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Activation of a nuclear localized-SIPK in tobacco cells challenged by cryptogein, an elicitor of plant defence reactions

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1 SUMMARY

2
3 When a plant cell is challenged by a well-defined stimulus, complex signal transduction
4 pathways are activated to promote the modulation of specific sets of genes and *in fine* to
5 develop adaptive responses. In this context, protein phosphorylation plays a fundamental role
6 through the activation of multiple protein kinase families. While the involvement of protein
7 kinases at the plasma membrane and cytosolic levels are now well documented, their nuclear
8 counterparts are still poorly investigated. In the field of plant defense reactions, no known
9 study has yet reported the activation of a nuclear protein kinase and / or its nuclear activity in
10 plant cells, although some protein kinases, e.g. MAPK, are known to be translocated into the
11 nucleus. In the present report we investigated the ability of cryptogein, a proteinaceous
12 elicitor of tobacco defense reactions, to induce different nuclear protein kinase activities. We
13 found that at least four nuclear protein kinases are activated in response to cryptogein
14 treatment in a time-dependent manner, some of them exhibiting Ca^{2+} -dependent activity. Our
15 study focused on one 47-kDa protein kinase with a Ca^{2+} -independent activity, closely related
16 to the MAPK family. After purification and microsequencing, this protein kinase was
17 formally identified as SIPK, a biotic and abiotic stress-activated MAPK of tobacco. We also
18 showed that cytosolic activation of SIPK is not sufficient to promote a nuclear SIPK activity,
19 the latter being correlated with cell death. In that way, this study provides evidences of a
20 functional nuclear MAPK activity involved in response to an elicitor treatment.

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1 INTRODUCTION

2
3 Plant cells challenged by stimuli develop adaptive responses based on the modulation
4 of the expression of specific sets of genes which activities are tightly controlled by signalling
5 cascades. However, with the exception of transcription factors, little information is available
6 regarding the signaling events operating in the nucleus [1]. Indeed, the possibility that this
7 organit displays specific transduction processes has been poorly investigated. One of the most
8 demonstrative studies highlighting the ability of the nucleus to perceive and to manage a
9 specific response according to the applied stimulus came from Pauly et al [2]. Using isolated
10 nuclei from *Nicotiana tabacum* BY2 cells expressing the nuclear calcium reporter aequorin,
11 these authors showed that plant cell nuclei are able to generate their own calcium signals
12 independently of the changes in calcium ion concentration occuring in the cytosol. These data
13 point out the ability of nuclei to perceive extranuclear stimuli and to convert it into an
14 appropriate nuclear response. A related and still poorly understood process concerns the
15 molecular mechanisms linking cytosolic events and the modulation of nuclear gene
16 expression. In other words, the question of how a specific signal is transmitted from the
17 cytosol to the nucleus remains largely unanswered.

18 Protein phosphorylation/dephosphorylation is a major prototypic posttranslational
19 modification regulating protein function [3]. Based on animal studies, it is commonly
20 assumed that once activated, plant nuclear protein kinases (PK) could modulate the activity of
21 target proteins including transcription factors leading, *in fine*, to the up- or down-regulation of
22 gene expression. However, whereas various studies predict a nuclear localization for several
23 PK based on primary sequence analysis (that is the presence of a nuclear localisation site; [4,
24 5]) and/or the nuclear localisation of overexpressed chimeric GFP-PK [6], the possibility that
25 the activity of these PK is modulated within the nucleus as well as their putative translocation
26 from the cytosol to the nucleus in physiological contexts have been examined in only few
27 cases [7, 8].

28 Cryptogein is a 10 kDa proteinaceous elicitor secreted by the oomycete *Phytophthora*
29 *cryptogea*, an avirulent pathogen of tobacco. Upon application to tobacco leaves, cryptogein
30 triggers the expression of defense-related genes, a hypersensitive response (HR) and a systemic
31 acquired resistance (SAR) against various pathogenic micro-organisms [9]. The molecular
32 mechanisms underlying the effects of cryptogein have been mainly investigated using tobacco
33 cell suspensions (for review see [10]). These studies highlight a key role for PK in
34 transducing the cryptogein signal. Notably, Lecourieux-Ouaked et al [11] demonstrated that
35 the elicitor induced within 5 minutes the phosphorylation of about 20 polypeptides, this
36 process being partly controlled by a primary calcium influx across the plasma membrane. The
37 identity of these polypeptides has not been reported. Cryptogein was also shown to trigger the
38 activation of cytosolic MAPK (mitogen-activated protein kinases), including WIPK (wound-
39 induced protein kinase), SIPK (salicylic acid-induced protein kinase) and Ntf4 [12-15] as well
40 as the activation of a 42-kDa PK operating downstream of anion effluxes [16]. Accordingly,
41 PK inhibitors efficiently suppressed cryptogein-induced events including ion fluxes, reactive
42 oxygen and nitrogen species production and gene expression (reviewed by [10, 13]). A role
43 for SIPK and WIPK in the elicitor-induced defense responses has been tentatively assigned.
44 Zhang et al [15] reported that staurosporine and K-252a, two Ser/Thr kinase inhibitors that
45 blocked WIPK activation, suppressed cell death, suggesting a role for WIPK in HR
46 developpement. A similar role for SIPK was also postulated on the basis that the
47 overexpression in tobacco plants of NtMEK2, a MAPK kinase (MAPKK) catalysing the
48 phosphorylation and subsequent activation of different MAPK including SIPK, led to the
49 developpement of spontaneous necrosis [17]. Complementary evidences were obtained by Liu
50 et al [18] and Menke et al [38], who showed that WRKY1, a transcription factor involved in

1 the control of cell death, was *in vitro* phosphorylated by SIPK. However, the conclusions
2 drawn from these experiments require attention. First, a main problem facing these studies
3 is the current lack of drugs capable of selectively acting on one specific PK. Second, the
4 hypothesis that NtMEK2 activates PK distincts from SIPK also involved in HR should not
5 be ruled out. Finally, it should be mentioned that a recent study showed that NbSIPK, the
6 *Nicotiana benthamiana* homologue of SIPK is located in the nucleus [19]. *In vitro* analysis
7 indicated that NbSIPK might be upregulated by the upstream nuclear MAPKK NbMKK1
8 which mediates *Phytophthora infestans* INF1 elicitor-mediated HR and non-host resistance to
9 *Pseudomonas cichorii*. The demonstration that NbSIPK is indeed one of the downstream
10 targets of NbMKK1 and is activated at the nuclear level in this biological model remains to be
11 demonstrated.

12 In the present study, we analyzed the ability of cryptogein to trigger the activation of
13 nuclear PK and partly addressed the question of the identity of these PK. We demonstrated that
14 cryptogein activates a set of different nuclear PK showing distinct kinetics of activation and
15 biochemical properties in term of Ca²⁺-dependence. One of these PK was firmly identified as
16 SIPK. Finally we provided arguments that the activation of SIPK at the nuclear level is
17 correlated to cell death.

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1 MATERIALS AND METHODS

3 Cell culture and treatment

4 *Nicotiana tabacum* cv Xanthi cell suspensions were cultivated as previously described [20].
5 Briefly, cell suspensions were maintained in Chandler's medium [21] on a rotary shaker (150
6 rpm, 25°C) under continuous light (photon flux rate 30–40 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Cells were
7 maintained in the exponential phase and subcultured 1 d prior to utilization. Treatment of cell
8 suspensions was carried out directly in the culture medium.

9 Cryptogein was purified as previously described [22], and prepared as a 100 μM stock
10 solution in water. Oligogalacturonates (degrees of polymerization from 25 to 30) were
11 provided lyophilized by Goëmar S.A. (Roscoff, France) and resuspended in water. All
12 chemicals were purchased from Sigma-Aldrich. Sorbitol, salicylic and butyric acid were
13 prepared in water. Gadolinium chloride (Gd^{3+}) and carboxy-PTIO (cPTIO) were prepared as
14 stock solution in water, except that carboxy-PTIO was dissolved extemporaneously;
15 diphenylene-iodonium (DPI), glibenclamide, and cycloheximide were dissolved in DMSO.
16 Chemicals used as inhibitors were added 10 min before elicitor treatment. When needed,
17 equivalent volumes of DMSO or water were added to control cells to ensure that they did not
18 interfere with the experiments. Final DMSO concentration did not exceed 0.25% (v/v). At
19 various times, 30 to 40-mL cells (typically 8–10 g fresh weight) were harvested by filtration,
20 quickly frozen in nitrogen liquid and stored at -80°C until use.

22 Preparation of nuclear extracts

23 All steps were performed on ice and centrifugations were done at 4°C . Cell samples
24 were ground in liquid nitrogen and thawed in 2 volumes of nucleus extraction buffer (NB1 ;
25 Tris-Mes 25 mM, pH 7.5, hexylene-glycol 0.5 M, EDTA 5 mM, EGTA 5 mM, DTT 10 mM,
26 NaF 10 mM, Na_3VO_4 1 mM, β -glycerophosphate 50 mM, PMSF 1 mM). The suspensions
27 were filtered through a nylon mesh with a porosity of 31 μm (Sefar AG, Heiden), and the
28 filtrate was centrifuged at 500 g for 10 min. An aliquot (500 μL) of the supernatant was
29 centrifuged at 21,000 g for clarification and kept as a “cytosolic fraction”. The initial pellet
30 was resuspended in 10 mL of NB1 and this suspension was layered on top of a 3 mL layer of
31 25% iodixanol (Optiprep via AbCys S.A., Paris) in NB1, and centrifuged at 3,000 g for 30
32 min. The pellet was then washed by centrifugation at 300 g for 10 min in 15 mL of NB2
33 buffer (NB1 without hexylene-glycol). After discarding the supernatant, the pelleted nuclei
34 were frozen in liquid nitrogen and stored at -80°C until use. Nucleus integrity was checked by
35 fluorescent microscopy using a double coloration with propidium iodide and DIOC₆(3) as an
36 endoplasmic reticulum dye. Nuclear-enriched fractions contamination rate was estimated by
37 measuring the activity of marker enzymes [23–25].

39 In-gel kinase activity assays

40 Nuclear proteins analyzed by electrophoresis were extracted using the TriReagent
41 (Molecular Research Center, Inc), following the instructions of the manufacturer. The nuclear
42 proteins were resuspended in an urea buffer (7 M urea, 2 M thiourea, 4 % CHAPS (w/v),
43 10 mM DTT) and diluted with at least 2 volumes of Laemmli buffer for in-gel kinase assays
44 or western blot experiments. In-gel kinase assays were performed as described previously
45 [14] using 20 μg of nuclear proteins. Separating SDS-polyacrylamide gels were embedded
46 with a PK substrate, i.e. histone-III_S (0.14 $\text{mg}\cdot\text{mL}^{-1}$) or MBP (0.12 $\text{mg}\cdot\text{mL}^{-1}$). Once the
47 phosphorylation reaction was performed, the gels were dried onto Whatmann 3MM paper and
48 exposed to Kodak XAR-5 films. Prestained size markers (Fermentas) were used to estimate
49 the apparent molecular mass of the PK.

1 Immunoblot Analysis

2 Nuclear and cytosolic proteins from control and cryptogein-treated cells were
3 submitted to 10 % SDS-PAGE and transferred to nitrocellulose membrane. The membrane
4 was blocked for 2 hrs at room temperature in TBST buffer (10 mM Tris, pH 7.5, 100 mM
5 NaCl, and 0.1% Tween 20) containing 1% bovine serum albumin (BSA), and then incubated
6 overnight at 4°C in the same buffer containing the primary antibodies anti-SIPK raised
7 against the specific SIPK N-terminal peptide [14]. After incubation and several washings with
8 TBST, the membrane was then incubated with the appropriate horseradish peroxidase-
9 conjugated secondary antibodies (Bio-Rad). Detection was carried out using an ECL Western
10 detection kit (Cell Signaling).

11 Immunoprecipitation

12 Nuclear proteins from untreated and cryptogein-treated cells were extracted by adding 2
13 volumes of lysis buffer (NB1 supplemented with 0.5 M NaCl, 0.5 MgCl₂) to the nuclei. After
14 ultracentrifugation (100 000 g, 1 h), immunoprecipitation using the nuclear proteins contained
15 in the supernatant was carried out as previously described [26]. Immunoprecipitated proteins
16 were analyzed by in-gel kinase assays using histone H1S as substrate, in the presence of Ca²⁺.
17

18 Purification of PK47 and mass spectrometry sequencing

19 Nuclei obtained from 8 kg of tobacco cells were resuspended in 1 L of lysis buffer.
20 After 60 min on ice, the suspension was ultracentrifuged at 100,000 g for 1 hr at 4°C. Using a
21 30-kDa cut-off centricon, the extracted nuclear proteins were concentrated in the Q buffer
22 (Tris-HCl 25 mM, EDTA 2 mM, EGTA 2 mM, β-glycerophosphate 50 mM, MgCl₂ 25 mM,
23 DTT 2 mM, glycerol 5 % (v/v), PMSF 1 mM) and were loaded onto a 5-mL Q-Sepharose
24 anion exchange column (HiTrap Q HP column, Amersham BioSciences) equilibrated with the
25 same Q buffer. After washing with 25 mL of buffer Q, the proteins were eluted with a 40-mL
26 linear gradient of 0 to 700 mM NaCl in buffer Q. The PK47 activity eluted with about 300
27 mM NaCl.
28

29 The fractions containing the highest PK47 activity, as determined by in-gel kinase
30 assay using histone-H1S as a substrate, were pooled and concentrated in buffer Q with 500
31 mM NaCl and loaded onto a 1-mL hydrophobic interaction column (HiTrap phenyl-sepharose
32 HP column, Amersham BioSciences) equilibrated in the same buffer. Bound proteins were
33 eluted with a 20-mL linear gradient of 0 to 60 % ethylene-glycol and 500 to 0 mM NaCl in
34 buffer Q. The PK47 active fractions (still determined using in-gel kinase assay with H1S as
35 substrate) were then pooled and concentrated in buffer IP (HEPES-KOH 50 mM, pH 7.5,
36 EDTA 5 mM, EGTA 5 mM, Na₃VO₄ 1 mM, NaF 10 mM, β-glycerophosphate 50 mM,
37 glycerol 5 % (v/v), PMSF 1 mM). The proteins were then incubated with 20 μg of antibodies
38 directed against SIPK and 85 μL of packed volume of protein A-agarose beads (Sigma-
39 Aldrich), at 4°C for 12 h on a wheel. After washes, immunocomplexes were recovered in 30
40 μL of buffer IP added with 10 μL of 5-fold concentrated Laemmli sample buffer, and were
41 analyzed by 1D-electrophoresis. The gel was stained using a mass spectrometry-compatible
42 silver staining kit (Dodeca Silver Stain Kit, Bio-Rad). After 1D-electrophoresis, the PK47
43 corresponding band was excised and protein was characterized after trypsin in-gel digestion
44 as previously described [27]. The trypsin digest was separated and analysed by nanoLC-
45 MS/MS using an Ultimate 3000 system (Dionex, Amsterdam, the Netherlands) coupled to an
46 LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany).
47

48 Cell death

1 Cell death was estimated as previously described [28] by using neutral red as a vital
2 dye that accumulates in the acidic vacuole. Cells that did not accumulate neutral red were
3 considered dead. Five hundred cells were counted for each treatment.
4
5

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1 RESULTS

2 3 Cryptogein induces the activation of a set of nuclear protein kinases

4 In order to investigate cryptogein-activated nuclear PK, we first set up an optimized
5 protocol for the purification of nuclei from cultured tobacco cells. The extent of
6 contamination by other cellular compartments including the cytosol, the endoplasmic
7 reticulum, the Golgi apparatus and peroxysomes was followed by marker enzyme analysis.
8 All of the marker enzymes assayed were poorly detectable in the nuclear preparation, the
9 contamination rate being below 1% for each enzyme (Supplemental data 1). The integrity of
10 the nuclei was ensured by the use of a detergent-free protocol [29] and controlled by the
11 staining of enriched nuclei with DiOC6(3), an ER dye (data not shown). However, it should
12 be specified that a significant contamination by cell wall fragments was observed
13 (Supplemental data 2) and confirmed by ruthenium red and phloroglucinol staining of the
14 nuclear preparations. But changes in buffer conditions, ionic strength or pH value did not
15 significantly modify it. However the use of tobacco protoplasts as starting material was not
16 conceivable since cryptogein does not induce any known signal transduction events
17 (unpublished data).

18 To examine the putative involvement of nuclear PK in cryptogein signaling, tobacco
19 cell suspensions were treated for various times with 100 nM cryptogein and the corresponding
20 nuclear protein extracts were analysed by in gel kinase assay using histone IIIS as a general
21 phosphorylation substrate in presence of 0.5 mM Ca^{2+} . As shown in Fig. 1A, several nuclear
22 PK with molecular masses of 36, 55 and 60 kDa (named PK36, PK55 and PK60, respectively)
23 were constitutively activated in control cells. In nuclear protein extracts prepared from
24 cryptogein-treated tobacco cells, 3 additional PK exhibiting a molecular mass of 42, 47 and
25 80 kDa (named PK42, PK47 and PK80) were activated. Furthermore, the activity of the
26 PK36, or of another PK with the same apparent molecular mass, was significantly increased
27 following cryptogein treatment. A fifth PK with a molecular mass of 75 kDa also showed a
28 rise of activity in response to the elicitor. However, in contrast to PK36, PK42, PK47 and
29 PK80, its activation was not systematically observed, as well as the enhanced activity of PK
30 with the same molecular mass than the PK55 and PK60, detected in cryptogein-elicited cells.

31 In response to cryptogein, PK36, PK42, PK47 and PK80 displayed distinct kinetics of
32 activation. PK36 activation was detected after 30 min of cryptogein treatment and lasted for
33 12 hrs; PK42 was slightly activated after 3 to 6 hrs, depending on the experiments, peaked at
34 9 hrs and remained detectable up to 12 hrs of treatment; PK47 was activated within 5 to 10
35 min following cryptogein application, and showed a high level of activity for at least 12 hrs;
36 PK80 was activated after 30 min of cryptogein treatment and peaked at 3 hrs before returning
37 to basal level within 9 hrs (Fig. 1A).

38 To further characterize the biochemical features of the cryptogein-activated nuclear
39 PK, their Ca^{2+} -dependence was assessed. For this purpose, nuclear proteins were analyzed by
40 in-gel kinase assay using histone IIIS as substrate (a preferred one for CDPK) but without
41 Ca^{2+} in the phosphorylation buffer (Fig. 1B). These conditions allow the detection of strictly
42 Ca^{2+} -independent PK activities. In the absence of Ca^{2+} , only the activities of PK36, PK47 and
43 PK60 were still detected. In contrast, the elicitor-triggered activation of PK42 and PK80 as
44 well as the constitutive activity of PK55 were completely suppressed, highlighting the
45 requirement of Ca^{2+} for their full activity. To confirm that these PK activities were not due in
46 part to cell wall contaminations, nuclear proteins were prepared after lysis of the nuclei by a
47 high salinity buffer and ultracentrifugation to remove DNA, nuclear envelopes and cell wall
48 fragments. In these conditions we obtained the same PKs pattern (Fig. 1C), indicating that all
49 PK activities described previously correspond to nuclear proteins.

1 Taken together, these data highlight the ability of cryptogein to promote the activation
2 of several nuclear PK, displaying distinct kinetics of activation and Ca^{2+} -dependence.

4 **Cryptogein activates a nuclear PK related to MAP kinases**

5 Amongst Ca^{2+} -independent PK, MAPKs are the most studied PK involved in
6 signalling pathway leading to defense responses. Based on the findings that cryptogein
7 induces the activation of cytosolic MAPKs including WIPK, SIPK and Ntf4 [12-14] and that
8 MAPKs are translocated into the nucleus upon activation in various mammalian transduction
9 processes [30], we investigated whether some of the cryptogein-induced nuclear PK could
10 correspond to MAPKs. Therefore, nuclear proteins were analyzed by in gel kinase assay using
11 myelin basic protein (MBP), a preferential phosphorylation substrate compared to histone
12 IIIS, in the absence of Ca^{2+} to favour the detection of Ca^{2+} -independent MAPK activities [31].
13 As shown in Fig. 2, only the PK36, PK47 and PK60 were able to phosphorylate MBP, the
14 PK47 and PK60 activities being more intense than those observed using histone IIIS as a
15 substrate.

16 The PK47 displayed the same apparent molecular mass than SIPK [14]. We therefore
17 investigated whether the PK47 activity could correspond to SIPK, or to a closely related PK.
18 For this purpose, nuclear protein extracts from untreated and cryptogein-treated tobacco cell
19 suspensions were immunodetected using a polyclonal antibody raised against SIPK [14]. As a
20 control, the same experiment was carried out using cytosolic extracts. As expected,
21 immunostaining revealed a reactive polypeptide of 47 kDa in the cytosolic protein fractions,
22 corresponding to SIPK (Fig. 3A). The immuno-reactive band was observed in both the control
23 and cryptogein-treated cytosolic extracts, indicating that SIPK is constitutively produced as
24 previously reported [13]. In the nuclear extracts, the antibodies detected a polypeptide with an
25 apparent molecular mass of 47 kDa, suggesting that SIPK could be also present in the nuclei
26 from both control and cryptogein-treated cells. However, we were unable to detect any
27 significant accumulation of SIPK in the nucleus that could have suggested a translocation in
28 response to cryptogein treatment. Intriguingly, in the nuclear fractions, besides the 47-kDa
29 polypeptide, a 45-kDa polypeptide was also detected, this last one showing the most reactive
30 signal, but the immuno-detection of the P45 was not reproducible.

31 To determine if the 47-kDa polypeptide carries the PK47 activity, nuclear extracts
32 from untreated and cryptogein-treated cells were immunoprecipitated with specific anti-SIPK
33 polyclonal antibodies, and the resulting immunocomplexes were analyzed with the in-gel
34 kinase assay technique using histone IIIS as a substrate and in the presence of Ca^{2+} . These
35 conditions were used because they allowed the detection of a higher number of nuclear PK
36 activities as compared to MBP in the absence of Ca^{2+} (Fig. 1 versus Fig. 2). As shown in Fig.
37 3B, only the PK47 activity was clearly immunoprecipitated from the nuclear extracts of
38 cryptogein-treated cells. No signal could be detected at 45 kDa, avoiding us to conclude on its
39 putative activity, especially to determine whether it could correspond to an inactive form of
40 SIPK. Work is in progress to identify P45. Therefore, this data strongly suggests that the
41 nuclear PK47 corresponds to SIPK and although it is present both in control and cryptogein-
42 treated cells, its activity is only observed in response to cryptogein treatment.

44 **Purification and identification of SIPK from nuclear protein-enriched extracts**

45 To confirm the assumption that the PK47 corresponds to SIPK, we undertook its
46 purification from 70 mg of nuclear proteins corresponding approximately to 8 kg of
47 cryptogein-treated tobacco cells. The nuclear proteins were first loaded on a HiTrap Q anionic
48 exchange column and each collected fraction was analyzed by in-gel kinase assay. As shown
49 in Fig. 4A, several nuclear PK showing an induced activity in response to cryptogein, mainly
50 the PK50-60, were eluted within the flowthrough. Other PK of interest, including the PK47,

1 were retained by the column and subsequently eluted by increasing NaCl concentrations. The
2 fractions containing the PK47 activity were pooled, loaded on a HiTrap Phenyl hydrophobic
3 column and subsequently eluted with increasing concentrations of ethylene glycol and
4 decreasing concentrations of NaCl. The hydrophobic chromatography did not permit an
5 efficient fractionation of the nuclear PK activities as most of them were collected both in the
6 flowthrough and the eluted fractions (Fig. 4B). In contrast, activity of the P47 was specifically
7 detected in eluted fractions. The corresponding fractions were pooled, desalted, concentrated
8 and finally immunoprecipitated using the specific anti-SIPK polyclonal antibodies. The
9 immunoreactive polypeptides were loaded on a preparative SDS-PAGE gel and the band
10 corresponding to PK47 was excised for microsequencing (supplemental data 3).

11 The LC-MS/MS analysis of the tryptic digest from the immunopurified protein
12 allowed the sequencing of four internal tryptic peptides, which unambiguously identified the
13 PK47 protein as SIPK (Fig. 5). The predicted molecular mass of SIPK is 45.1 kDa and
14 calculated pI 5.53. Three of the sequenced peptides are shared with Ntf4, but the fourth ruled
15 out the possibility that the purified PK47 could correspond to Ntf4. The MS/MS identification
16 of SIPK from nuclear extracts confirmed the data obtained in immunoblot and
17 immunoprecipitation experiments (Fig. 3), and demonstrated that SIPK is present and active
18 in response to cryptogein in the nuclei of treated tobacco cells.

19 20 **Characterisation of the nuclear SIPK activation**

21 The cryptogein-induced signaling events acting upstream of cytosolic SIPK activation
22 have been investigated in several studies. It was reported that SIPK activation is strictly
23 dependent on the elicitor-triggered influx of extracellular Ca^{2+} [14]. In contrast, anion
24 effluxes, NADPH oxidase-derived ROS and NO did not appear as essential components of the
25 cryptogein-induced signaling pathway leading to the activation of SIPK [32]. To assess the
26 involvement of signalling events in transmitting the cryptogein signal contributing to the
27 activation of the nuclear SIPK, a pharmacological approach was performed.

28 The possible influence of the cryptogein-induced Ca^{2+} influx was investigated by
29 blocking plasma membrane Ca^{2+} permeable channels using Gd^{3+} . This compound has been
30 shown to block the entrance of Ca^{2+} and subsequent Ca^{2+} -dependent events in cryptogein-
31 treated cells [33]. As shown in Fig. 6, Gd^{3+} completely suppressed nuclear SIPK activation.
32 To determine a putative link between nuclear SIPK activation and cryptogein-induced anion
33 effluxes, ROS and NO production, we next examined the effects of the plasma membrane
34 anion channel inhibitors glibenclamide and niflumic acid (data not shown) [16], the NADPH
35 oxidase inhibitor DPI [20] and the NO scavenger cPTIO [33]. These compounds had no
36 inhibitory effects on the elicitor-triggered nuclear SIPK activation. Finally, the effect of
37 cycloheximide, a widely-used inhibitor of protein synthesis, was also tested. Here too,
38 cycloheximide did not impair the activation of the nuclear SIPK induced by cryptogein in
39 cultured tobacco cells, suggesting that nuclear SIPK activation did not require prior *de novo*
40 synthesis of SIPK itself or of other proteins acting upstream of its activation. This data fits
41 well with those reported in the immunoblot (Fig. 3A) in which the amount of nuclear SIPK
42 was comparable in both control and cryptogein-treated cells.

43 Together, these results indicate that SIPK is first regulated at the post-translational
44 level in the cytosol and the nucleus and that similarly to its cytosolic counterpart, the
45 activation of the nuclear SIPK is strictly dependent on the elicitor-trigger Ca^{2+} influx but
46 independent of anion effluxes, ROS and NO production.

47 48 **Activation of SIPK by various stimuli**

49 Various stimuli, including biotic and abiotic stresses, activate cytosolic MAPK
50 including SIPK [34]. However to our knowledge, no study analysed whether a cytosolic

1 MAPK activation is paralleled by its activated form in the nucleus. We therefore investigated
2 the ability of stimuli known to trigger the activation of SIPK in the cytosol to promote an
3 active SIPK in the nucleus. We tested as stimuli (i) oligogalacturonates (OGs), non-necrotic
4 elicitors of tobacco defence reactions [14]; (ii) salicylic acid (SA, [35]); (iii) butyric acid (BA,
5 [26]); and (iv) hyperosmotic shock triggered by sorbitol [36]. As previously reported (see
6 above references), all these compounds provoked SIPK activation in the cytosolic extracts,
7 but with different kinetics (Fig. 7). OGs triggered a transient activation of SIPK, peaking at 10
8 min and decreasing to basal levels within 60 min. Sorbitol also produced a very transient
9 activation of SIPK, which peaked at 5 min and disappeared within 30 min. Similar data were
10 obtained in response to SA but with a more extended kinetics. When used at 1 mM, BA used
11 to acidify the cytosol [26] induced a slower activation of SIPK, occurring after 30 min of
12 treatments and lasting for at least 2 hrs. Higher concentrations of BA (that is 5 mM) induced a
13 faster, stronger and long-lasting activation which occurred within 5 min and was maintained
14 for at least 2 hrs.

15 When the nuclear activity of SIPK was assessed, its activation was detected only in
16 response to 5 mM BA (Fig. 7). In this condition, a slight activation occurred within 10 min.
17 The activation became stronger after 60 min where it reached a level of activation similar to
18 those observed in response to cryptogein. Interestingly, in contrast to 250 mM sorbitol, 1 mM
19 BA, SA and OGs, only cryptogein and 5 mM BA triggered a significant cell death within 24
20 hrs, reaching 35 % and 100 %, respectively.

21 Taken as a whole, these data indicate that the presence of the activated SIPK in the
22 nucleus depends on the applied stimuli and is promoted by cell death-triggering signals.
23 Furthermore, they suggest that the cytosolic activation of SIPK is not systematically
24 paralleled by the presence of its activity in the nucleus, confirming that detection of active
25 SIPK in the nucleus is not due to cytosolic contamination or mechanical translocation when
26 cells are harvested.
27

1 DISCUSSION

2 The possibility that nuclear PK could be specifically activated in plant cells challenged
3 by biotic stresses has been suggested but not firmly established [8, 37, 38]. The data presented
4 in this report support for the first time the conclusion that biotic stresses promote the
5 activation of nuclear PK. Indeed, compared to control cells, at least four PK, that is PK36,
6 PK42, PK47, and PK80, showed increased PK activities in nuclear protein extracts prepared
7 from cryptogein-treated tobacco cells. Each PK was activated in a specific time-dependent
8 manner, varying from min in the case of PK47 to several hours in the case of PK42. Several
9 lines of evidence indicate that these activities did not reflect a contamination by cytosolic PK.
10 G6PDH activity, a marker of the cytosolic and plastidic compartments [39], was extremely
11 low in the nuclear fraction. An ambiguous situation came from the analysis of the activity of
12 SIPK which was similarly detected in response to cryptogein treatment in both the cytosol and
13 the nucleus. However, the observation that activated SIPK was specifically observed in
14 cytosolic but not in nuclear extracts prepared from tobacco cells exposed to OGs,
15 hyperosmotic stress, SA or 1 mM BA provided further arguments ruling out the hypothesis
16 that SIPK activity in the nuclear extracts could result from a cytosolic contamination.

17 The nuclear PK activated in response to cryptogein showed distinct biochemical
18 features. PK activities of the PK50-55 and PK80 were strictly Ca^{2+} -dependent, suggesting that
19 those were probably due to CDPK or CCaMK [40]. Several representatives of these families
20 have already been found to be located in the nuclear compartment. A study investigating the
21 subcellular localization of several *A. thaliana* CDPK isoforms fused to GFP showed that at
22 least two of them were constitutively nuclear [4]. A well-known CCaMK, DMI3, involved in
23 nodulation in *Medicago truncatula*, also exhibits a nuclear localization when fused to GFP
24 [41]. With the exception of nuclear CCaMK-like activities in response to temperature stress
25 [42], the activities of such PK in the nuclear compartment has not yet been reported.

26 PK47 displayed features of MAPK including a comparable molecular mass and an
27 efficiently phosphorylation activity toward MBP in a Ca^{2+} -independent manner. Supporting
28 this data, through combining immunological and biochemical purification approaches, we
29 identified PK47 as the MAPK SIPK, providing the first evidence that MAPK are active
30 within the nucleus of plant cells challenged with a biotic stress. Experiments with plasma
31 membrane Ca^{2+} channel inhibitors indicate that Ca^{2+} influx from the extracellular space is
32 required for nuclear SIPK activation in cryptogein-treated cells. In contrast, neither anion
33 effluxes nor ROS and NO production appeared to be necessary for nuclear SIPK activation.
34 Interestingly, such regulation resembles those already described for cytosolic SIPK [14, 16,
35 26]. By using various stimuli known to activate SIPK, our results also clearly demonstrated
36 that the cytosolic activation of SIPK is a prerequisite but is not sufficient to induce the activity
37 of the nuclear SIPK.

38 Taken together, these findings raise the question of whether SIPK nuclear activity
39 results from the translocation of the activated cytosolic enzyme from the cytosol to the
40 nucleus or whether it results from the direct activation of the nucleus enzyme. Results from
41 the western blot analysis performed using antibodies raised against SIPK indicated that this
42 PK is expressed both in the cytosol and in the nucleus of control and cryptogein-treated
43 tobacco cells. Accordingly, using a chimeric SIPK-GFP construct under the control of the
44 cauliflower mosaic virus 35S promoter, Menke et al [38] showed that SIPK is present both in
45 the cytosol and the nucleus of tobacco BY2 cells. Similar results were obtained in *Nicotiana*
46 *benthamiana* cells overexpressing a NbSIPK-GFP construct [19], but it was also shown in this
47 case that a constitutively nuclear MAPKK, NbMKK1, interacts with and could be responsible
48 for the nuclear activation of NbSIPK. However, both studies did not provide evidence that
49 SIPK could be active at the nuclear level and/or translocated from the cytosol. Two
50 hypothesis are conceivable to explain the activation of a nuclear localized SIPK: (i) SIPK is

1 phosphorylated and activated in the cytosol by a MAPKK, presumably NtMEK2 [43], and
2 then translocated into the nucleus; (ii) SIPK could also be phosphorylated at the nuclear level
3 by a nuclear MAPKK, as suggested for NbMKK1, a nuclear MAPKK able to phosphorylate
4 in vitro NbSIPK [19]. In this case how his MAPKK could be activated in the nucleus remains
5 undetermined. More generally, few studies have underlined the possibility that cytosolic
6 MAPK could be translocated to the nucleus. For instance, in parsley cell suspensions, it was
7 shown by immunolabelling that two MAPKs, PcMPK3 and PcMPK6 are translocated to the
8 nucleus upon stimulation by the elicitor Pep13 [8]. Similarly, two *A. thaliana* MAPKs,
9 AtMPK3 and AtMPK6, were shown to be translocated from the cytosol to the nucleus upon
10 ozone treatment [37]. Here too, the demonstration that these MAPK are active in the nucleus
11 has not been provided.

12 Although several studies point to the presence of plant MAPKs in the nucleus, their
13 roles in this compartment have been poorly investigated to date. Indeed few substrates of
14 these PK have been identified. Amongst those, WRKY1, the only known substrate for SIPK
15 to date, was shown to be implicated in HR-like cell death in association with SIPK [38].
16 Similarly, Cheong et al [44] found that BWMK1, a rice nuclear MAPK, could activate by
17 phosphorylation the transcription factor OsEREBP1 which could lead to *PR*-genes activation.
18 More generally several transcription factors were evidenced to act downstream of MAPKs
19 [45-49], accrediting the fact that the nuclear activity of SIPK we showed is related to the
20 regulation of transcription. It is then of major importance to identify the putative nuclear
21 targets of SIPK and the associated plant genes.

22 In summary, this work greatly extends previous studies describing the involvement of
23 PK in plant defence by providing the first evidence of a functional nuclear MAPK activity
24 involved in response to an elicitor treatment. Work in progress aims at investigating whether
25 SIPK is directly activated within the nucleus or whether it is translocated from the cytosol to
26 the nucleus in its activated form.

27

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1 **Figure legend:**
2

3 **Figure 1: Cryptogein activates several nuclear protein kinase activities.**

4 Tobacco cells (*Nicotiana tabacum* cv. Xanthi) were treated with 100 nM cryptogein for
5 various times. The corresponding nuclear extracts were prepared and PK activities were
6 analyzed by in-gel kinase assay with histone IIIS as a substrate. The phosphorylation reaction
7 was performed in the presence of 0.5 mM CaCl₂ (A) or without Ca²⁺ in the medium (2 mM
8 EGTA) (B); protein kinase patterns from nuclear proteins extracted using the TriReagent
9 (lane 1) or the high salinity buffer (lane 2) protocols (C). The arrow on the right indicates the
10 different PK activities. These experiments were repeated five times with similar results.
11

12 **Figure 2: Cryptogein activates a nuclear protein kinase related to MAP kinases.**

13 Tobacco (*Nicotiana tabacum* cv. Xanthi) cells were treated with 100 nM cryptogein for
14 various times. The corresponding nuclear extracts were prepared and PK activities were
15 analyzed by in-gel kinase assay with MBP as a substrate, a preferred one for MAP kinase.
16 The phosphorylation reaction was performed without Ca²⁺ in the medium (2 mM EGTA).
17 This experiment is representative of three independent ones.
18

19 **Figure 3: The nuclear PK47 is a protein kinase closely related to SIPK.**

20 (A) Tobacco cells were treated with 100 nM cryptogein for the indicated times. Cytosolic and
21 nuclear extracts were prepared from harvested cells and proteins were separated on the same
22 10 % polyacrylamide gel. After transfer on nitrocellulose membrane, the immunoblot was
23 performed using antibodies directed against the N-terminal part of SIPK. The black arrow
24 indicates the band corresponding to SIPK. Molecular mass markers are indicated on the left.
25 (B) Nuclear extracts containing 100 µg proteins from untreated control (Cont.) or cells treated
26 30 min with 100 nM cryptogein (Cry) were immunoprecipitated with 5 µg antibodies directed
27 against SIPK. Kinase activity of the immunocomplexes were then determined by in-gel kinase
28 assay using histone IIIS as a substrate, in presence of 0.5 mM CaCl₂ in the reaction buffer.
29 The PK47-corresponding activity is indicated on the right. Molecular mass markers are
30 indicated on the left.
31

32 **Figure 4: Enrichment of the nuclear PK47**

33 Seventy milligrams of nuclear proteins extracted from tobacco cells treated with 100 nM
34 cryptogein for 3 hrs were used to enrich the PK47 activity through 2 chromatography steps.
35 (A) Each fraction obtained after an anion exchange chromatography was analyzed by in-gel
36 kinase assay using histone IIIS as a substrate, in the presence of 0.5 mM CaCl₂. The arrow
37 indicates the activity corresponding to PK47. Molecular weights are indicated on the left. (B)
38 Each fraction after the hydrophobic chromatography was analyzed by in-gel kinase assay
39 using histone IIIS as a substrate, in the presence of 0.5 mM CaCl₂. The arrow indicates the
40 activity corresponding to PK47. Molecular weights are indicated on the left.
41

42 **Figure 5: Identification of PK47 as SIPK.**

43 SIPK and Ntf4 aminoacid sequences were aligned. For Ntf4, only divergent aminoacids are
44 indicated. The sequences of the four tryptic peptides obtained by mass spectrometry are
45 indicated by the black lines over SIPK sequence. The aminoacid of the fourth microsequenced
46 peptide highlighted in grey discriminate SIPK from Ntf4. Roman numerals indicate the 11
47 major conserved subdomains of serine/threonine protein kinases. Dotted line in subdomain VI
48 indicates the catalytic site, the conserved residues essential for catalysis are marked with a
49 black dot. The phosphorylated residues in the MAPK activation motif TEY are indicated with
50 the asterisks.

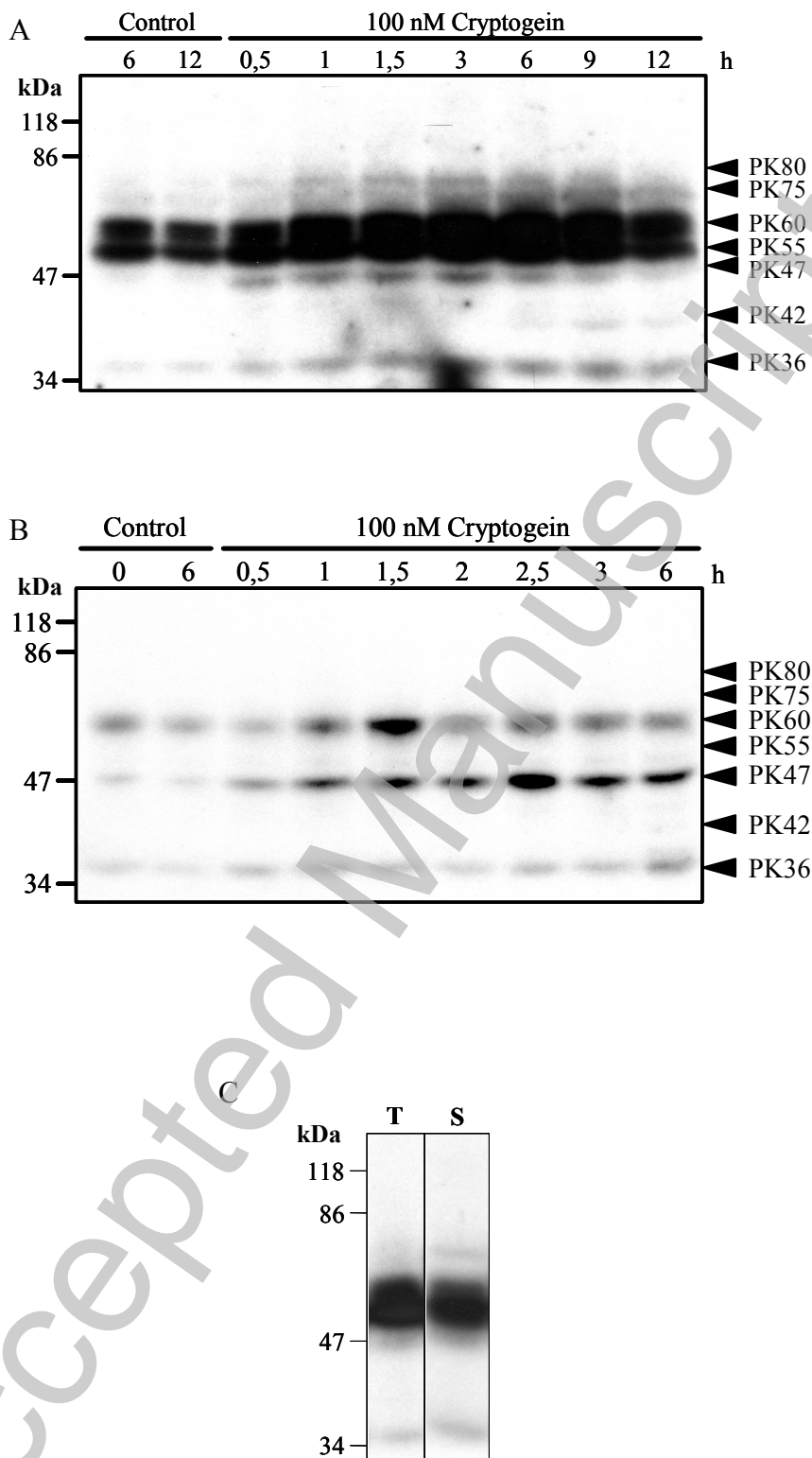
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2 **Figure 6: Integration of SIPK nuclear activity in cryptogein signalling pathway.**
3 (A) SIPK activity was first estimated in response to cryptogein alone. Control (Cont.) and
4 treated cells (Cry) were harvested at the indicated times and SIPK activity was analyzed in
5 nuclear extracts by in-gel kinase assay using MBP as a substrate in absence of Ca^{2+} (2 mM
6 EGTA in the reaction buffer).

7 (B) Control or cryptogein-treated (Cry) cells were pre-treated 10 min with different inhibitors:
8 2 mM gadolinium chloride (Gd^{3+}), 10 μM diphenylene-iodonium (DPI), 200 μM
9 glibenclamide (Gli), 500 μM carboxy-PTIO (cPTIO) and 10 μM cycloheximide (CHX). Cells
10 were harvested at the indicated times, and SIPK activity was analyzed by in-gel kinase assay
11 using MBP as a substrate in absence of Ca^{2+} (2 mM EGTA in the reaction buffer).
12

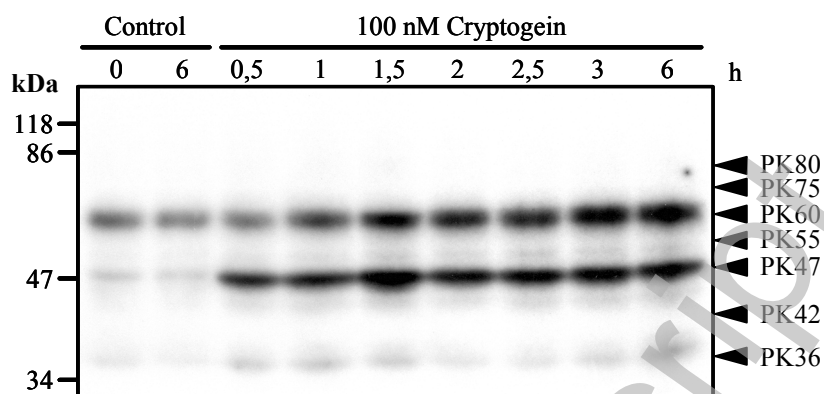
13 **Figure 7: Activity of SIPK in the cytosol and nucleus in response to different stimuli.**

14 Different treatments, known to activate SIPK in the cytosol, were applied to cells: 100 $\mu\text{g}\cdot\text{mL}^{-1}$
15 oligogalacturonates (OGs), 250 μM salicylic acid (SA), 1 and 5 mM butyric acid and
16 250 mM sorbitol. Control and treated cells were harvested at various times, and cytosolic
17 (Cyt) and corresponding nuclear (Nuc) extracts were prepared. In-gel kinase assays were
18 performed to analyze SIPK activity in these extracts, with MBP as a substrate in absence of
19 Ca^{2+} (2 mM EGTA in the reaction buffer).

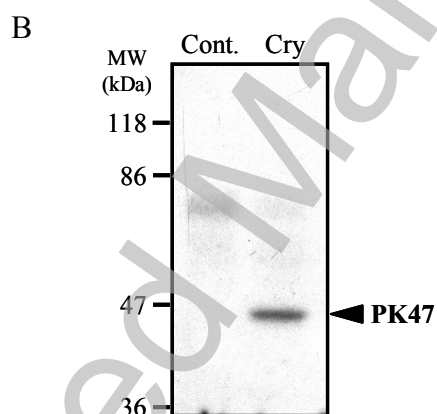
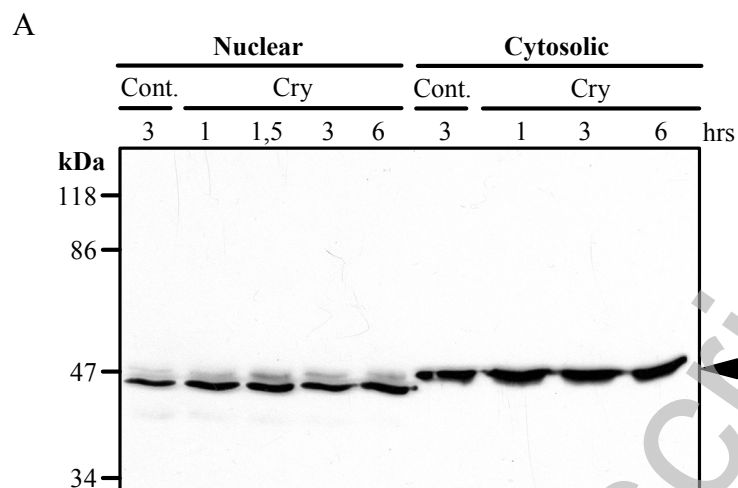
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Dahan et al - Figure 1

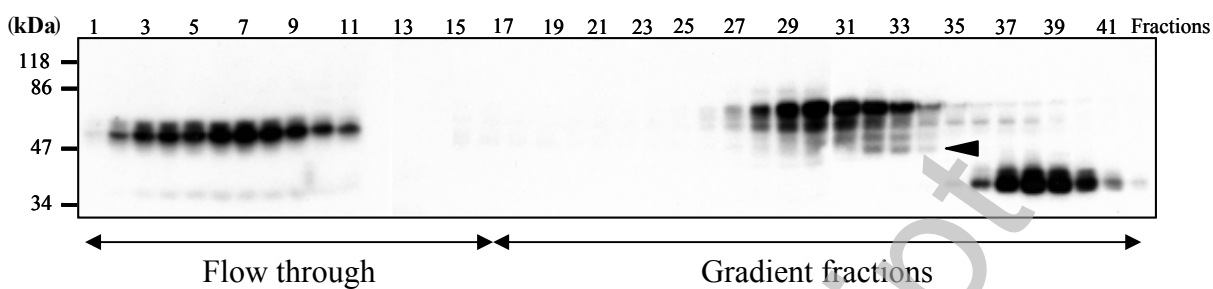


Dahan et al - Figure 2

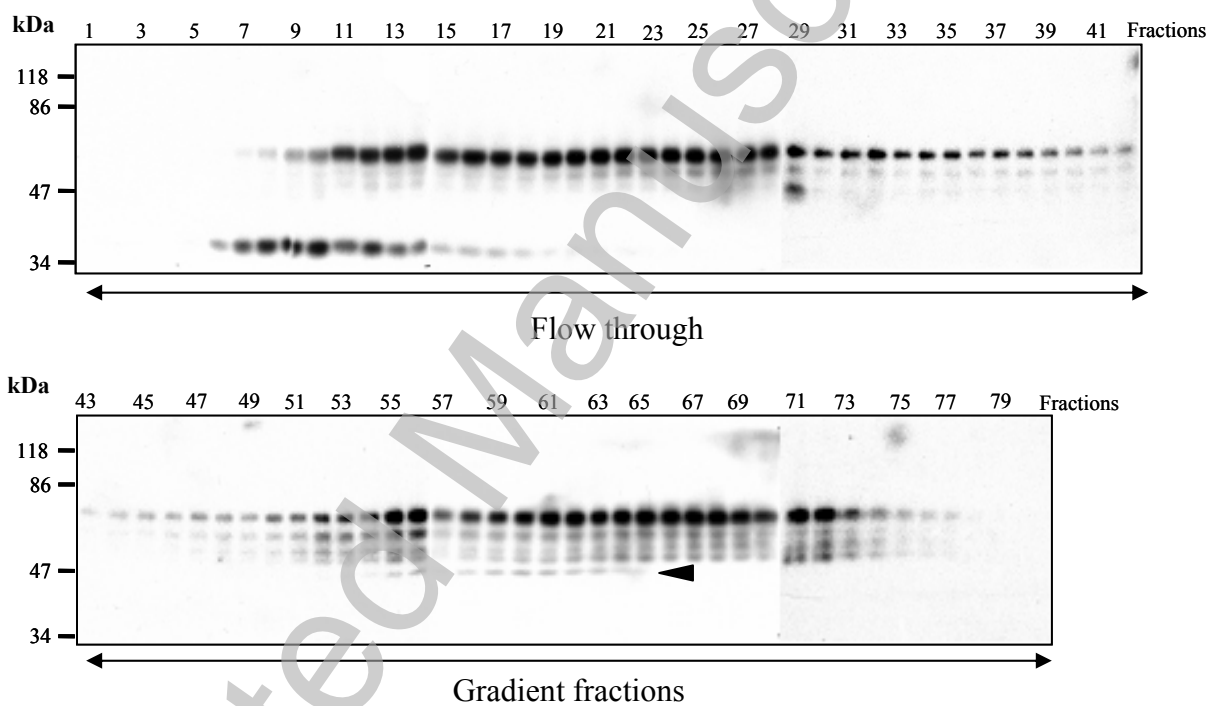


Dahan et al - Figure 3

A



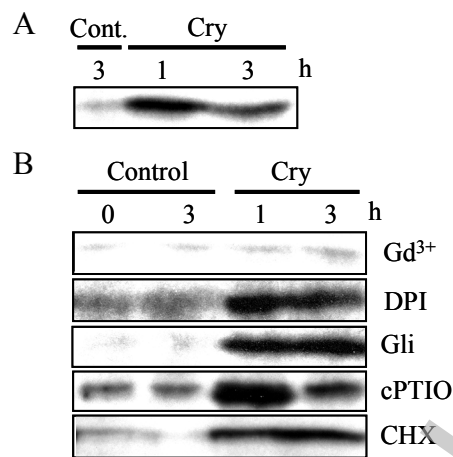
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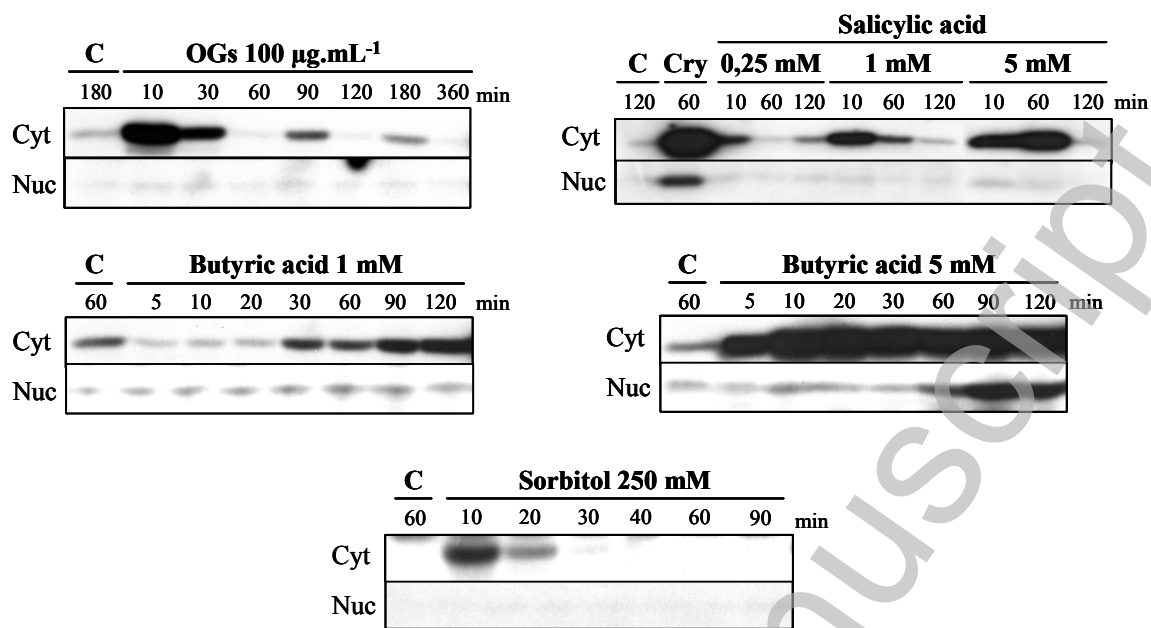
Dahan et al - Figure 4

NtSIPK	MDGSGQQTDT	MMSDAGAEQP	PTAPQPVAGM	DNIPATLSHG	GRFIQYNIFG	NIFEVTAKYK	PPILPIGKGA	[70]
Ntf4	---PAH---	V---AGQ--	APPS-----I	-----	-----	-----	---M-----	[70]
							I	
NtSIPK	YGIVCSALNS	ETIENVAIKK	IANAFDNKID	AKRTLREIKL	LRHMDHENIV	AIRDIIPPPQ	REAFNDVYIA	[140]
Ntf4	-----	--N-H-	-----	-----	-----	-----	-----	[140]
		II		III		IV		
NtSIPK	YELMDTDLHQ	IIRSNQGLSE	EHCQYFLYQI	LRGLKYIHSA	NVLHRDLKPS	NLLLNANCDL	KICDFGLARV	[210]
Ntf4	-----	-----	-----	-----	-----	-----	-----	[210]
	V				VI		VII	
NtSIPK	TSETDFMTEY	VVTRWYRPE	LLLNSSDYTA	AIDVWSVGC	FMELMDRKPL	FPGRDHVHQL	RLIMELIGTP	[280]
Ntf4	-----	-A-	-----	-----	-----	-----	-L-	[280]
		VIII		IX			X	
NtSIPK	SEAEMEFLNE	NAKRYIRQLP	LYRRQSFTK	FPHVHPTAID	LVEKMLTFDP	RRRITVEGAL	AHPYLNSLHD	[350]
Ntf4	-----	-----	-----V-	-----N-A-	-----	-----D-	-----T-	[350]
					XI			
NtSIPK	ISDEPICMTP	FSFDPEQHAL	TEEQMKELIY	RESLAFNPEY	QHM			[393]
Ntf4	-----V-	-N-----	-----	-----G-	-----			[393]

Dahan et al – Figure 5



Dahan et al - Figure 6



Dahan et al - Figure 7