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Copper excess triggers phospholipase D activity in wheat roots

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Abstract

Wheat seedlings (*Triticum durum* Desf.) were incubated in 100 μM Cu^{2+} for different periods of time ranging from 1 min up to 16 h. Following metal addition a rapid intake of copper ions into the roots was observed. Cu^{2+} induced an accumulation of both phosphatidic acid and phosphatidylbutanol within 1 min of incubation, the latter indicating a very rapid induction of phospholipase D (PLD) activity. The highest PLD stimulation was detected after 2 h from copper addition and decreased almost to the initial value at increasing times. Cycloheximide treatment of roots lowered phosphatidylbutanol accumulation because of a reduced PLD activity. The expression profile of a *T. durum* putative PLD-encoding gene showed a peak after 1 h of treatment as well, indicating that enhanced gene expression contributed to the increase in PLD activity. In the absence of copper ions, roots treated with the G protein activator mastoparan showed increases in phosphatidic acid and phosphatidylbutanol similar to those detected with the metal. PLD activity was also stimulated by cholera toxin. Two putatively G protein α subunit encoding sequences were isolated and no significant differences in transcription activity following Cu^{2+} addition were observed. In copper-treated roots an early production of superoxide generated both by total and membrane-bound NADPH oxidase occurred. The G protein inhibitor suramin as well as the PLD antagonist 1-butanol abolished copper-induced superoxide production.

Keywords: *Triticum durum*; Gramineae; Durum wheat; Copper; Lipids; Phosphatidic acid; Phospholipase D; Reactive oxygen species

1. Introduction

Membrane lipid deterioration is often related to stress injuries (Navari-Izzo et al., 1993; Quartacci et al., 2001, 2002; Berglund et al., 2002; Calucci et al., 2003), but membrane phospholipids (PL) constitute a dynamic system that may generate signal molecules as well. PL play an important role in early signalling cascades in animal cells, but also in plants hydrolysis of PL couples signal perception and synthesis of lipid-derived messengers mediating plant defence responses (Zhu, 2002; Zhang et al., 2003; Testerink et al., 2004; Yamaguchi et al., 2004, 2005).

It has been suggested that one of the roles of phospholipase D (PLD, EC 3.1.4.4) is to provide an intracellular signal for cell activation (Park et al., 2004; Wang, 2005). This signal is believed to be phosphatidic acid (PA), which is a minor lipid representing not more than 5% of the total PL of membranes. PLD catalyses the hydrolysis of structural PL such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) at the terminal phosphate diester bond leading to the formation of PA and a free head group (Zhang et al., 2003). PLD has the unique ability to transfer the phosphatidyl group of its substrate, instead to water, to a primary alcohol forming phosphatidyl alcohols. Thus, by adding *n*-BuOH to living cells, PLD activity can be measured by the amount of phosphatidylbutanol (PBut) formed in vivo (Testerink and Munnik, 2005). PA can also be indirectly formed by phospholipase C (PLC), which

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hydrolyses the PL phosphatidylinositol 4,5-biphosphate to give diacylglycerols (DAG), which are immediately phosphorylated by DAG kinase to PA.

In plants PLD exerted a positive role in triggering downstream ABA responses (Zhang et al., 2004). Also drought, cold and hyperosmotic stress activated PLD with consequent transient increases in PA levels (Frank et al., 2000; Munnik et al., 2000; Ruelland et al., 2002). Nod factors, which stimulate nodulation in legumes, induced PLD activity within minutes (den Hartog et al., 2003). Multiple forms of PLD were also activated in response to wounding (Wang et al., 2000), and a very rapid accumulation of PA in wounded leaves and in the neighbouring non-wounded leaves of tomato seedlings was reported (Lee et al., 1997). In addition, PA plays a critical role in mediating plant response to H_2O_2 and stress tolerance (Zhang et al., 2003; Yamaguchi et al., 2004, 2005). On the whole, these results suggest the involvement of PLD and PA turnover in the early signalling process in response to different stresses (Testerink et al., 2004; Zhang et al., 2005).

Plant cells generate reactive oxygen species (ROS) when subjected to environmental stress. The NADPH oxidase complex catalyses the NADPH-dependent production of superoxide anions. It is well established that the presence of a PA-specific protein kinase mediates the activation of NADPH oxidase in animals and neutrophils (Palicz et al., 2001). In plants a positive relationship between PA and ROS production was also demonstrated (Sang et al., 2001; Yamaguchi et al., 2003, 2004). Recently, the effect of PA on ROS production has been studied, suggesting that PA may be an important regulator of ROS generation and cell death during stress and plant defence responses (Park et al., 2004; Yamaguchi et al., 2005).

Due to its widespread industrial and agricultural use, copper toxicity is one of the major environmental concerns in modern society, affecting plant productivity and human health. Cu^{2+} – which performs one-electron oxireduction reactions – catalyses the formation of ROS that are capable of initiating peroxidation of unsaturated fatty acids in membranes (Navari-Izzo et al., 1999; Quartacci et al., 2000, 2001). In contrast to the detoxification mechanisms, the heavy metal signal transduction pathway in higher plants has not been well examined. Copper-induced cell death of rice roots was partially blocked by a mitogen-activated protein kinase (MAPK) inhibitor, suggesting that the kinase cascade may play a role in the metal induced-signalling pathway (Yeh et al., 2003), as confirmed by the observation that distinct MAPKs were activated in response to cadmium and copper stress (Jonak et al., 2004).

The role of lipid signalling in plants as well as the involvement of excess metals in early lipid degradation remain poorly investigated. The aim of the present study was to evaluate the role of lipids in the early signalling activation in wheat roots exposed to Cu^{2+}, this plant organ being the first to sense the damaging metal effects. In particular, PLD activation, at both biochemical and molecular levels, was evaluated to verify whether it represents an early event in the accumulation of PA. In response to copper-induced PLD activation the generation of ROS was monitored as well.

2. Results

The time course of Cu^{2+} accumulation into the wheat root cells showed a remarkable increase in the metal already after 1 min (Fig. 1). Copper ion uptake showed a sharp increase up to 15 min reaching a maximum value at the end of the experimental period. PLD-derived PBut increased in a dose-dependent manner from 2.5 up to 100 μM Cu^{2+} (Fig. 2), evidencing an about 5-fold stimulation in comparison with untreated roots in which PBut level represented 0.3 mol% of total phospholipids. Higher Cu^{2+} concentrations did not cause any further PBut accumulation.

The PL amount decreased throughout the whole experimental period, showing an about 40% reduction after 6 h of copper treatment (Table 1). As regards the individual PL, after 2 h of treatment Cu^{2+} caused a reduction of PC, PE and phosphatidylinositol levels. As a consequence, the PC to PE molar ratio did not show significant changes. In contrast, the amount of PA showed a remarkable increase up to 2 h, starting to decrease after this period. No significant increase in lysophosphatidylcholine, a product of phospholipase A activity, was detected before 16 h. The fatty acid composition of total PL showed an increase in linoleic and linolenic acids during the first 2 h of copper treatment that resulted in a higher unsaturation degree (Table 2).

Copper ions elicited the production of PA in a time-dependent manner (Fig. 3A). The accumulation of PA was activated within 1 min and doubled after 15 min, the accumulation going on till 2 h, when the stimulation was maximal (5.4-fold). After this period PA level decreased. Our results show that at least part of PA was formed via the PLD pathway, because PBut was generated as well (Fig. 3B). PBut accumulation increased approximately 2-fold within 10 min. It reached a maximum after 2 h, when

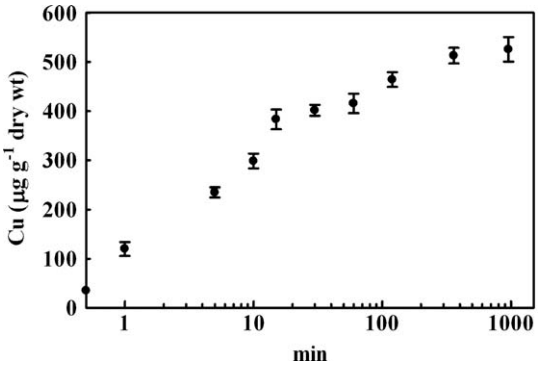

Fig. 1. Time course of copper ion accumulation in wheat roots. Wheat seedlings were grown for 14 days at 0.25 μM Cu^{2+} (control) and then incubated in 100 μM $CuSO_4$ for different periods of time. Results are the means of three replicates each analysed twice ± s.e. ($n = 3$).

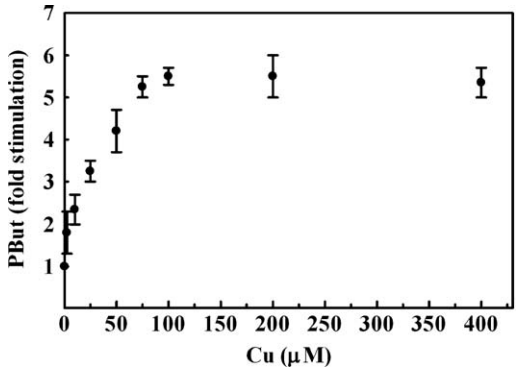

Fig. 2. Dose-dependent response of phosphatidylbutanol (PBut) to copper in wheat roots. Wheat seedlings were grown for 14 days at 0.25 μM Cu^{2+} (control) and then incubated with increasing $CuSO_4$ concentrations. Results are the means of three replicates each analysed twice ± s.e. ($n = 3$).

it peaked to 7-fold, starting thereafter to decrease. In the presence of cycloheximide, which is known to inhibit de novo protein synthesis, PBut accumulated to a lesser extent showing at 120 min a 3.7-fold increase in comparison with control roots (Fig. 3B).

Mastoparan (MP) is a G protein-activating amphipatic peptide that mimicks the intracellular domain of membrane-spanning receptors (de Vrije and Munnik, 1997) and as such accumulates in membranes, which may result in their permeabilisation. In our study the presence of MP did not affect negatively membrane permeability as wheat roots incubated with 25 μM MP for 24 h did not show any increase in ion leakage (leachate conductivity: control 0.39% h^{-1}, MP-treated 0.38% h^{-1}) neither membranes became permeable to Evans Blue dye, both results indicating membrane integrity following MP treatment. In the absence of copper ions, after 2 h of MP treatment PBut and PA accumulation increased 5- and 4.5-fold, respectively, compared to the control (Fig. 3C). When the inactive MP analog – MP 17 – was used, no increases in PBut or PA were observed (data not shown). In comparison with PLD basal activity, Cu^{2+}-treated microsomal membranes showed a 3.5-fold increase in the enzyme activity (Fig. 3D). Cholera toxin (CT), which is known to activate certain G proteins by ADP ribosylation of the G α subunit at its intrinsic GTPase site (Munnik et al., 1995), had a similar effect by itself and in combination with copper ions. The addition of the NADPH oxidase antagonist diphenyleneiodonium (DPI) (de Jong et al., 2004) lowered PBut accumulation by about 35% compared to Cu^{2+} treatment (Fig. 3D).

Table 2
Fatty acid composition of phospholipids (mol%) of wheat roots exposed to copper excess

Fatty acid	Minutes				
	0 (control)	15	120	360	960
14:0	1.8 ± 0.1	1.6 ± 0.3	2.0 ± 0.3	4.2 ± 0.4	2.4 ± 0.3
16:0	44.0 ± 2.2	40.9 ± 2.4	25.2 ± 1.5	29.0 ± 0.9	38.0 ± 1.7
16:1	0.9 ± 0.2	1.0 ± 0.1	1.3 ± 0.3	1.5 ± 0.3	1.5 ± 0.4
18:0	1.8 ± 0.2	1.3 ± 0.3	2.1 ± 0.1	2.3 ± 0.4	2.6 ± 0.3
18:1	5.6 ± 0.3	4.1 ± 0.2	4.7 ± 0.5	3.0 ± 0.3	5.6 ± 0.5
18:2	32.9 ± 0.4	36.1 ± 1.9	41.5 ± 2.2	37.5 ± 1.4	32.9 ± 2.7
18:3	13.0 ± 0.7	15.0 ± 0.6	23.2 ± 1.1	22.5 ± 2.0	17.0 ± 0.5
DBI	2.3	2.7	5.4	4.1	2.8

Seedlings were grown for 14 days at 0.25 μM Cu^{2+} (control) and then treated with 100 μM $CuSO_4$ for different periods of time. Results are the means of three independent experiments each analysed twice ± s.e. ($n = 3$). DBI, double bond index.

A *Triticum durum* putative PLDα-encoding cDNA fragment (*TdPLD1*, 917 bp, Accession No. AJ880008), including the 3′ end of coding portion and 3′-UTR, was isolated and used to verify whether this gene is transcriptionally activated by copper ions. The translated sequence showed 91%, 80% and 78% similarity to PLD proteins of *Oryza sativa* (Morioka et al., 1997), *Craterostigma plantagineum* (Frank et al., 2000), and *Arabidopsis thaliana* (Dyer et al., 1995), respectively. Northern blot hybridizations showed that this PLD-encoding gene is constitutively expressed in root tissues. However, a significant increase in transcript levels was observed after 1 h of Cu^{2+} treatment (Fig. 4).

Two putative *T. durum* G protein α subunit encoding cDNA fragments, *TdGA1* (269 bp, Accession No. AJ879999) and *TdGA2* (274 bp, Accession No. AJ880000), were also isolated, showing 98.9% (for *TdGA1*) and 98.5% (for *TdGA2*) sequence similarities to the corresponding

Table 1
Phospholipid amount (μmol g^{-1} dry weight) and composition (mol%) of wheat roots exposed to copper excess

Phospholipid	Minutes				
	0 (control)	15	120	360	960
PC	41.2 ± 2.4	38.5 ± 1.9	26.4 ± 0.7	30.7 ± 1.0	35.6 ± 1.3
PE	29.7 ± 1.2	26.9 ± 1.1	20.7 ± 1.5	25.3 ± 0.8	28.2 ± 1.1
PG	9.0 ± 0.5	9.2 ± 0.5	9.5 ± 0.5	9.5 ± 0.5	8.6 ± 0.6
PI	7.3 ± 0.4	5.0 ± 0.4	3.9 ± 0.3	5.7 ± 0.6	5.0 ± 0.4
PS	6.0 ± 0.5	6.3 ± 0.4	7.6 ± 0.6	7.1 ± 0.3	5.2 ± 0.7
PA	5.1 ± 0.6	12.7 ± 1.3	30.6 ± 2.0	19.4 ± 1.5	12.7 ± 1.1
LPC	1.7 ± 0.3	1.4 ± 0.2	1.3 ± 0.4	2.3 ± 0.7	4.7 ± 0.5
PC/PE	1.4	1.4	1.3	1.2	1.3
PL amount	1.3 ± 0.2	1.2 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.8 ± 0.1

Seedlings were grown for 14 days at 0.25 μM Cu^{2+} (control) and then treated with 100 μM $CuSO_4$ for different periods of time. Results are the means of three independent experiments each analysed twice ± s.e. ($n = 3$). PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; LPC, lysophosphatidylcholine.

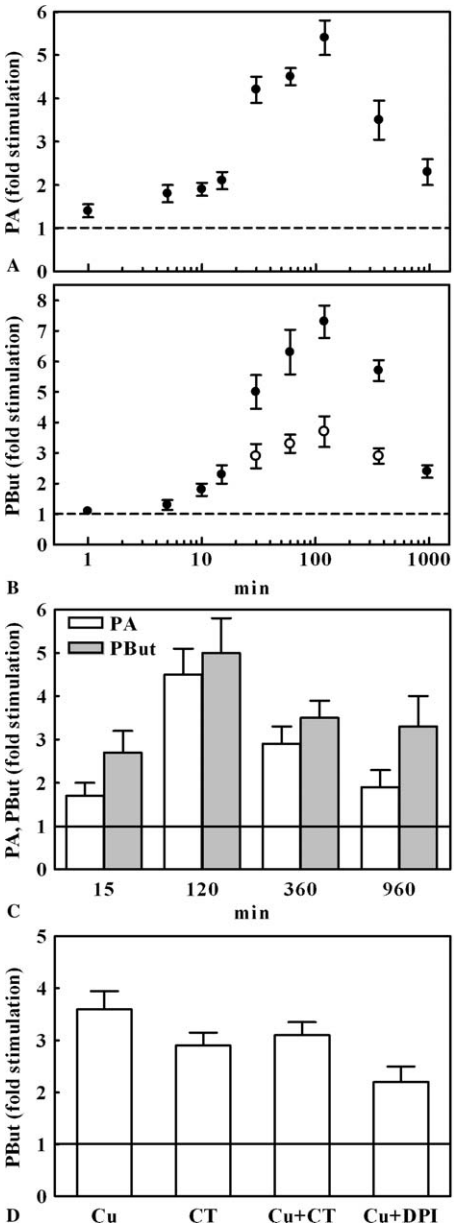

Fig. 3. Effects of copper, mastoparan, cholera toxin and diphenyleneiodonium on phosphatidic acid (PA) and phosphatidylbutanol (PBut) accumulation in wheat roots. (A, B) Copper treatment. Wheat seedlings were grown for 14 days at 0.25 μM Cu^{2+} (control) and then incubated in 100 μM $CuSO_4$ for different periods of time in the presence of 0.2% *n*-BuOH (closed symbols) and BuOH plus cycloheximide (open symbols). Cycloheximide (20 μg ml^{-1}) was added to the solution 1 h before copper treatment. At each time point PA or PBut are expressed as fold stimulation in comparison with controls (5.1 and 0.3 mol% of total phospholipids, respectively) incubated in 0.2% *n*-BuOH (horizontal lines). (C) Mastoparan (MP) treatment. Wheat seedlings were grown for 14 days at 0.25 μM Cu^{2+} (control) and then incubated in 25 μM MP for different periods of time in the presence of 0.2% *n*-BuOH. At each time point PA and PBut are expressed as fold stimulation in comparison with controls incubated in 0.2% *n*-BuOH (horizontal line). (D) In vitro treatment. Wheat seedlings were grown for 14 days at 0.25 μM Cu^{2+} (control). The isolated root microsomal membranes were incubated for 15 min in the presence of 0.2% *n*-BuOH, 50 μg ml^{-1} cholera toxin (CT), 20 μM Cu^{2+} and 15 μM diphenyleneiodonium (DPI). PBut amounts are expressed as fold stimulation in comparison with controls incubated in 0.2% *n*-BuOH (PLD basal activity, horizontal line). Results are the means of three replicates each analysed twice ± s.e. ($n = 3$).

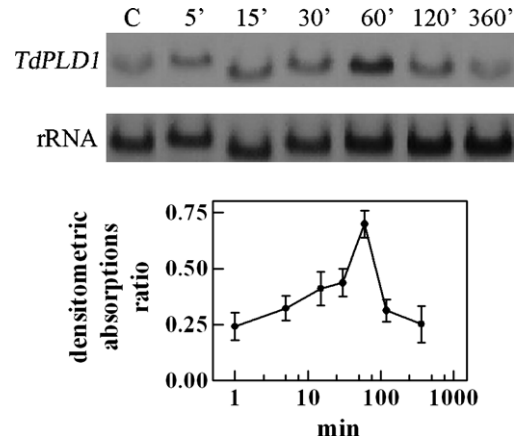

Fig. 4. Northern blots of RNAs isolated from roots of wheat plantlets. Wheat seedlings were grown for 14 days at 0.25 μM Cu^{2+} (control, C) and then incubated in 100 μM $CuSO_4$ for different periods of time. Twenty micrograms of total RNA per sample were loaded. Filters were hybridised with digoxigenin labelled *TdPLD1* probe. A *Phaseolus coccineus* 25S rDNA fragment was used as a control probe (rRNA). Densitometric absorption of hybridisation bands were measured and reported as the mean ratio (of five replicates ± s.e.) between *TdPLD1* and rRNA absorption values.

cDNAs of *Triticum aestivum* (Shakhavat-Hossain et al., 2003). Because *TdGA1* and *TdGA2* were substantially undetectable in RNA by Northern blot analysis, RT-PCR was used to examine the relative expression profiles (Fig. 5). Both genes resulted constitutively expressed in root tissues, as expected. The transcript levels of *TdGA1* were always higher than those of *TdGA2*. Differences in transcript levels during Cu^{2+} treatment were not significant.

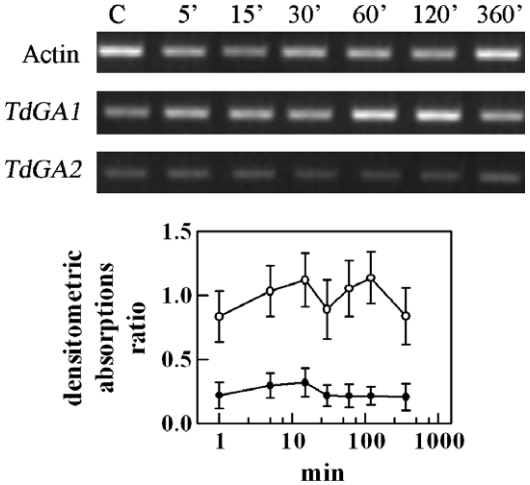

Fig. 5. RT-PCR analysis of *TdGA1* and *TdGA2* mRNA expression in roots of wheat plantlets. Wheat seedlings were grown for 14 days at 0.25 μM Cu^{2+} (control, C) and then incubated in 100 μM $CuSO_4$ for different periods of time. A *T. durum* actin-encoding gene was used as a marker for tissue unspecific gene expression. Negative images of electrophoresed gels were analysed densitometrically. Densitometric absorption of bands are reported as the mean ratio (of three replicates ± s.e.) between *TdGA1* or *TdGA2* and actin absorption values (open circles: *TdGA1*, closed circles: *TdGA2*).

NADPH-dependent superoxide generation was affected by Cu^{2+} in a time-dependent manner (Fig. 6). Compared to the control, the superoxide production by total NADPH oxidase (soluble and membrane-bound) increased by ca. 1.5-fold within 1 min of treatment, as observed for PA (Figs. 3A and 6A). The increase lasted 5 min, the formation of superoxide returning after 10 min to the level of the untreated roots, and further decreasing up to 16 h. Due to copper ion excess, also membrane-bound NADPH oxidase activity increased by 1.9-fold within 1 min of treatment (Fig. 6B), showing the same time course of total NADPH oxidase and decreasing at very low levels at the end of the experiment. As far as other ROS are concerned, no increase in hydrogen peroxide was evident during the whole experimental period (data not shown). In comparison with the control, the addition of exogenous dioleoyl-PA to untreated microsomal membranes induced a ca. 2.5-fold stimulation of the NADPH oxidase activity (Fig. 6C). Copper ions caused a 1.8-fold increase in superoxide formation, whereas DPI drastically inhibited such production. In the presence of Cu^{2+}, also suramin – which is known to uncouple G proteins from receptors (Zhao and Sakai, 2003) – and the PLD antagonist 1-BuOH abolished almost completely superoxide production (Fig. 6C).

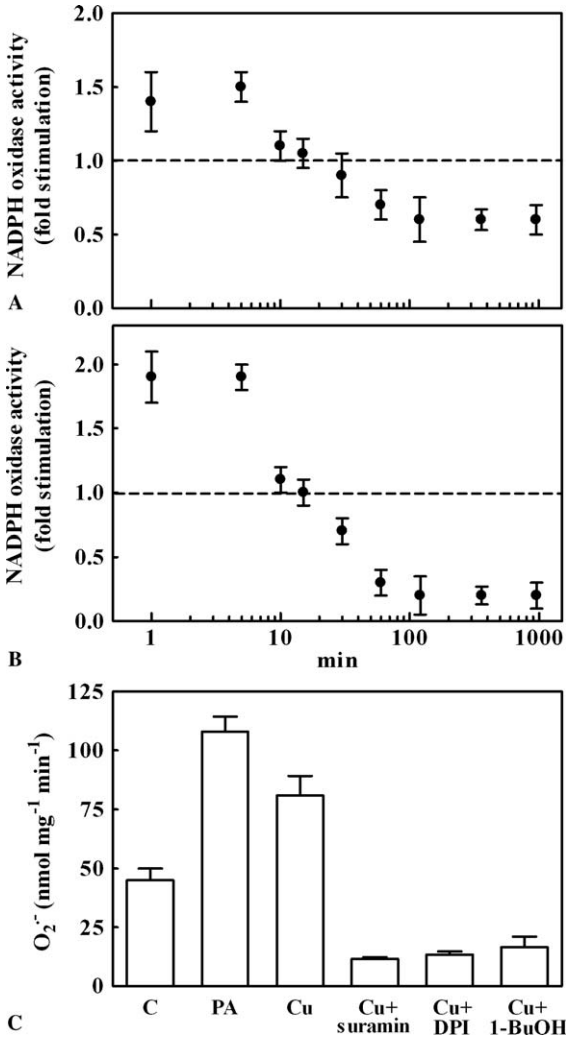

Fig. 6. Effect of copper on the NADPH-dependent oxidase activities of wheat roots. (A) Total (soluble and membrane-bound) and (B) membrane-bound NADPH-dependent oxidase activities. Wheat seedlings were grown for 14 days at 0.25 μM Cu^{2+} (control) and then incubated in 100 μM $CuSO_4$ for different periods of time. At each time point NADPH-dependent superoxide formation is expressed as fold stimulation in comparison with controls. (C) In vitro superoxide formation. Wheat seedlings were grown for 14 days at 0.25 μM Cu^{2+} (control, C). The isolated root microsomal membranes were incubated in the presence of copper (20 μM), PA (100 μM), suramin (100 μM), DPI (15 μM) and 1-BuOH (0.3%). Suramin, DPI and 1-BuOH were added to the reaction mixture 10 min before copper supply. Results are the means of three replicates each analysed twice ± s.e. ($n = 3$).

3. Discussion

Copper concentration (100 μM Cu^{2+}) was chosen on a rooting test basis as the highest metal concentration that did not cause any decrease in wheat root length and dry weight during the whole treatment. During the exposure to copper ions plants did not show any visible sign of injury and survived the longest incubation period. The metal concentration used in the present experiment was the concentration at which Yeh et al. (2003) observed in rice suspension cells the maximal level of MAPK transcripts involved in the copper-induced signalling pathway even though an induction of MAPK was also observed at lower copper concentrations (Yeh et al., 2003; Jonak et al., 2004).

In roots of wheat Cu^{2+} treatment did not change the PC to PE molar ratio (Table 1), differently from a previous experiment in which roots treated with copper for 11 days showed remarkable decreases in both the PC to PE ratio and the saturation degree (Quartacci et al., 2001) that resulted in more tightly packed bilayers and adaptive reduced membrane permeability (Berglund et al., 2002; Calucci et al., 2003). Thus, in contrast to long-term stress-associated lipid degradation, the alteration of PL composition observed in this study does not seem to be related to membrane damage, but rather may be interpreted as a chemical modification involved in the signalling response to an external stimulus. It has been suggested that both PL degradation and signalling could be carried out by different PLD enzymes or the same PLD could exert both actions, depending on the duration and/or severity of the stress (Wang, 2000). Besides PA, in stress conditions also lysophospholipids could be formed (Meijer and Munnik, 2003; Wang, 2004), but this is not our case since an accumulation of lysophosphatidylcholine occurred only after 16 h, suggesting that PLD activation precedes the rise in lysophospholipids carried out by A-type phospholipases.

Phosphatidylation of BuOH can be used for an in vivo assay of PLD in plant tissues because the formation of PBut is specific for PLD activity. Since alcohols can themselves activate PLD (Munnik et al., 1995) we used a concentration of *n*-BuOH (0.2%) that caused little enzyme activation in the untreated roots (PBut level 0.3% of total

PL). Particularly striking and unexpected for intact plants was the rapidity of root PLD activation. Indeed, our results show that Cu^{2+} significantly activated PLD within 1 min from the onset of the treatment. The accumulation of both PA and PBut (Fig. 3A and B) increased during the first 2 h of copper treatment decreasing thereafter. A general property of intracellular signals is that any increase in concentration is transient so that the signal level, after decreasing, can increase again in response to another stimulus. The rapid and transient kinetics of PA and PBut is typical of second messengers released by activation of phospholipases. The activation of PLD might be a general mechanism in stress conditions (Xiong et al., 2002; Yamaguchi et al., 2005) and seems also involved in signal transduction of physiological events (Wang, 2005). Our results are consistent with these reports, so we suggest that the activation of PLD in wheat roots treated with copper ions is part of the response of these plants to copper stress.

Increased PLD activity usually results from stimulation of pre-existing PLD isoforms rather than de novo synthesis of the enzyme, since in many cases PLD-encoding genes are constitutively transcribed (Frank et al., 2000; Wang, 2005). However, increase in PLD-encoding transcripts following different stress stimuli as wounding, low temperature, salts, drought, and hormones has been also reported (Wang, 2005). For example, in *C. plantagineum* two PLD genes were isolated, *CpPLD1* and *CpPLD2*, the former constitutively transcribed, the latter induced (after 2 h) by dehydration (Frank et al., 2000). In rice, two PLD-encoding genes were constitutively expressed and also induced by wounding (McGee et al., 2003). In our experiments, a *T. durum* putative PLD encoding sequence (*TdPLD1*) resulted constitutively expressed in roots. However, a significant increase in the transcript levels was observed after 1 h of incubation (Fig. 4). Such data suggest that the early PLD activation (within minutes) is related to conformational changes, and that the highest PBut peak observed after 2 h of Cu^{2+} treatment (Fig. 3B) can be in part related to an increase in the number of PLD isoforms, as indicated by the higher gene transcript level after 1 h (Fig. 4) and by the reduced PBut accumulation following cycloheximide treatment of roots (Fig. 3B) due to inhibition of de novo protein synthesis.

In animal cells, G protein-activated PLD activity in absence of stress has been known since long time (Exton, 1997). We used the G protein agonists MP and CT to get insight into the possible involvement of G proteins in the regulation of PLD activity in wheat roots. MP and CT were able to mimic the Cu^{2+}-related response of PLD (Fig. 3C and D), whereas the antagonist MP 17 was not effective in increasing the accumulation of PA or PBut in comparison with the untreated plants. This response is in agreement with other findings which demonstrated the effect of G protein activators on PLD stimulation in *Dianthus caryophyllus*, *Chlamydomonas* cells and in *C. plantagineum* (Munnik et al., 1995; de Vrije and Munnik, 1997; Frank et al., 2000). PLD may be directly coupled to a G protein (de Vrije and Munnik, 1997) by an interaction between the enzyme and the canonical α subunit of the G protein (Zhao and Wang, 2004) or, as reported for animal cells (Exton, 1997), MP might directly activate PLD. It may indirectly be suggested that G protein-regulated events are likely involved in copper-stimulated PLD signalling even though a recent report indicates that, at least in plants, MP has also the ability to activate MAPK signalling without requiring the involvement of a canonical heterotrimeric G protein (Miles et al., 2004).

G protein activation is related to the binding between an agonist (i.e. an activating ligand) and its specific G protein-coupled receptor which leads to the conversion of an inactive G protein to its active conformation (Perfus-Barbeoch et al., 2004). To determine if such activation may be also related to increased expression of G protein encoding genes, we isolated two putative G protein α subunit-encoding genes of *T. durum* (*TdGA1* and *TdGA2*) – based on two corresponding genes whose sequence is available in *T. aestivum* (Shakhavat-Hossain et al., 2003) – to verify whether these genes are induced by copper ions. RT-PCR experiments showed that *TdGA1* and *TdGA2* are both constitutively expressed and their expression does not change significantly during Cu^{2+} treatment. It is worth noting that the expression profiles of *TdPLD1* and *TdGA1* or *TdGA2* are uncoupled. Thus, even though copper induction of G protein gene expression does occur, other G protein-encoding genes should be involved in copper-mediated PLD activation. Further studies are in progress to isolate other G protein-encoding genes.

Besides producing lipid-derived messengers, PLD can also participate to plant defence responses through modulation of NADPH-oxidase activity, which is involved in ROS production (Fig. 6A and B). ROS may be both damaging agents contributing to stress injury and signals inducing ROS scavengers and other protective mechanisms, depending on the intensity and duration of their production (Xiong et al., 2002). While NADPH oxidase-mediated ROS formation has been found to activate Ca^{2+} channels (Foreman et al., 2003), ROS can also be sensed directly by key signalling proteins such as tyrosine kinases/phosphatases which modulates MAPK pathways (Xiong et al., 2002; Yamaguchi et al., 2004).

The dose–response relationship between an alcohol such as EtOH and PLD stimulation showed that above 250 mM EtOH (0.9%, v/v) an inhibition of PLD activity occurs (Ella et al., 1995). In accordance with this observation, exposure of wheat roots to 5% EtOH for 15 min inhibited PA formation and superoxide generation (data not shown), suggesting a likely involvement of PLD in the response of NADPH oxidase to copper. In tomato cells treated with race-specific fungal elicitors, phosphorylation and translocation of plant p47-*phox*, a substrate for the PA-activated protein kinase in animals (Palicz et al., 2001), have been found to occur (Xing et al., 1997). The depletion of PLDα in *Arabidopsis* decreased both PA and superoxide accumulation, while the addition of PA promoted the synthesis of

superoxide in PLDα-depleted plants (Sang et al., 2001), even though the relationships with the defence responses were not examined. Furthermore, it has been suggested that PA may regulate the ROS-generating machinery assembling active ROP small G protein (a family of Rac-like GTPases) into the active NADPH oxidase complex (Park et al., 2004). In suspension rice cells, PLD and PA played a role in promoting the elicitor-induced biphasic generation of ROS (Yamaguchi et al., 2005).

PA is a non-bilayer lipid favouring hexagonal phase formation. In accordance with Sang et al. (2001), also in Cu^{2+}-treated roots a rapid and localised production of PA may have activated complex subunits increasing the NADPH oxidase-dependent generation of superoxide (Fig. 6A and B). The very low superoxide formation around 2 h of copper treatment might be explained by the fact that Cu^{2+} ions altered membrane lipids and, in particular, PL acyl unsaturation (Tables 1 and 2) with consequences on lipid–protein interactions. However, the relatively high exogenous PA concentration (100 μM) – which in our experiment determined a 42% stimulation of the NADPH oxidase activity – might not represent the in vivo true level at which stimulation occurs. It might be suggested that enzyme-surrounding lipid levels rather than their total concentrations are effective in enhancing an enzyme activity (Sang et al., 2001). For this reason it is likely that the PA concentration required to increase superoxide production in vivo would be much lower than 100 μM. The addition of exogenous PA led to a higher superoxide formation than copper incubation probably because PA was closely in contact with the membrane suspension and exerted a direct effect on the complex.

The suppression of superoxide formation by suramin and by DPI (Fig. 6C) is an indication that ROS produced by NADPH oxidase are involved in Cu^{2+}-induced PLD activation. Furthermore, the observations that DPI lowered but not suppressed PBut accumulation and that the PLD antagonist 1-BuOH almost inhibited superoxide formation (Fig. 3D) may indirectly suggest an involvement of different PLD isoforms in copper response. Indeed, specific PLDs operate in different steps in plant oxidative stress pathways; whereas PLDα1 promotes ROS production, PLDδ has been seen to act downstream of ROS and to protect plants against ROS-induced cell death (Zhang et al., 2005; Testerink and Munnik, 2005). At the moment it is not understood how ROS generated in response to stimuli can function as signalling molecules. Recently, it has been hypothesised that ROS formation can activate expression of genes encoding protein kinases that take part to the signal transduction pathway linking oxidative burst to diverse downstream responses (Rentel et al., 2004). It was also observed that stress-stimulated production of ROS (H_2O_2) activates plasma membrane-associated PLDδ through an increase in the cytosolic Ca^{2+} level (Zhang et al., 2003).

In conclusion, our study shows that the level of PA, a compound that is now accepted to play a major role in signalling, was early increased with a very rapid kinetics in Cu^{2+}-treated wheat roots and suggests the involvement of PLD in copper signal transduction, whose higher activity is linked both to induction of pre-existing isoforms and to transient enhanced gene expression.

4. Experimental

4.1. Plant material

Wheat seedlings (*T. durum* Desf. cv Adamello) were grown in hydroponic culture with continuous aeration in a growth chamber with day/night temperature of 21 °C/16 °C, a 16 h photoperiod, a photon flux density of 400 μmol m^{-2} s^{-1} and 70–75% relative humidity. Light was provided by fluorescent tubes (Osram L140W/20) and incandescent lamps (Philips 25 W). Seeds were surface-sterilised for 10 min with NaClO (approximately 2% of active chlorine), rinsed in distilled H_2O and imbibed for 16 h with running tap water. The seeds were then placed on a floating layer of Perlite in plastic pots filled with a half-strength aerated Hoagland's No. 2 solution containing 0.25 μM Cu^{2+} and renewed every 3 days (Hoagland and Arnon, 1950). At day 14 roots of intact plants were washed with distilled H_2O, and plants were treated as reported below.

4.2. Phospholipase D activity

PLD (EC 3.1.4.4) activity of roots was measured as the production of in vivo phosphatidylbutanol essentially as described by de Vrije and Munnik (1997). Roots of intact plants were incubated in 5 mM MOPS–NaOH (pH 6.0) containing 100 μM Cu^{2+} as $CuSO_4$ and 0.2% *n*-BuOH, whereas control roots were treated only with 0.2% BuOH. Both set of plants were incubated for different periods of time (from 1 min up to 16 h) with continuous stirring at room temperature. Cycloheximide (20 μg ml^{-1}) was added to the solution 1 h before copper treatment. In the dose-dependent experiments Cu^{2+} was added at concentrations ranging from 2.5 up to 400 μM and intact plants were incubated for 15 min before PBut determination in roots. For PLD assay in the presence of MP or MP 17 (Peninsula Laboratories, Belmont, CA, USA) treatments were performed adding the peptides at a concentration of 25 μM in the absence of copper ions. In order to distinguish between PLD activation induced by MP and BuOH, in each experiment control roots were incubated for the different time points with 0.2% BuOH in the absence of MP and MP 17. At the end of the treatments roots were washed, harvested and used immediately. The in vitro effects of CT and DPI (Sigma, St. Louis, MO, USA) on PLD activity were evaluated after incubating microsomal membranes for 15 min in the presence of 0.2% BuOH and, when present, 50 μg ml^{-1} CT, 20 μM Cu^{2+} and 15 μM DPI.

4.3. Copper content

Copper ion content was determined after wet-ashing of the desorbed roots in conc. HNO_3 and measured by atomic absorption spectrophotometry. The desorption was performed incubating the fresh roots in 5 mM $Pb(NO_3)_2$ for 30 min with continuous stirring.

4.4. Solute leakage

In order to determine whether MP induced permeabilisation of membranes, intact roots of wheat were treated with 25 μM MP for 24 h. Thereafter, solute leakage was measured as previously described (Navari-Izzo et al., 1993). Briefly, roots were immersed in distilled H_2O and gently shaken. Conductivity of the diffusate was determined after 1 and 3 h. The total conductivity was determined after immersion of the roots in liquid N_2. Conductivity was expressed as the percentage of solute leakage per hour relative to total conductivity of the roots.

4.5. Isolation of microsomal membranes

Fresh roots were ground with a chilled pestle in a homogenisation buffer (1:3, w/v) containing 50 mM Tris–HCl (pH 7.5), 10 mM KCl, 1 mM EDTA, 0.5 mg ml^{-1} bovine serum albumin, 0.5 mM phenylmethylsulfonyl fluoride, and 10 mM β-mercaptoethanol at 4 °C. The homogenate was centrifuged at 10,000*g* for 5 min and the supernatant was centrifuged further at 100,000*g* for 60 min in order to obtain the microsomal fraction. All operations were carried out at 4 °C. Protein content was determined by the Bradford method (1976). The effect of PA on superoxide production was evaluated adding 100 μM dioleoyl-PA (Sigma, St. Louis, MO, USA) to the extraction buffer prior to homogenisation.

4.6. Extraction and analysis of lipids

Lipids were extracted from roots by addition of boiling *iso*-PrOH followed by $CHCl_3$–MeOH (2:1, v/v) containing butylhydroxytoluol (50 μg ml^{-1}) as an antioxidant. The solvent mixture was then washed with 0.88% KCl to separate the $CHCl_3$ phase. The upper H_2O phase was re-extracted with $CHCl_3$, the $CHCl_3$ phases combined and dried under a stream of N_2. Total lipids were fractionated into neutral lipid, glycolipid and PL fractions on Sep-Pak cartridges (Waters, Milford, MA, USA) according to Quartacci et al. (2001). Separation of individual structural PL was performed by TLC (Silica Gel 60, Merck, Damstadt, Germany) with a solvent mixture containing $CHCl_3$–MeOH–HOAc–H_2O (85:15:10:3.5). After development, bands were located with iodine vapours. Individual lipids were identified by co-chromatography with authentic standards. Quantitative analysis of individual PL was performed measuring their phosphorous content with KH_2PO_4 as standard. All procedures were performed in the presence of silica gel from TLC. PBut was isolated from the other PL developing TLC plates in the organic upper phase of a solvent mixture composed by ethyl acetate–*iso*-octane–HOAc–H_2O (13:2:3:10). After development, bands were located with iodine vapours and PBut identified by co-chromatography with an authentic standard (Avanti Polar Lipids, Alabaster, AL, USA). Quantitative analysis of PBut was performed as reported above. The fatty acid methyl ester derivatives from total PL were obtained as previously described (Quartacci et al., 1997) and separated by GC on a Dani 86.10 HT gas chromatograph equipped with a 60 m × 0.32 mm SP-2340 fused silica capillary column (Supelco Sigma–Aldrich, St. Louis, MO, USA) coupled to a flame ionisation detector (column temperature 175 °C). Both the injector and detector were maintained at 250 °C. Nitrogen was used as the carrier gas at 0.9 ml min^{-1} with a split injector system (split ratio 1:100).

4.7. NADPH-dependent superoxide production

The determination of the NADPH-dependent superoxide-generating activity in microsomal membranes isolated from Cu^{2+}-treated roots was carried out as described by Van Gestelen et al. (1997) by measuring the rate of superoxide dismutase-inhibitable reduction of nitroblue tetrazolium using NADPH as electron donor. In order to measure maximal enzyme activity in all tests 0.02% (w/v) Brij 58 (eicosaethyleneglycol hexadecylether, Sigma–Aldrich) as a detergent was added. The reaction mixture consisted of 50 mM Tris–HCl buffer (pH 7.5), 0.25 M sucrose, 0.1 mM nitroblue tetrazolium and 50–100 μg proteins. After 1-min pre-incubation the reaction was started by the addition of 0.1 mM NADPH and the A_{530} changes were followed for 2 min. Rates of superoxide generation were calculated using an extinction coefficient of 12.8 mM^{-1} cm^{-1}. In in vitro experiments on untreated microsomal membranes, final concentrations in the reaction mixture were 20 μM for Cu^{2+}, 15 μM for DPI, 100 μM for suramin and PA, and 0.3% for 1-BuOH. Microsomal membranes were incubated with DPI and suramin for 10 min before copper addition.

4.8. RNA isolation

For total RNA extraction, root tissues were ground in liquid N_2 with a MES-guanidine hydrochloride-containing buffer and RNA was isolated using two PhOH–$CHCl_3$ extractions and an HOAc–EtOH precipitation procedure (Logemann et al., 1987).

4.9. Isolation of PLD- and G protein-encoding sequences

PLD- and G protein-encoding sequences were isolated by reverse transcriptase-polymerase chain reaction (RT-PCR) on total RNA isolated from *T. durum* plantlet roots. Three micrograms total RNA were heated for 10 min at 70 °C and retrotranscribed in a 20 μl volume reaction using

500 μM each deoxynucleotide triphosphate, 0.5 μM of the antisense oligonucleotide as primer (5′-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTT-3′, Invitrogen, Carlsbad, CA, USA), 1 × RT-Buffer (Invitrogen), 10 mM DTT, 200 U M-MLV reverse transcriptase (Invitrogen). The reaction was stopped by incubating at 70 °C for 15 min. Retrotranscribed cDNA was used for PCR amplification. To isolate cDNA homologous to a PLD-encoding gene (*TdPLD1*), one primer was designed based on a conserved PLD domain in the EMBL database (5′-TGGCAAAGGAGAACRATGGAGATG-3′) and used as sense primer coupled to the antisense primer 5′-GGCCACGCGTCGACTAGTACTTTTTTTTTTTT-TTTT-3′ (Invitrogen). To isolate G protein α subunit encoding gene fragments, primers were designed on corresponding sequences of *T. aestivum* (*TaGA1* and *TaGA2*, Shakhavat-Hossain et al., 2003). Since *TaGA1* and *TaGA2* have very similar sequences, except for a 21 bp insertion in *TaGA2*, two different antisense primers were designed corresponding to this gene portion, which allow to distinguish between the two genes: 5′-TAGCTCCATCATACAGTATTTTG-3′ for *TdGA1*, and 5′-ACAGTATCCTTTCCCAGTATTG-3′ for *TdGA2*. The same sense primer was used for both *TdGA1* and *TdGA2*: 5′-CAGCAGACCTCACTCCGTAA-3′. PCR was performed in 25 μl volume reaction, using 1 μl of retrotranscribed cDNA, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 0.2 μM of each primer, 1 × Thermophilic Buffer (Promega, Madison, WI, USA), 2.5 U *Taq*-DNA polymerase (Promega). The reaction mixture was heated at 94 °C for 3 min, then 30–35 cycles, each consisting of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C, were used. The final step at 72 °C was extended for 7 min. The amplified fragments were cloned into a pGEM-T Easy plasmid vector (Promega). The cloned fragments were sequenced by the dideoxy chain termination method. Experiments of amplification of genomic DNA, cloning and sequencing of the genes were repeated three times. Three clones were sequenced per experiment: no sequence variation was observed.

4.10. Northern blot analysis

Total RNAs (20 μg) were separated by 1% formaldehyde agarose gel electrophoresis and blotted onto positively charged nylon membranes (Roche, Basel, Switzerland). The integrity and the equal amount of RNA loading were confirmed by ethidium bromide staining and subsequent densitometric image analysis and by hybridisation with a *Phaseolus coccineus* rDNA probe. The ribosomal probe was an *Eco*RI-*Sau*3A 25S-rDNA fragment of clone pPH1, subcloned in pBluescript II SK+ and labelled as the other probes. Hybridisations were performed using *TdPLD1* labelled RNA probe. The probe was digoxigenin-labelled using the DIG RNA labelling kit SP6/T7 (Roche). After hybridisations in 50% deionised formamide, 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0), 2% blocking reagent (Roche), 0.02% SDS, and 0.1% SLS, filters were washed twice in 2 × SSC, 0.1% SDS for 15 min at room temperature, once in 1 × SSC, 0.1% SDS for 30 min at 68 °C and once in 0.3 × SSC, 0.1% SDS for 30 min at 68 °C. Detection of hybridisation signals was performed using the DIG-Nucleic Acid Detection Kit (Roche) according to manufacturer's instructions. Northern blots were repeated five times. Images of Northern blots were analysed densitometrically. Densitometric absorptions are reported as the mean ratio between *TdPLD1* and rDNA absorption values.

4.11. RT-PCR experiments

Semi-quantitative RT-PCR was used to determine expression profiles of *TdGA1* and *TdGA2*. The same primers designed for sequence isolation (see above) were used. Total RNA was isolated as described above and contaminant DNA was removed from samples by digestion with RQ1 DNAse (Promega). RNA was then purified by PhOH–$CHCl_3$ extraction, EtOH-precipitated and solubilized in diethylpyrocarbonate-treated water. Purified RNA was reversely transcribed as described above and then inactivated at 70 °C for 15 min. The second strand synthesis was continued in a total volume of 25 μl using hot lid PCR under the following conditions: 30 cycles at 94 °C for 20 s, at 55 °C for 30 s, at 72 °C for 20 s with elongation at 72 °C for 5 min. As positive controls, two primers were designed based on the sequence of different plant actin genes: 5′-AGCAACTGGGATGATATGGAGAA-3′ (sense) and 5′-ATTTCCCGCTCGGCCGTTGT-3′ (antisense). These primers were used to perform RT-PCR as described above. To estimate template amounts the RT-PCR was stopped during the exponential phase of amplification, maintaining initial differences in target transcript amounts. PCR products were separated in agarose gels. RT-PCR experiments were repeated three times. Negative images of electrophoresed gels were analysed densitometrically. Densitometric absorptions of bands are reported as the mean ratio between *TdGA1* or *TdGA2* and actin absorption values.

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