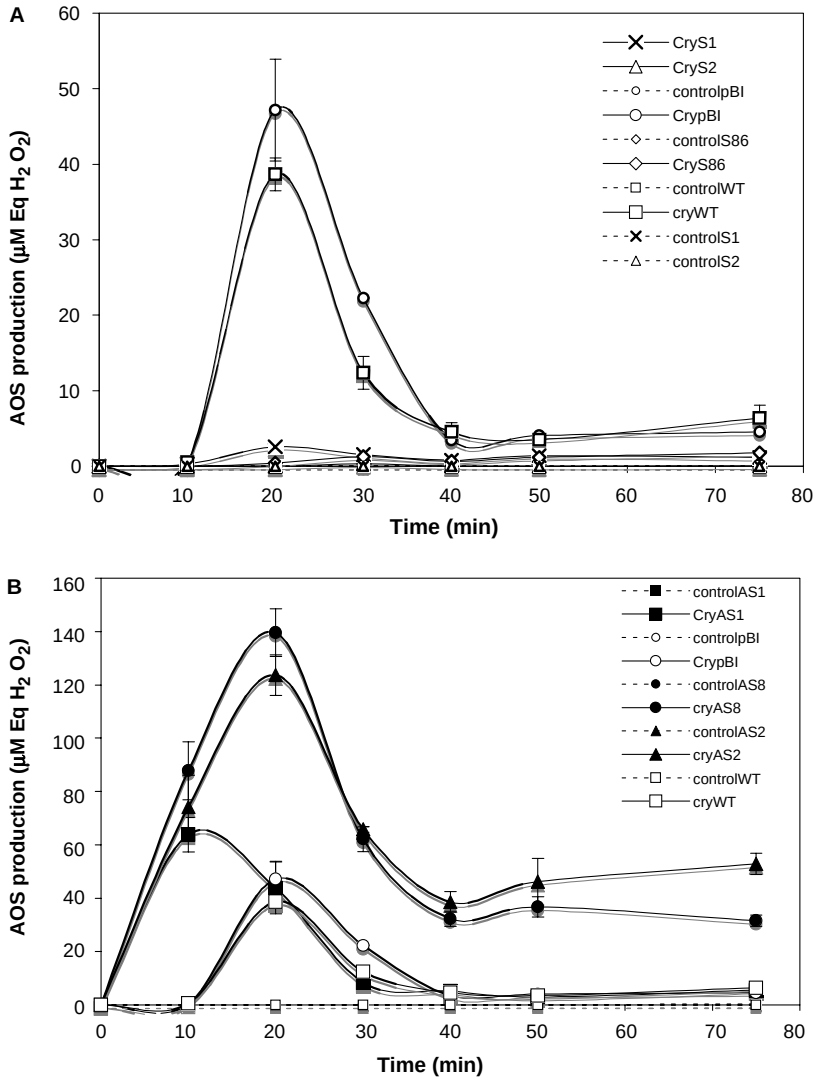

Fig. 3. Effect of cryptogein on AOS production. At zero time of the experiment, cryptogein (50 nM) was added to each suspension culture and AOS production was measured every 10 min. Wild-type (WT) tobacco cells and cells transformed with the empty vector pBI were used as controls. AOS production was measured on sense (S) transgenic tobacco cells (A) or antisense (AS) transgenic tobacco cells (B). Experiments were performed in triplicate and results are expressed as mean values.

were used to analyze expression of tobacco proteasome *β1din* subunit and NADPH oxidase *NtrbohD*, following treatment with cryptogein. S8(6) and AS15(8) were choosed as the most representative of sense and antisense transformed lines, respectively. Fig. 4 shows the accumulation of *β1din* mRNAs in BY-2 wild-type tobacco cells within 30 min following elicitation with cryptogein (consistent with previous results, [33]). Whilst *β1din* mRNA was constitutively and strongly expressed in *β1din* sense transformed cells, none was detected in the *β1din* antisense cells. In the same cells, *NtrbohD* mRNA strongly accumulated after elicitation in BY-2 wild-type cells and in antisense *β1din* transformed cells, whereas only a slight accumulation was observed in sense *β1din* transformed cells. These results suggest that expression of the oxidase NtrbohD is negatively affected at the transcriptional level in the tobacco cells overexpressing the β1din subunit of 20S proteasome following elicitation.

3.5. Expression of β1din and NtrbohD genes in elicited tobacco cells transformed with antisense constructs of NtrbohD

Since expression of β1din was shown to be dependent on production of AOS, following elicitation of defense reactions with crytogein, we used Gp3, a tobacco cell line transformed with an antisense construct of *NtrbohD*, unable to produce AOS and where both NtrbohD mRNA and protein remained undetected even after elicitation [16], to investigate RNA expression of *β1din* during elicitation with cryptogein. When used as a negative control, the probe *NtrbohD* confirmed the absence of the corresponding mRNA (Fig. 5). In addition, levels of *β1din* mRNA remained low, even after elicitation showing that in the absence of NtrbohD, the expression level of *β1din* was not increased. This confirms the tight link between induction of the oxidase NtrbohD, the main producer of active AOS during elicitation and the induction of β1din, a β1 subunit of 20S proteasome involved in plant defense reactions.

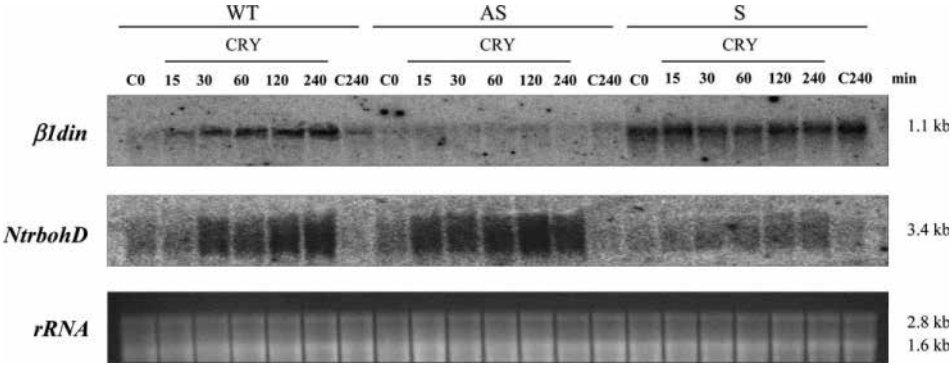

Fig. 4. Northern-blot analysis of *β1din* and *NtrbohD* expression in wild-type (WT), sense (S) and antisense (AS) transgenic tobacco cells treated (CRY) or untreated (C0) with cryptogein. Total RNA (15 μg) was analyzed and probed with ^{32}P labeled restriction fragments corresponding to *β1din* or *NtrbohD* encoding regions; rRNA is shown as a control.

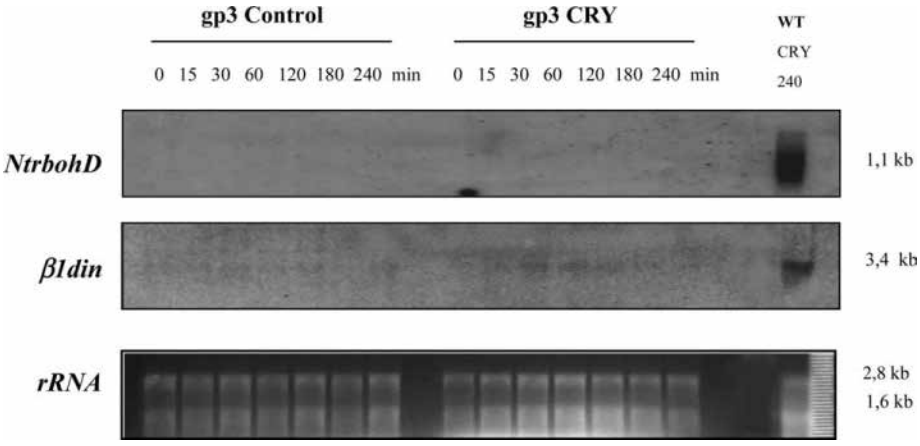

Fig. 5. Northern-blot analysis of *β1din* and *NtrbohD* expression in the gp3 tobacco cell line transformed with an antisense construct of *NtrbohD*, treated (CRY) or untreated (Control) with cryptogein. Total RNA (15 μg) was analyzed and probed with ^{32}P labeled restriction fragments corresponding to *β1din* or *NtrbohD* encoding regions. rRNA is shown as a control.

4. Discussion

The rapid and intense production of AOS is believed to contribute to several disease resistance strategies and thus, the mechanism and regulation of their biosynthesis have been extensively studied (for reviews see [15,38]). We previously demonstrated that NtrbohD, an enzyme similar to gp91^{phox}, the NADPH oxidase of mammalian neutrophils, was responsible for the production of AOS in tobacco cells treated with the fungal elicitor cryptogein [16]. However, if some characteristic features are conserved between these plant and animal oxidases, the similarity of their regulation remains questionable as the sites in neutrophil gp91phox known to interact with p47phox and p67phox (regulators of gp91phox) are not conserved in NtrbohD. To date, no direct evidence for sequences presenting significant homology with p47phox and p67phox exists in the entire *Arabidopsis* genome database and, in tobacco, only a small G protein, Ntrac5, was shown to act as a negative regulator of NtrbohD [39]. So, the particular way of regulation of this plant oxidase remains to be elucidated. Since we previously cloned *β1din*, a gene encoding a β1 subunit of proteasome, induced rapidly after elicitation of plant defense reactions and also induced by hydrogen peroxide [21,33], it was interesting to explore the possibility that proteasome, especially proteasome comprising the β1din subunit could be involved in the regulation of NtrbohD.

Firstly, we have shown in this work that inhibition of the proteasome with MG132, leads to an increased production of AOS following elicitation of tobacco cells with cryptogein, suggesting that proteasome could indeed be involved in the regulation of AOS production. Many processes in plant growth and development (for review see [40]), but also responses to biotic and abiotic stresses could be proteolysis-dependent. Over 5% of the *Arabidopsis thaliana* genome encodes for components of the ubiquitin–proteasome system [41] although it is only in recent years that the importance of regulated proteolysis, and more specifically the ubiquitin–proteasome system in the control of plant development [42] and defense [43] has been recognized. Recently, analysis of the Arabidopsis 26S proteasome revealed the presence of multiple isoforms of each subunit [44]. The authors demonstrate that each

isoform assembles into the mature particle. The incorporation of paralogous subunits raises the interesting possibility that plants synthesize multiple 26S proteasome types with unique properties and/or target specificities. The tight link previously observed between production of AOS and induction of *β1din* after elicitation of defense reactions leads to speculate that the NADPHoxidase, NtrbohD, could be a target for proteasomes modified by integration of β1din subunits. Therefore, further investigation on the involvement of "β1din comprising proteasomes" in the regulation of NtrbohD, was carried out using transgenic tobacco cells transformed with sense or antisense constructs for *β1din*. These experiments confirmed this hypothesis since overexpression of *β1din* lead to a strong decrease of AOS production following cryptogein treatment. As *β1din* sense and antisense transformations were shown to be associated with modifications of two of the three main peptidasic activities of proteasome (PGPH and trypsin-like), a specific proteolysis of NtrbohD by β1din comprising proteasomes may explain the transient activity of these plant NADPHoxidase in AOS production occuring after elicitation. Furthermore, NtrbohD, induced by cryptogein is located in plasmamembrane [16] and performing a two-hybrid system using the N-terminal part of NtrbohD, the same authors found specific partners of this plant oxidase amongst which ubiquitin (Accession No. AJ309010, personal communication). This could significate that NtrbohD was ubiquitinated for proteolysis by proteasome, may be preferentially by β1din comprising proteasomes, but this specific proteolysis remains to be demonstrated.

The most obvious results obtained in this work concerned the negative regulation of NtrbohD at the transcriptional level. We showed that overexpression of *β1din* was correlated with transcriptional inactivation of *NtrbohD* and that knock-out of *NtrbohD* was correlated with transcriptional inactivation of *β1din*. The decreased AOS production could then result in an inactivation of *β1din* expression.

Taken together, these results suggest that proteasomes comprising the β1din subunit could directly or indirectly negatively regulate induction of NtrbohD, the intrinsic plasma membrane oxidase responsible for the AOS production following elicitation of defense reactions in tobacco by a loop of regulation presented in Fig. 6. As previously shown [10,11,16,45], the first event involves binding of cryptogein to its receptor (step 1), triggering a cascade of transduction pathways (step 2) leading to activation of AOS production via the induction of the NADPHoxidase, NtrbohD (step 3), NtrbohD remaining undetectable in WT tobacco cells before treatment with cryptogein. In this work, we confirm that cryptogein via AOS production induces *β1din* (step 4) [21,32,33], and we demonstrated that β1din negatively regulates expression of NtrbohD at the transcriptional level (step 5) and so reduces AOS production. Inactivation of NtrbohD could also arise from a specific direct proteolysis by the proteaome as mentioned above (step 6). In this model, step 5 and step 6 could contribute complementarily to the regulation of NtrbohD and so to the limitation of AOS production and *β1din* induction by feedback. So, the present results demonstrate for the first time that β1din proteasome regulates the expression of NtrbohD, homologue to the flavocytochrome of the neutrophil NADPH oxidase responsible for the AOS production following elicitation of defense reactions.

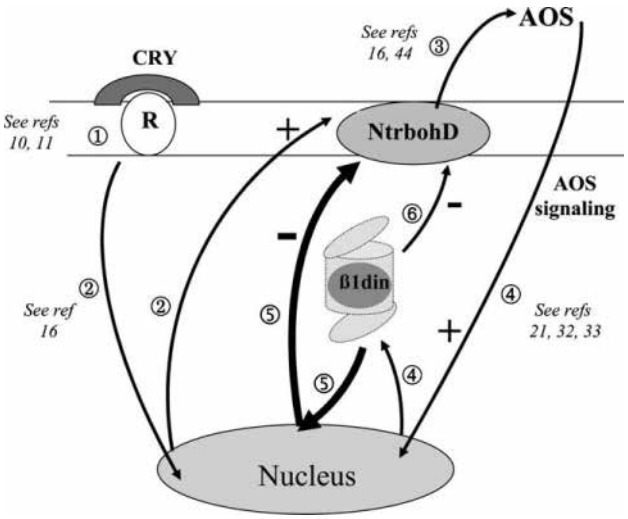

Fig. 6. Loop of regulation presenting the role of *β1din* as a negative regulator of NtrbohD during elicitation of plant defense reactions. (1) Binding of cryptogein to its plasmalemma receptor, (2) signaling pathway leading to transcriptional activation of *NtrbohD*, (3) AOS production, (4) signaling pathway leading to activation of *β1din*, (5) signaling pathway leading to transcriptional inactivation of *NtrbohD* demonstrated in this work (in thick arrows), (6) possible direct specific proteolysis of NtrbohD by β1din comprising proteasomes.

Elucidation of the fine mechanisms underlying this regulation is likely to require the study of the "transcription factors hypothesis". Indeed, roles in transcription for proteasome mediated proteolysis have been already identified. In animals, an overlap between the transcriptional activation domain and sequences targeting for ubiquitin–proteasome mediated degradation was found for several transcription factors such as fos, jun, p53, β catenin and myb [46]. Further investigations are in progress in order to identify transcription factors involved in this pathway. Such a change in transcriptional activity may be achieved trough the oxidation of components of signalling pathways that activate transcription factors or by modifying a redox-sensitive transcription factor directly (for review see [38,47]). Concerning direct activation of signal transduction pathways by redox-sensitive transcription factors, Mou et al. [48] reported the redox regulation of NPR1 (non-expressor of PR1), an essential regulator of plant SAR. During a SAR response, NPR1 initially present in the cytosol as an oligomer is reduced to a monomeric form that accumulates in the nucleus and activates gene expression. Potential H_2O_2 responsive elements were identified by microarray analysis of H_2O_2-induced gene expression in Arabidopsis [49]. Other components of the signalling cascades that mediate responses to AOS remain to be discovered such as NF-κB homologs, since a NF-κB box has previously been localized in the promoter of β1din [21].

Nevertheless, this work provides new insights into the regulation of AOS production occurring during HR and for the first time, assigns a precise role to the proteasome during early signal transduction processes associated with plant defense responses. This loop of regulation is probably a part of a multiple components system finely regulating AOS production in order to activate genes and limit cell death. Extensive studies are required to better understand the implication of the ubiquitin–proteasome mediated proteolysis in plant defense reactions. Future research should concentrate on

the identifying upstream signal or upstream signaling components involved in this regulation process.

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