FEBS Letters 581 (2007) 4205–4211

A role for phospholipase A in auxin-regulated gene expression

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Received 25 April 2007; revised 8 June 2007; accepted 11 July 2007

Available online 3 August 2007

Edited by Michael R. Sussman

Abstract Auxin increases phospholipase A_2 activity within 2 min (Paul, R., Holk, A. and Scherer, G.F.E. (1998) Fatty acids and lysophospholipids as potential second messengers in auxin action. Rapid activation of phospholipase A_2 activity by auxin in suspension-cultured parsley and soybean cells. Plant J. 16, 601–611) and the phospholipase A inhibitors, ETYA and HELSS, inhibit elongation growth of etiolated Arabidopsis hypoctyls (Holk, A., Rietz, S., Zahn, M., Quader, H. and Scherer, G.F.E. (2002) Molecular identification of cytosolic, patatin-related phospholipases A from *Arabidopsis* with potential functions in plant signal transduction. Plant Physiol. 130, 90–101). To identify the mode of action, rapid auxin-regulated gene expression was tested for sensitivity to these PLA_2 inhibitors using seedlings expressing β-glucuronidase (GUS) under the control of the synthetic auxin-responsive promoter DR5. ETYA and HELSS inhibited the auxin-induced increases in GUS activity, the steady-state level of the corresponding GUS mRNA and the mRNAs encoded by four other auxin-induced genes, IAA1, IAA5, IAA19 and ARF19. Factors that bind to the auxin response elements of the DR5 promoter and thereby regulate gene expression are regulated by a set of proteins such as Aux/IAA1 whose abundances are, in part, under control of E3 ubiquitin ligase SCF complexes. To investigate this mechanism further, the effect of ETYA on Aux/IAA1 degradation rate was examined using seedlings expressing Aux/IAA1:luciferase fusion proteins. In the presence of cycloheximide and excluding synthesis of IAA1:luciferase, ETYA had no apparent effect on degradation rates of IAA1, either with or without exogenous auxin. Therefore, the E3 ubiquitin ligase SCF^{TIR1} complex is an unlikely direct target of the PLA inhibitor. When cycloheximide was omitted, however, the inhibitors ETYA and HELSS blocked a sustained auxin-induced decrease in its steady-state level, indicating an unknown target capable to regulate Aux/IAA protein levels and, hence, transcription.

Keywords: Aux/IAA protein; Auxin; Degradation; Phospholipase A; TIR1

1. Introduction

The DR5 promoter is a synthetic, auxin-activated promoter often employed to report indirectly the local endogenous auxin level [1–6]. The DR5 promoter is composed of five tandem hexamer TGTCTC elements derived from the auxin-activated *GH3* promoter that were identified as required for its auxin responsiveness [7]. Auxin-response factors (ARF) bind to these hexamers to form complexes with their transcription cofactors, the Aux/IAA proteins. The formation of ARF-Aux/IAA complexes, when binding to DNA of the artificial DR5 promoter, as well as to naturally auxin-activated promoters leads to specific gene repression [8]. The ARF-Aux/IAA regulated promoters behave as primary response targets i.e. they are fast auxin-regulated promoters [9–13]. Examples of primary auxin-regulated genes are other members of the *GH3* gene family as well as members of the *SAUR* gene family; members contain auxin-regulated cis elements within their promoters although primary auxin response has not yet been confirmed for all family members [1].

Transcription from this group of promoters is usually derepressed by proteolysis of Aux/IAA proteins [14,15]. Proteolysis is initiated by auxin- and SCF-dependent ubiquitination of IAA proteins, an E3 ubiquitin-ligase complex of Skp1-Cullin-F-box-Rbx proteins [16–23]. Central for auxin-dependent ubiquitination is a ternary complex of the F box protein, either TIR1 or a related AFB, Aux/IAA protein, and auxin in that these F box proteins are both an auxin receptor and SCF complex is the first hormone-regulated enzyme capable of modifying a transcription cofactor activity critical for gene activity. Hence, this is a short signal transduction chain from signal to molecular site of action [15,24,25]. If the two gene families of *ARF* and Aux/*IAA* genes prove to be the only transcription factors for auxin primary gene regulation, this model may explain the entire primary gene regulation by auxin.

Interestingly, a number of auxin-regulated enzymatic activities or processes are known which are faster than can be explained by changes in the corresponding gene transcription. This means that those proteins pre-exist in target cells prior to the stimulus and are not newly synthesized, rather they are activated/regulated by post-translational reactions (e.g. phosphorylation), independent of gene regulation, as is typical for many signal transduction chains. There are several examples of post-translational regulation by auxin: (1) the activation of phospholipase A (PLA) activity occurs within 2 min after auxin application [26,27], (2) the regulation of channel activity occurs within 3 min [28,29], (3) the activation of MAP kinase occurs within 5 min [30], (4) the activation of

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; ETYA, 5,8,11,14-eicosatetraynoic acid; HELSS, tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one; IAA, indolyl-3-acetic acid; 1-NAA, 1-naphthaleneacetic acid

0014-5793/$32.00
doi:10.1016/j.febslet.2007.07.059

rac-like GTPases occurs within 5 min [31], and (5) the inhibition of endocytosis occurs within 10 min [32]. All these responses are difficult to explain by a mechanism based solely on transcriptional regulation such as is initiated by TIR1/AFB-dependent ubiquitination [24,25]. Therefore, we hypothesize that these and likely other responses are triggered by a receptor other than the TIR1 family because the very rapid activation of PLA is difficult to explain by a mechanism requiring transcriptional regulation of the *PLA* genes. While the kinetics of auxin activation of Aux/IAA degradation is detectable within 2 min [21], it is slower than auxin activation of PLA [27]. Moreover, PLA can also be activated by elicitors within 1–2 min [33,34] suggesting a post-translational mechanism. Taken together, these results indicate a pathway for PLA activation different than the one starting with SCF-dependent ubiquitination.

Advances in our understanding of auxin signaling have made use of Yokonolide B (YkB; also known as A82548A), a spiroketal-macrolide, and terfestatin (terphenyl-β-glucoside), inhibitors of auxin-activated transcription of the DR5 promoter [3,6,35]. In addition, sirtinol, a constitutive activator of auxin action was identified from a screen of a large chemical library [36]. YokB and terfestatin inhibit Aux/IAA degradation and appear to act on steps other than TIR1 family-dependent ubiquitination. In contrast, sirtinol activated IAA degradation in the absence of exogenous auxin [36] and subsequent work determined that sirtinol is converted in vivo into an active auxin [37]. Another example is the MAPKK inhibitor, 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one, which inhibits the auxin-activated promoter BA3, suggesting that a MAPK cascade acts upstream of transcription factor activity-regulation by the TIR1 family [30].

PLA inhibitors were used to inhibit auxin-specific elongation [38,39]. Two PLA inhibitors were subsequently used to inhibit purified patatin-like PLA and elongation growth of etiolated Arabidopsis seedlings [39]. To address the mechanism of action of these PLA inhibitors, we tested if the target is one that acts on or interferes with components of the regulatory network of the Aux/IAA proteins, their degradation, and/or their action as transcription factors. To our knowledge, these results provide the first evidence that the signal pathway for the activation of PLA by auxin culminates in Aux/IAA proteins. Furthermore, these results indicate that this new pathway runs independently of TIR1-dependent ubiquitination and the regulation of auxin-activated genes suggesting parallel pathways, although possibly linked.

2. Materials and methods

2.1. Growth conditions

The Arabidopsis ecotype Col-0 was used as the wild type in all experiments. The DR5-*GUS* construct and transgenic plants were described [7]. Seedlings were grown for 5 d at 23 °C in the dark in half-strength Murashige and Skoog [40] liquid medium (Life Technologies/Gibco-BRL, Cleveland) supplemented with 1% (w/v) sucrose, with shaking at 60 rpm in 12-well plates. The plants were treated by hormones and inhibitors for 2 h or mock treated in the same medium containing 0.5% (v/v) dimethyl sulfoxide (DMSO) without pretreatments. The phospholipase A inhibitors (ETYA: 5,8,11,14-eicosatetraynoic acid; HELSS: tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one or haloenol suicide substrate), and hormones 2,4-dichlorophenoxy acetic acid, indolyl-3-acetic acid, 1-naphthalene acetic acid were diluted from DMSO stock solutions. Transgenic plants ectopically expressing a IAA1:luciferase fusion protein from the *UBQ10* promoter were produced and grown as described [19]. Briefly, seeds were surface-sterilized, stratified for 2–4 d and grown in liquid half-strength Murashige–Skoog medium in a continuous white light growth chamber at 22 °C. Approximately 80 seeds were grown in Petri dishes (5 cm) in 1 mL medium for 7–8 days until they had 2–4 primary leaves.

2.2. Histochemical GUS activity

Histochemical GUS staining was performed by incubating whole seedlings in GUS staining buffer containing 1 mM 5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid (X-Gluc; Duchefa, Haarlem, Netherlands), 50 mM NaH_2PO_4 (pH 7.0), 0.5% Triton-X, and 0.5 mM potassium ferri- and ferrocyanide. After vacuum infiltration for 1 min, the seedlings were incubated at 37 °C for 24 h [41] then cleared by extraction of the seedlings in 70% ethanol. Plants were scanned by an Epson perfection 1250 scanner at 1200 dpi resolution. Each experiment was performed at least three times.

2.3. RT-PCR gene expression analysis

Total RNA was isolated from seedlings and treated with DNase according to the manufacturer's protocol (NucleoSpin RNA Plant Kit, Macherey & Nagel, Düren, Germany). Initiated by an oligo(dT)12–18 primer, first-strand cDNA was synthesized from each 3 μg of total RNA according to the manufacturer's protocol (RevertAid™ H Minus First Strand cDNA Synthesis Kit, Fermentas, Burlington, Canada) . The *ACTIN2* mRNA (gene accession At3g18780) was used to normalize RT-PCR amplification with the primers (+) 5-GGT AAC ATT GTG CTC AGT GGT GG-3- and (−) 5-AAC GAC CTT AAT CTT CAT GCT GC-3 (fragment size 162 bp). Semi-quantitative PCR was performed using 500 ng of cDNA as template in a PCR standard reaction mix of (final volume 100 μl) containing 1 U TaKaRa Ex Taq™ (Takara, Shiga, Japan) Taq-DNA-polymerase. Forty cycles were performed and 10 μl PCR reaction mix was taken at the cycles 20, 25, 27, 30 and 35 to check when the reaction is in logarithmic range. Conditions for amplification were 94 °C for 30 s, 55–65 °C for 30 s, and 72 °C for 30 s. The following primers were used: GUS: (+) 5′-TGA ACC GTT ATT ACG GA-3′ and (−) 5′-CCA TGC ACA CTG ATA C-3′ (fragment size 162 bp); IAA1 (gene accession no. At4G14560) (+) 5′-CGG AGC ACA AGA ACA AC-3′ (−) 5′-ATG GAA CAT CAC CGA CCA AC-3′ fragment size 381 bp; IAA5 (gene accession At1g15580.1): 5′-AAG AGT CAA GTT GTG GGT TGG C-3′ and 5′-AAT GCA GCT CCA TCT ACA CTC ACT-3′ (fragment size 106 bp); IAA19 (gene accession At3g15540.1): 5′-GAG CAT GGA TGG TGT GCC TTA T-3′ and 5′-TTC GCA GTT GTC ACC ATC TTT C (fragment size 138 bp); *ARF19* (gene accession At1g19220.1): 5′-ACA AAG GTT CAA AAA CGA GGG TCA-3′ and 5′-CGA TGG CCC TCG AAT GAT AAT GTA A-3 (fragment size 429 bp). Ten microlitre of each PCR assay were separated in 2% agarose gels and nucleic acids were stained by ethidium bromide. Initial experiments in GUS staining and quantification of the GUS mRNA expression were at UNC Chapel Hill and expanded and continued at the University of Hannover. The experiments were performed three or four times. Densitometry was done with the program Gel-Pro Analyzer 4.5 and the area density tool therein. For each actin band and the corresponding gene of interest-band a rectangular area was quantified and an equivalent background band from the same gel was subtracted. The value for actin was set as 100% and the corresponding band for each pair calculated accordingly.

2.4. Luciferase assays

Both at 18–24 h and at 2 h prior to initiation of a time course, the medium was exchanged with 0.5 mL fresh medium. ETYA or HELSS was added from stock solutions in DMSO as a 10-fold concentrated solution (50 μL). Hormone was added as a 50-fold concentrated solution (10 μL) in growth medium to the seedlings. Cycloheximide was dissolved in growth medium and added as a 10-fold concentrated solution having a final concentration of 0.2 mg/mL. The respective controls received DMSO or growth medium. The final DMSO concentration was 0.1%. Plants were pre-treated with inhibitors for 30 min and by hormone for 2 h to allow for pre-equilibration of the state of the E3 ligase and then cycloheximide initiated the measurement of degradation rates (Fig. 3). For the time-course experiments without cycloheximide (Fig. 4), compounds were added at $t = 0$ and steady states of

Aux/IAA1:luciferase were measured. At the indicated times, seedlings were rapidly blotted on absorbent paper and frozen in liquid nitrogen. Luciferase activity assays and protein measurements were performed as previously described [42,43]. Duplicate or triplicate samples were taken for each data point. Each experiment was performed 3–4 times with similar results.

3. Results

3.1. PLA inhibitors block the long-term auxin-dependent induction of gene transcription

Both PLA inhibitors, HELSS and ETYA, inhibited auxin-induced GUS activity in intact etiolated seedlings within the same concentration range as they were previously shown to inhibit elongation growth in etiolated seedlings and the enzymatic activity of the purified PLA enzyme [39,44]. The inhibitors elicited a similar effect for the three tested auxins, 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene-1-acetic acid (1-NAA), and indolyl-3-acetic acid (IAA) (Fig. 1A–C). PLA inhibitors had no effect on cytokinin (10 μM zeatin) induction of the cytokinin-activated promoter of the *ARR5* gene (results not shown) [45]. To determine the nature of this inhibition of GUS activity, mRNA levels for GUS were compared between control and treated seedlings. In parallel with the decrease in GUS activity, mRNA encoding GUS also declined. This result indicated that the effect of PLA inhibitors is not inhibition of GUS activity, but rather reflects reduced GUS protein.

To confirm that PLA inhibitors block auxin-induced transcription of endogenous genes in addition to a transgene with a synthetic auxin response element, the mRNA levels of of *GUS* and four auxin-regulated genes (*IAA1*, *IAA5*, *IAA19*, and *ARF19*) were quantified by RT-PCR after 2 h treatment with auxin. As shown in Fig. 2A, the activity of all promoters was up-regulated by 10 μM 2,4-D and this stimulation by auxin was decreased in a concentration-dependent fashion by HELSS and ETYA. When densitometrically related to actin the expression of these genes in the presence of auxin was inhibited by about 50% and more (representing one PCR cycle) (Fig. 2B). The lower regulation both by auxin and inhibitors of *IAA19* and *ARF19* may be explained by different regulatory properties of promoters of individual genes and by the known transient timing of the mRNA accumulation [9,10]. Thus, treatment of seedlings with HELSS and ETYA reduces mRNA levels for both synthetic and native auxin response elements.

3.2. PLA inhibitors do not affect the apparent short-term auxin-dependent decrease in IAA1 degradation rate but elevate Aux/IAA1 levels after longer treatment

Because the DR5 promoter and several *Aux/IAA* gene promoters are regulated by the amount of Aux/IAA proteins, the influence of the PLA inhibitors on the degradation rate

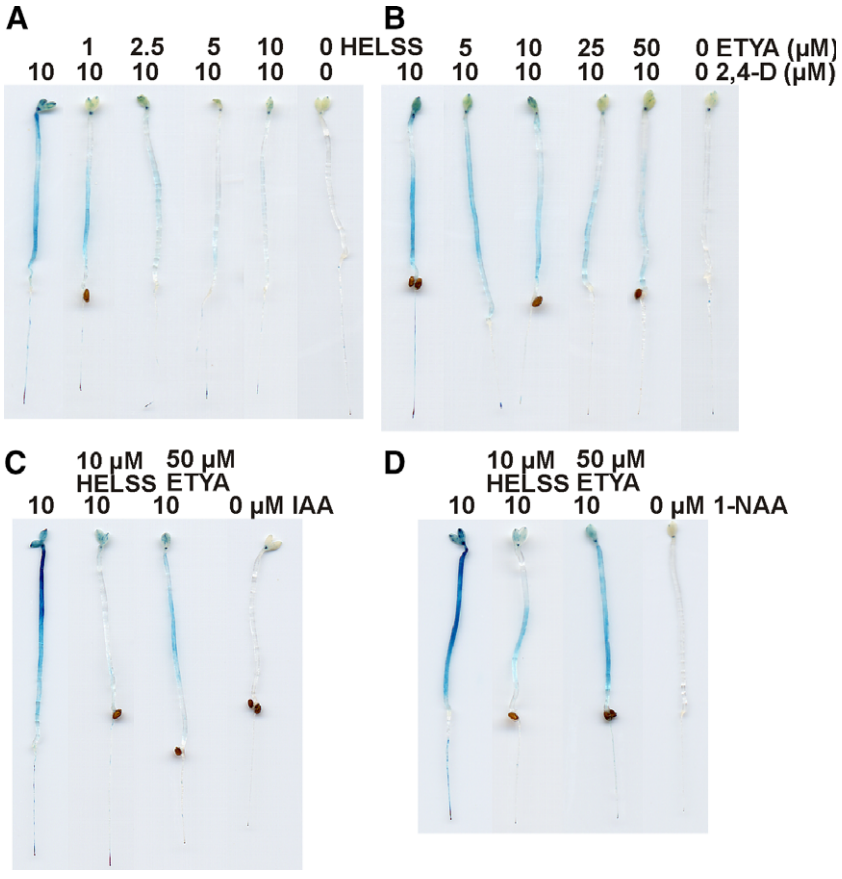

Fig. 1. Effect of PLA inhibitors on auxin-induced *DR5*::*GUS* expression. (A) Effect of 10 μM 2,4-D and increasing concentrations of 0–10 μM HELSS. (B) Effect of 10 μM 2,4-D and increasing concentrations of 5–50 μM ETYA. (C) Effect of 10 μM IAA and 10 μM HELSS or 50 μM ETYA. (D) Effect of 10 μM 1-NAA and 10 μM HELSS or 50 μm ETYA. Etiolated 3-d old seedlings were treated for 2 h with the indicated auxins and inhibitors and processed for GUS activity as described. Effects on elongation growth were not observed in that time span.

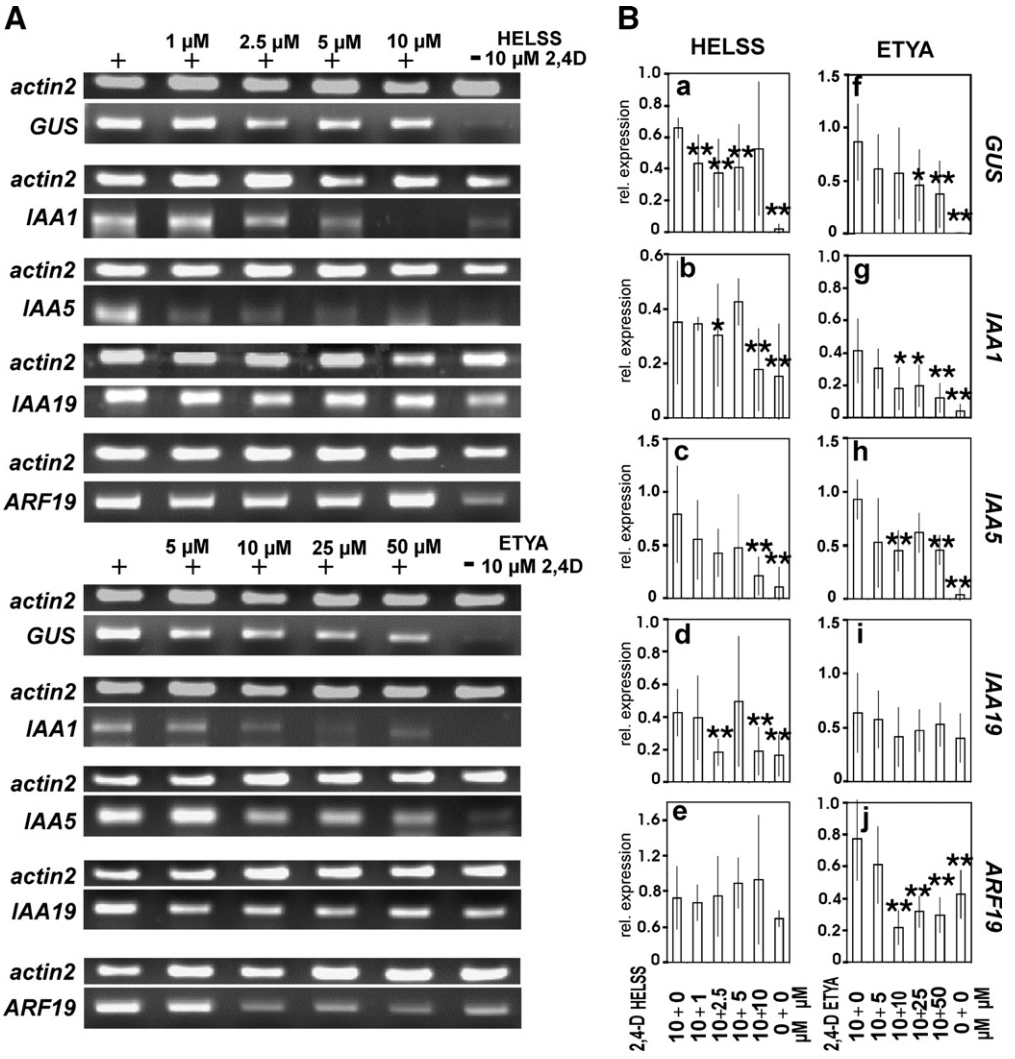

Fig. 2. Inhibition of auxin-activated gene expression by PLA inhibitors. (A) Semiquantitative estimation of mRNA by RT-PCR of *DR5::GUS*, *IAA1*, *IAA5*, *IAA19*, and *ARF19*. (B) Densitometric quantification of gel bands from experiments as shown in (A) ($n = 3$; S.D.; significant with respect to values for 10 µM 2,4-D: $^{**}P < 0.05$; $^{*}P < 0.10$; in *t*-test). Values for corresponding actin bands were set as 100% and all others calculated relative to this. Etiolated 3-d old seedlings were treated for 2 h with or without 10 µM 2,4-D plus PLA inhibitors at the indicated concentrations. RNA was indirectly determined using reverse-transcribed PCR with the indicated gene-specific probes as described in Section 2.

of Aux/IAA protein was tested with an Aux/IAA1:luciferase fusion protein and a truncated Aux/IAA17–luciferase fusion protein as artificial substrates [19,42,43]. The biosynthesis of these fusion proteins was under the control of the *UBQ10* 5′ flanking region [42] so that the biosynthesis was not linked to auxin action [21]. To measure the degradation rate of Aux/IAA1:luciferase, seedlings were pretreated for 30 min with ETYA and then for an additional 2 h with 10 µM 2,4-D or solvent vehicle. Cycloheximide (0.2 mg/ml), which blocks protein synthesis, was added to allow determination of the half-life of the Aux/IAA1:luciferase hybrid protein-degradation. ETYA treatment had a slight but variable effect when compared to the basal and auxin-regulated (10 µM 2,4-D) degradation rates of Aux/IAA1:luciferase (Fig. 3). Exogenous 10 µM 2,4-D stimulated the degradation rate of Aux/IAA1:luciferase as shown previously [21] and of a truncated Aux/IAA17-luciferase [46]. The magnitude of the auxin-induced increase of degradation rates, however, was the same, either in the presence of PLA inhibitor or without. In summary, the auxin modulation of Aux/IAA1 degradation rate is insensitive to the presence of PLA inhibitor.

Time course experiments without cycloheximide were conducted to reveal possible differences in kinetics of the regulation of the steady-state levels of Aux/IAA1:luciferase. When 10 µM 2,4-D or 10 µM 2,4-D plus 75 µM ETYA were added, the steady state levels of Aux/IAA1:luciferase were quickly down-regulated within 10–20 min with a similar velocity but then rose again (Fig. 4A). Later at 60–120 min, the rise was, on average, higher in the presence of ETYA. After 80–120 min another slow drop occurred, lasting at least several hours (not shown). Using 25 µM HELSS in the presence of 10 µM 2,4-D, the initial drop was again nearly identical with or without inhibitor (Fig. 4B) but at 120–180 min the Aux/IAA1:luciferase activity in the presence of inhibitor was higher than without. The rise in the presence of PLA inhibitors later in the time course may explain why Aux/IAA protein levels can be increased by PLA inhibitors and, in this way, probably

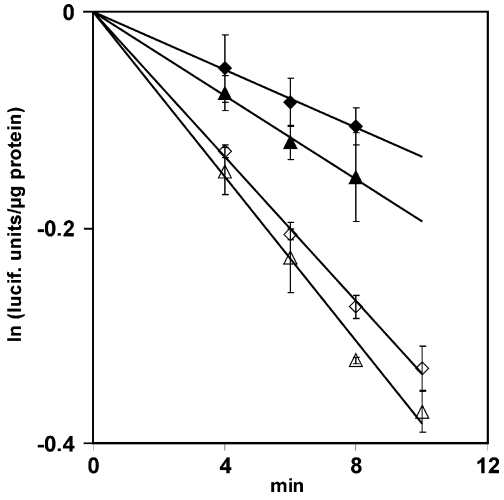

Fig. 3. Effect of 2,4-D and PLA inhibitor ETYA on Aux/IAA1:luciferase fusion protein degradation rates. Degradation rate of Aux/IAA1:luciferase protein was determined in intact seedlings using cycloheximide chase assay [42,43] after pre-treatment of seedlings with mock solutions, PLA inhibitor, auxin, or both. Filled diamond: mock; filled triangle: 75 µM ETYA; open diamond: 5 µM 2,4-D open triangle, 5 µM 2,4-D + 75 µM ETYA. Lines were calculated and fitted by the program excel.

inhibit transcription of auxin-regulated genes as shown in Figs. 1 and 2.

4. Discussion

Three auxin-relevant processes are inhibited by PLA inhibitors: (1) auxin activation of PLA [27], (2) auxin-dependent elongation [38,39], and (3) auxin-induced mRNA transcription of several genes (this work). To further explore the mechanism of inhibition, the effects of PLA inhibitors on Aux/IAA proteolysis were determined. The results suggest that PLA operates independently of, or in parallel to, auxin-mediated changes in SCF F-box TIR1- or AFB-IAA protein interactions. The possibility of two different ways of regulating *Aux/IAA* genes is provided by the comparison of brassinolide and auxin: in the presence of brassinolide, *IAA5* and *IAA19* RNA accumulate slowly and steadily over 24 h but auxin activates transcription within 1–2 h transiently [47,48].

Two methods were used to assess the effect of PLA inhibitors on auxin acceleration of Aux/IAA proteolysis. Pretreatment with either PLA inhibitor had no effect on Aux/IAA1:luciferase degradation rates when measured directly in the presence of cycloheximide. Without cycloheximide, the rapid effect of auxin on Aux/IAA1:luciferase activity previously observed [21] was unaffected by treatment with PLA inhibitor. Aux/IAA1:luciferase activity drops rapidly after auxin application, consistent with exogenous auxin increasing Aux/IAA1-luciferase degradation rate. PLA inhibitor had no effect on this rapid drop which is mediated by TIR1 [19–23]. However, examination of the steady-state levels of Aux/IAA1:luciferase over a longer time course revealed an interesting aspect. By 60–120 min after auxin addition in the presence of the PLA inhibitors, the steady state level of Aux/IAA1 increased. This PLA inhibitor effect on Aux/IAA1:luciferase accumulation independent of proteolysis is not understood. Since rapid activation of PLA by auxin was detectable within 2–5 min [26,27] the TIR1 receptor family is unlikely a receptor for the auxin in the PLA pathway. Evidence to link PLA to another receptor, at this point, is lacking and suitable mutants should be the future tool to shed light on this question.

Acknowledgements: Peter Pietrcyk provided excellent technical assistance in transcription and GUS analyses and Stefan Binder helped with the densitometry analysis. G.S. was supported by the DAAD by a travel grant for work at the University of North Carolina at Chapel Hill. G.S. thanks his wife Birgit for financial support during his sabbatical at the University of California at Davis and the members of the Callis lab for discussions and support. Work in the lab of G.S. was partially supported by a grant from the BMBF-DLR

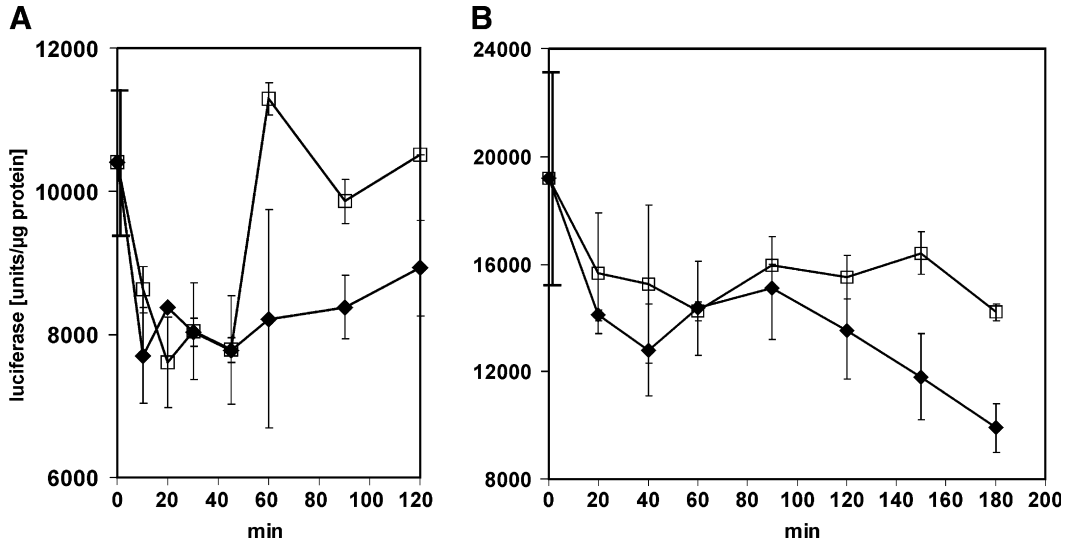

Fig. 4. Time course of the steady-state level of Aux/IAA1:luciferase activity in seedlings treated by 10 µM 2,4-D alone (filled diamonds) or by 10 µM 2,4-D with added phospholipase A inhibitor (A) 75 µm ETYA or (B) 25 µM HELSS (both open squares). Steady state levels, which combine the summed effect of both synthesis and degradation, were determined by the addition of auxin and omitting cycloheximide.

(50WB0325), in the lab of A.M.J. by grants from the NIGMS (GM65989-01), the DOE (DE-FG02-05er15671), and the NSF (MCB-0209711), and in the lab of J.C. by a Grant from the NSF (IBN-0212659).

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