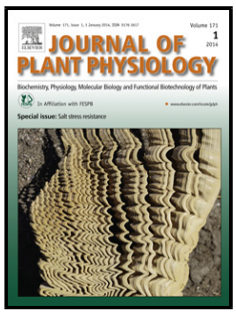

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The tomato phosphatidylinositol-phospholipase C2 (*Sl*PLC2) is required for defense gene induction by the fungal elicitor xylanase

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Abstract

The tomato [*Solanum lycopersicum* (*Sl*)] *phosphatidylinositol-phospholipase C* (*PI-PLC*) gene family is composed of six members, named *SlPLC1* to *SlPLC6*, differentially regulated upon pathogen attack. We have previously shown that the fungal elicitor xylanase rapidly induces nitric oxide (NO), which is required for PI-PLCs activity and downstream defense responses in tomato cell suspensions. Here, we show that all six *SlPLC* genes are expressed in tomato cell suspensions. Treatment of the cells with xylanase induces an early increase in *SlPLC5* transcript levels, followed by a raise of the amount of *SlPLC2* transcripts. The production of NO is required to augment *SlPLC5* transcript levels in xylanase-treated tomato cells. Xylanase also induces *SlPLC2* and *SlPLC5* transcript levels *in planta.* We knocked-down the expression of *SlPLC2* and *SlPLC5* by virus-induced gene silencing. We found that *Sl*PLC2 is required for xylanase-induced expression of the defense-related genes *PR1* and *HSR203J*.

Keywords: defense gene; phospholipid signalling; pathogen-associated molecular pattern**;** phosphatidic acid, nitric oxide

Abbreviations

cPTIO, 2-(4-carboxyphenylalanine) 4,4,5,5 tetramethylimidazoline-1-oxyl-3-oxide, potassium salt; DAF-FM-DA, 3-Amino,4-aminomethyl-2',7'-difluorescein

diacetate; DAG, diacylglycerol; DGK, diacylglycerol kinase; HR, hypersensitive response; IP_3, inositol 1,4,5-trisphosphate; NO, nitric oxide; PA, phosphatidic acid; PAMP, pathogen-associated molecular pattern; PIP_2, phosphatidylinositol 4,5-bisphosphate; PI-PLC, phosphatidylinositol-phospholipase C; PR, pathogenesis-related; ROS, reactive oxygen species; RT-qPCR, reverse transcribed – quantitative polymerase chain reaction; TRV, tobacco rattle virus.

Introduction

Phosphatidylinositol-phospholipase C (PI-PLC) catalyzes the hydrolysis of phosphoinositides, mainly phosphatidylinositol bisphosphate (PIP_2), to produce inositol trisphosphate (IP_3), a Ca^{2+} mobilizing second messenger, and diacylglycerol (DAG), which is further phosphorylated by DAG kinase (DGK) to produce phosphatidic acid (PA) (Arisz et al., 2009). In plants, PA plays a role as a second messenger in a broad array of processes (Testerink and Munnik, 2005). The induction of PI-PLC/DGK activity and the resulting accumulation of PA is an early plant defense response as evidenced upon treatment of plant cells with pathogen-associated molecular patterns (PAMPs) (Laxalt and Munnik, 2002), which are conserved compounds of pathogenic microbes, or race-specific pathogen effectors that are perceived by immune receptors present in resistant plants. For example, the fungal PAMP xylanase and the race-specific effector Avr4 of *Cladosporium fulvum*, rapidly trigger PI-PLC/DGK activity in cells of tomato and *Cf-4*-expressing tobacco, respectively (van der Luit et al., 2000; de Jong et al., 2004; Laxalt et al., 2007; Lanteri et al., 2011). It is well documented that PI-PLC/DGK activation induces downstream plant defense responses like reactive oxygen species (ROS) production, expression of defense genes and cell death (Yamaguchi et al., 2003; de Jong et al., 2004; Yamaguchi et al., 2005; Andersson et al., 2006; Chen et al., 2007; Laxalt et al., 2007; Raho et al., 2011; Wang et al., 2012).

Plant PI-PLCs are encoded by a gene family. There are nine *PI-PLC* genes in the *Arabidopsis thaliana* genome (Mueller-Roeber and Pical, 2002), whereas in tomato (*Solanum lycopersicum, Sl*) Vossen et al. (2010) characterized a *PI-PLC* gene family composed of six members, named *SlPLC1*

to *SlPLC6*. The expression levels of the various *SlPLCs* were found to be differentially regulated in *C. fulvum*-inoculated resistant and susceptible tomato plants (Vossen et al., 2010). By performing silencing assays, it was demonstrated that *Sl*PLC6 is required for resistance to *C. fulvum*, *Verticillium dahliae* and *P. syringae*, while *Sl*PLC4 is specifically involved in the induction of a hypersensitive response (HR) triggered upon Avr4 perception by the Cf-4 resistance protein (Vossen et al., 2010). Based on this evidence, the authors concluded that there are different PI-PLC isoforms participating in the signalling during different pathogen perception in plant defense.

Nitric oxide (NO) is a free radical that acts as a second messenger in various signalling pathways (Romero-Puertas et al., 2004; Gaupels et al., 2011). Rapid accumulation of NO has been observed in plants in response to pathogen perception (Gaupels et al., 2011) and treatment with xylanase or chitosan triggers NO production in tomato cell suspensions within minutes (Laxalt et al., 2007; Raho et al., 2011). Similarly, it was observed that *C. fulvum* Avr4 induces NO production in *Cf-4*-expressing tobacco cells (Laxalt and Joosten, unpublished data). Downstream of NO, plant defense responses are triggered through post-translational modifications of proteins like S-nitrosylation of cysteins present in the proteins (Gaupels et al., 2011).

Cross-talk between NO and PA was recently reported to occur in different plant responses, including those triggered by biotic stress (Distéfano et al., 2010). It was demonstrated that incubation of tomato cells with the NO-specific scavenger cPTIO inhibits PI-PLC/DGK activity and downstream defense responses induced by xylanase or chitosan treatment (Laxalt et al., 2007; Raho et al., 2011). Similarly, PA production via PI-PLC/DGK was inhibited in *Cf4-*

expressing tobacco cells treated with Avr4 in the presence of cPTIO (Laxalt and Joosten, unpublished data). In addition, it was reported that S-nitrosylation events are involved in xylanase-induced PA production (Lanteri et al., 2011). These results indicate that NO is required for the induction of PI-PLC/DGK activity during the activation of plant defense.

The goal of this work was to identify the *SlPLC* genes of tomato involved in the NO-related signalling pathway induced by xylanase. We studied *PLC* gene expression in cell suspensions and plants. In silenced tomato plants, we studied the role of *Sl*PLC2 and *Sl*PLC5 in xylanase-induced plant defense gene expression.

Materials and Methods

Chemicals

Xylanase from the fungus *Trichoderma viride* was purchased from Fluka (Buchs, Switzerland). The fluorescent probe 3-amino,4-aminomethyl-2',7'-difluorescein diacetate (DAF-FM-DA) and the NO scavenger 2-(4-carboxyphenylalanine) 4,4,5,5 tetra-methylimidazoline-1-oxyl-3-oxide, potassium salt (cPTIO) were purchased from Molecular Probes (Eugene, OR).

Tomato cell suspensions and treatments with xylanase and cPTIO

Tomato cell suspensions (*Solanum lycopersicum* line Msk8) were grown at 25°C in the dark in Moorashige Skoog medium as previously described (Felix et al., 1991). For xylanase and cPTIO treatments, two mL of tomato cell suspensions grown for 4–5 days after transfer to fresh medium were treated

with 100 µg.mL^{-1} of xylanase or cell-free medium as control, in the absence or presence of 0.5 or 1 mM cPTIO, and incubated over a period of 1 or 8 h. Assays were done in 3 cm ∅ Petri dishes for subsequent quantification of NO production and RNA isolation.

Quantification of NO Production by Fluorometry

Ninety µL volumes of tomato cells treated as previously indicated were carefully pipetted into the wells of a Greiner 96-well plate containing 10 µL of DAF-FM-DA (1 µM final concentration). The production of green fluorescence due to NO generation was measured in a Fluoroskan Ascent microwell plate fluorometer (Thermo Electron Company, Vantaa, Finland) using Chroma (ChromaTechnology Corp, Rockingham, VT, USA) filters D480-40 and D525-30 for excitation and emission, respectively. Fluorescence (arbitrary units, AU) was measured over a period of 30 min at 1 min interval. The calculated slope ($R^2 \geq 0.99$) was employed to represent the rate of NO production over the 30 min period. Relative NO production levels were calculated from the obtained NO production rates in relation to control treatments in the absence of cPTIO. Three technical replicates were included for each experiment.

Plant material and treatment with xylanase

MM-Cf0 tomato plants were grown in soil under a 16 h light / 8 h dark regime, at 21°C and 70% relative humidity. Leaflets from the third and fourth compound leaves of five–week-old plant were treated with xylanase by infiltration of a 2.5 mg.mL^{-1} solution of the compound with a needleless syringe and leaves were harvested at 24h after infiltration.

Virus-induced gene silencing (VIGS) assays

For VIGS the pTRV-RNA1 and pTRV-RNA2 vectors were used. An insert of 138 bp corresponding to the 3'-UTR of *SlPLC2* was amplified using the forward primer 5'-GAGGTACCGTAGATCTTGAAAAGGGAGC-3' and the reverse primer 5'-GAGGATCCCCATCAGTCTGTGTGTACTCT-3'. For TRV:*SlPLC5*, a sequence of 70 bp of the 3'-UTR of *SlPLC5* was amplified employing the forward primer 5'-GAGGTACCGAACCTCCAAAGTTACTTTCC-3' and the reverse primer 5'-GAGGATCCATCCGTCATTTTGGTACATG-3'. The primers introduce flanking *Kpn*I and *Bam*HI restriction sites (underlined in the primer sequences) which were used to clone each UTR fragment into pTRV-RNA2. The integrity of the inserts of the resulting clones was confirmed by DNA sequencing, after which they were transformed to *Agrobacterium tumefaciens* strain GV3101. The cotyledons of 10–day-old tomato seedlings were agroinfiltrated (OD600 = 2) with a 1:1 mixture of the pTRV-RNA1- and the pTRV-RNA2-derived constructs. Subsequent plant treatments were performed four weeks post-TRV inoculation.

cDNA synthesis and quantitative PCR analysis

Total RNA was extracted using Trizol as described by the manufacturer (Invitrogen, NY, USA). Complementary DNA (cDNA) was synthesized using MMLV reverse transcriptase (RT) from Promega (Madison, USA) and an oligo-dT primer on 1 µg of total RNA as a template. The cDNA was diluted to a final volume of 200 µL and 2.5 µL was used for quantitative PCR (qPCR). The Fast Universal SYBR Green Master mix from Roche (Mannheim, Germany) was

employed, using a Step-one Real-time PCR machine from Applied Biosystems (California, USA). The standard amplification program was used. The nucleotide sequences of the specific primers for qPCR analysis of *SlACT*, *SlPLC1* to *SlPLC6* and *SlPR1a* were previously reported by Vossen et al. (2010). For *SlHSR203J* the primers used were 5'-GATGTAGTTTCCGGTTGGCTTAC-3' (forward primer) and 5'-GAAGTCGTCATGCGGTGGAACAG-3' (reverse primer). Stepone Software v2.1 (Applied Biosystems) was used to analyze the transcript amounts of *SlPLC1*-*SlPLC6*, *SlHSR203J*, *SlPR1a* and *SlACT*.

Results

We first investigated the basal *SlPLC* transcript levels in order to find out whether all described *SlPLCs* were expressed in untreated cell suspensions. For this, we employed a set of *SlPLC1* to *SlPLC6* gene-specific primers previously used by Vossen et al. (2010). Fig. 1 shows that the six members of the *SlPLC* family are all expressed in untreated tomato cell suspensions. *SlPLC3* and *SlPLC4* transcript levels were about 20% of the transcript levels of tomato actin (*SlACT*), whereas *SlPLC1* transcripts showed the lowest abundance (0.04% of *SlACT*) (Fig. 1). Transcript levels of *SlPLC2*, *SlPLC5* and *SlPLC6* relative to *SlACT* reached about 2%, 1% and 0.6%, respectively (Fig. 1).

Xylanase-induced PI-PLC activation occurs within minutes in tomato cells, and is likely to be regulated at the post-transcriptional level (van der Luit et al., 2000; Laxalt et al., 2007; Lanteri et al., 2011). To test whether *SlPLCs* are regulated by xylanase at a transcriptional level, tomato cell suspensions were treated with xylanase over a period of 1 or 8 h. Subsequently, total RNA was extracted and RT-qPCR analysis was performed to determine the expression profile of *SlPLC1* to *SlPLC6. HSR203J*, which is an HR gene marker, and the pathogenesis-related (PR) protein-encoding gene *PR1a* were used as a read-out for transcriptional defense-related gene activation, both of which have been previously reported as xylanase-induced defense-related genes (Laxalt et al., 2007; Gonorazky et al., 2008). As shown in Fig. 2A, transcript levels of *SlPLC5* increased upon treatment with xylanase compared to the control treatment within 1 h of incubation. *SlPLC2* transcript accumulation increased within 1h of xylanase treatment, however this was not statistically significant (Fig. 2A).

SlPLC2 transcript levels significantly augmented after 8 h of treatment (Fig. 2A). *SlHSR203J* transcript levels already increased within 1 h of incubation, and *SlPR1a* transcripts augmented after 8 h of incubation with xylanase (Fig. 2B). Transcript levels of *SlPLC1*, *SlPLC3*, *SlPLC4* and *SlPLC6* were not augmented upon treatment with xylanase at the taken time points (Fig. 2A).

We studied whether NO modulates the xylanase-induced gene expression of *SlPLC2* and *SlPLC5*. Xylanase triggers a rapid accumulation of NO in tomato cell suspensions (Laxalt et al., 2007; Lanteri et al., 2011), however, this response has only been analyzed during the first 30 min of treatment (Laxalt et al., 2007; Lanteri et al., 2011). Thus, we first measured NO production in tomato cell suspensions after 1 or 8 h of incubation with or without xylanase, employing a fluorometer and the NO-specific fluorophore DAF-FM-DA. Fig. 3A shows that xylanase triggers an increase in NO accumulation in relation to the control. This response was inhibited in the presence of the NO-specific scavenger cPTIO in a dose-dependent manner (Fig. 3A). Then we studied the expression pattern of *SlPLC2* and *SlPLC5* in control and xylanase-treated cells in the presence or absence of cPTIO, upon incubation over a period of 1 or 8 h. The transcript levels of *SlHSR203J* and *SlPR1a* as genes of which the regulation is NO-dependent were included in the analysis (Yamamoto et al., 2004; Laxalt et al., 2007). The increase in *SlPLC5* transcript levels was reduced by cPTIO in a dose-dependent manner during the treatment with xylanase (Fig. 3B). Similar results were obtained for *SlHSR203J* and *SlPR1a* (Fig. 3B). Expression levels of *SlPLC2* were not suppressed by cPTIO in xylanase-treated cells (Fig. 3B). However, cPTIO *per se* already induced *SlPLC2* transcript levels after 8 h of incubation (Fig. 3B). Thus, no conclusions

255 can be made regarding the role of NO in xylanase-induced *SlPLC2* expression. *SlPLC1*, *SlPLC3*, *SlPLC4* and *SlPLC6* levels lower than 2 fold for cPTIO treatments (supplemental Fig. 1). These results indicate that NO is involved in the induction of *SlPLC5* transcription in xylanase-treated tomato cells.

The expression of the *SlPLC* family was analyzed in tomato leaflets 260 infiltrated with xylanase for 24 h. We quantified the transcript levels of *SlHSR203J* and *SlPR1a* as well. Transcript levels were calculated relative to water infiltration. Fig. 4 shows that, also in tomato plants, xylanase enhances the transcript levels of *SlPLC2* and *SlPLC5* and of the defense-related genes *SlHSR203J* and *SlPR1a*.

265 In order to investigate which PLC isoform could be involved in the xylanase-induced defense signaling pathway, we knocked-down the expression of *SlPLC2* or *SlPLC5* by tobacco rattle virus (TRV)-induced gene silencing (VIGS) in tomato plants. 3'-UTR regions of *SlPLC2* and *SlPLC5* were inserted into RNA2 of TRV, resulting in the recombinant constructs TRV:*SlPLC2* and 270 TRV:*SlPLC5.* As a negative control we used a TRV with the *β-glucuronidase* (*GUS*) gene (TRV:*GUS*), which has no homologs in plants. We analyzed the *SlPLC2* and *SlPLC5* transcript levels in the various TRV-inoculated plants after xylanase treatment. Fig. 5A shows that there is a clear reduction in *SlPLC2* transcript levels in xylanase-treated leaflets taken from the tomato plants 275 inoculated with TRV:*SlPLC2* demonstrating *SlPLC2* knock down. Figure 5B shows a similar result for *SlPLC5* silencing, albeit to a lesser extent than was observed for *SlPLC2* (Fig. 5B). Generally, VIGS in tomato leaves remains patchy and does not completely abolish the target gene expression (Liu et al., 2002). It was therefore concluded that partial knock down of *SlPLC2* and

SlPLC5 expression was obtained. As a downstream response, we then analyzed the expression of *HSR203J* and *PR1a* in leaflets of the various TRV-inoculated plants at 24 h after the infiltration of xylanase. In plants inoculated with TRV:*SlPLC2*, xylanase-induced *HSR203J* and *PR1a* transcript levels were significantly lower as compared to TRV:GUS plants (Fig. 5A). In plants inoculated with TRV:*SlPLC5*, expression levels of both xylanase-induced defense-related genes were not significantly different when compared to their expression levels in TRV:*GUS*-inoculated plants (Fig. 5B). These results point to a role of *Sl*PLC2 in the activation of plant defense-related gene expression in xylanase-treated leaves.

Discussion

A property of signalling enzymes in general is that treatments that activate them often rapidly enhance expression of their genes. The response could be a positive feedback mechanism to prime the cell for further stimulation (Yamamoto, 1998; Hirt, 1999). Therefore we studied the *SlPLC* gene expression for the identification of the PLC activated by xylanase and required for downstream signalling in tomato. We show that xylanase induces an increase in *SlPLC2* and *SlPLC5* transcript levels in tomato cell suspensions and leaflets. Then, we show that knocking down the expression of *SlPLC2* in tomato plants resulted in a reduction of defense-related gene expression after treatment with xylanase, indicating that *Sl*PLC2 is required for the induction of plant defense responses upon recognition of this PAMP. This implies that *Sl*PLC2 is involved in the signaling cascade activated by xylanase. Likely NO

modulates *Sl*PLC2 activation, but remarkably scavenging NO by cPTIO does not decrease the *SlPLC2* expression.

SlPLC2 and *SlPLC5* transcript levels increase in tomato cell suspensions upon xylanase treatment, whereas no changes were observed in the expression levels of the remaining *SlPLCs*. In leaves, *SlPLC2* and *SlPLC5* expression is also triggered by xylanase and *SlPLC2* showed the highest induction levels. Vossen et al. (2010) showed that *SlPLC2* transcript levels have the largest increase upon inoculation of resistant or susceptible tomato plants leaves with *C. fulvum*. Vossen et al. (2010) reported that expression of all six *SlPLCs* is enhanced during a compatible interaction between tomato and *C. fulvum* albeit with a different timing. *SlPLC1*, *SlPLC2*, *SlPLC4* and *SlPLC5* transcript levels also rise during an incompatible interaction. Altogether, these results point out that *SlPLC2* and *SlPLC5* transcript levels increase upon xylanase perception and inoculation of tomato with *C. fulvum,* which suggests a general role of *Sl*PLC2 and *Sl*PLC5 in the activation of plant defense responses.

NO production is rapidly induced (within 30 min) by xylanase treatment and required for PLC activation in tomato cell suspensions (Laxalt et al 2007; Lanteri et al 2011). Here we also demonstrate sustained NO production up to 8 h of treatment with xylanase. Sustained production of NO was described earlier in *A. thaliana* and soybean cells inoculated with an avirulent strain of *P. syringae* (Delledonne et al., 1998; Clarke et al., 2000), elicitin-treated tobacco cells (Yamamoto et al., 2004) and pearl millet seedlings treated with chitosan (Manjunatha et al., 2009). We demonstrate that NO production is required for xylanase-induced expression of *SlPLC5* and to our knowledge this is the first report that describes the involvement of NO in the regulation of *PLC* gene

expression. The role of NO in *SlPLC2* expression could not be assayed since the NO scavenger caused the *SlPLC2* transcript levels to increase. The fact that scavenging NO by cPTIO in non-treated leaves induces *SlPLC2* expression is intriguing and it remains elusive whether NO modulates PLC2 activity.

It was previously reported that xylanase-induced NO production enhances PA levels through activation of PI-PLC/DGK, which subsequently induces downstream defense responses (Laxalt et al., 2007). The specific contribution that *Sl*PLC2 and *Sl*PLC5 make to PA production upon xylanase treatment remains to be demonstrated. This is difficult to analyse in plants or leaflets, however d*e novo* PA production can be measured by $^{32}P_i$ radiolabelling in tomato cell suspensions (van der Luit et al., 2000; Laxalt et al., 2007; Lanteri et al., 2011; Raho et al., 2011). To obtain stably silenced tomato cell suspensions is a long-term aim. Future studies are indeed focused on the generation of stable tomato cell suspensions expressing constructs that result in knock-down or over expression of specific *SlPLC* genes to study the role of *Sl*PLC2 and *Sl*PLC5 in PA production during plant defense.

Consequently we transiently silenced *SlPLC2* and *SlPLC5* in tomato plants by VIGS. This technique allowed silencing of the gene of interest in leaves. VIGS of *SlPLC2* was shown to be effective as the expression of the target gene was efficiently knocked down. Upon VIGS of *SlPLC5*, only a minor reduction of the *Sl*PLC5 was observed in xylanase-treated leaflets, showing that here the silencing was less effective. Unfortunately, in leaflets it is not possible to assay the *in vivo* PLC activity upon xylanase perception (Laxalt and Munnik unpublished). Therefore, in order to address the role of the two PLCs in the PAMP response of plants, we determined the levels of the induction of defense-

related gene expression as a xylanase-induced downstream response. The transcript levels of *HSR203J* and *PR1a* did not change upon partial knock-down of *SlPLC5* in tomato leaflets, suggesting that SlPLC5 is either not required for xylanase-induced expression of defense-related genes or the level of silencing is not enough to show a phenotype. On the other hand, knock-down of the expression of *SlPLC2* clearly impaired the induction of *HSR203J* and *PR1a* by xylanase. This implies that *Sl*PLC2 plays an important role in the defense signaling cascade activated by xylanase.

In conclusion, the presented evidence indicates that xylanase perception induces an increase in *SlPLC2* and *SlPLC5* transcript levels, and that NO regulates *SlPLC5* expression in tomato cells. Unfortunately, we could not determine the role of NO in xylanase-induced *SlPLC2* expression. A functional genetic approaches consisting of knocking down *SlPLC2* and *SlPLC5* gene expression in tomato plants showed that *Sl*PLC2 is required for the activation of plant-defense gene expression. Thus, *Sl*PLC2 protein is a good candidate to be involved in the PA production triggered by the PAMP xylanase which is regulated by NO in the activation of plant defense responses.

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Figure legends

Figure 1. Relative transcript levels of the *SlPLC* genes in untreated tomato cell suspensions. Total RNA was isolated from tomato cell suspensions and the transcript levels of the six *SlPLC* genes were determined by RT-qPCR. Transcript levels are shown relative to *SlACT*, of which the level was set to 100%, and are plotted on a logarithmic scale. Error bars represent standard deviations of three independent experiments.

Figure 2. Relative transcript levels of the *SlPLC*, *SlHSR203J* and *SlPR1a* genes upon treatment of tomato cell suspensions with xylanase. Tomato cell suspensions were treated with cell-free medium (control) or with 100 µg.mL^{-1} xylanase for 1 or 8 h. Total RNA was subsequently isolated and the transcript levels of the *SlPLC* genes **(A)** and the defense-related genes *SlHSR203J* and *SlPR1a* **(B)** were measured by RT-qPCR. Transcript levels were first normalized to *SlACT* and are shown relative to control-treated samples, which were set to one. Error bars represent standard deviations of three independent experiments. Asterisks denote that means are significantly different from control treatments according to a t-test ($P< 0.05$).

Figure 3. NO production and relative transcript levels of *SlPLC2, SlPLC5*, *SlHSR203J* and *SlPR1a* genes upon xylanase treatment of tomato cell suspensions in the presence or absence of the NO scavenger cPTIO. Tomato cell suspensions were treated either with cell-free medium (control) or 100 µg.mL^{-1} xylanase for 1 or 8 h in the absence or presence of cPTIO at the indicated concentrations. **(A)** NO levels were determined using the DAF-FM-DA

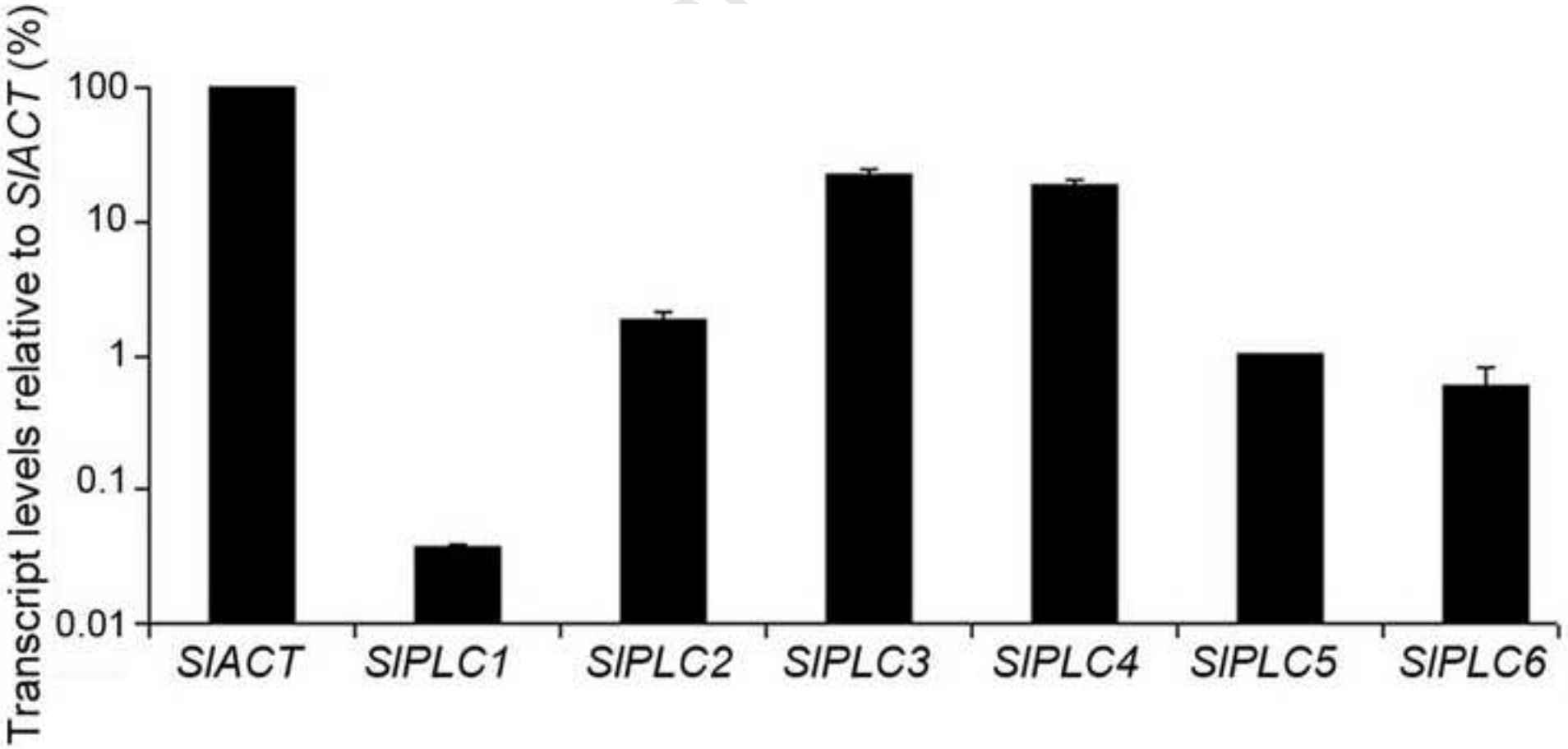
fluorescent probe. The fluorescence was determined in a microwell fluorometer and the rate of NO production over a period of 30 min was calculated and expressed relative to control treatments in the absence of cPTIO. **(B)** Total RNA was isolated and the transcript levels of *SlPLC2, SlPLC5* and the defense-related genes *SlHSR203J* and *SlPR1a* were measured by qRT-PCR. Transcript levels were first normalized to *SlACT* and are shown relative to control-treated samples in absence of cPTIO, which were set to one. Error bars represent standard deviations of three independent experiments. Asterisks denote that means are significantly different from control treatments according to a t-test ($P< 0.05$).

Figure 4. Relative transcript levels of the *SlPLC*, *SlHSR203J* and *SlPR1a* genes upon xylanase treatment of tomato leaflets. Tomato leaflets were infiltrated with water (control) or 2.5 $mg.mL^{-1}$ xylanase and harvested after 24 h. Total RNA was isolated and the transcript levels of the *SlPLC* genes **(A)** and the defense-related genes *SlHSR203J* and *SlPR1a* **(B)** were determined by RT-qPCR. Transcript levels were first normalized to *SlACT* and are shown relative to control-treated samples, which were set to one. Error bars represent standard deviations of three independent experiments. Asterisks denote that means are significantly different from control treatments according to a t-test ($P< 0.05$).

Figure 5. Relative transcript levels of *SlPLC*, *SlHSR203J* and *SlPR1a* genes upon xylanase treatment of leaflets of TRV:*SlPLC2*- and TRV:*SlPLC5*-inoculated tomato plants. Ten day old tomato seedlings were

inoculated with TRV:*GUS*, TRV:*SlPLC2 (A)* or TRV:*SlPLC5* (B) by agroinfiltration. After 4 weeks, tomato leaflets were infiltrated with water (control) or 2.5 $mg.mL^{-1}$ xylanase and harvested after 24 h. Total RNA was isolated and transcript levels of the indicated genes were determined by RT-qPCR. Transcript levels were first normalized to *SlACT.* The relative transcript levels of xylanase-treated TRV:*GUS*-inoculated plants were set to 100% and the transcript levels determined in the other treatments are expressed as a percentage of this. Error bars represent standard deviations of three independent experiments. Asterisks denote that means are significantly different from xylanase-treated, TRV:*GUS*-inoculated plants according to a t-test ($P < 0.05$).

Figure 1


Transcript levels relative to SlACT (%)
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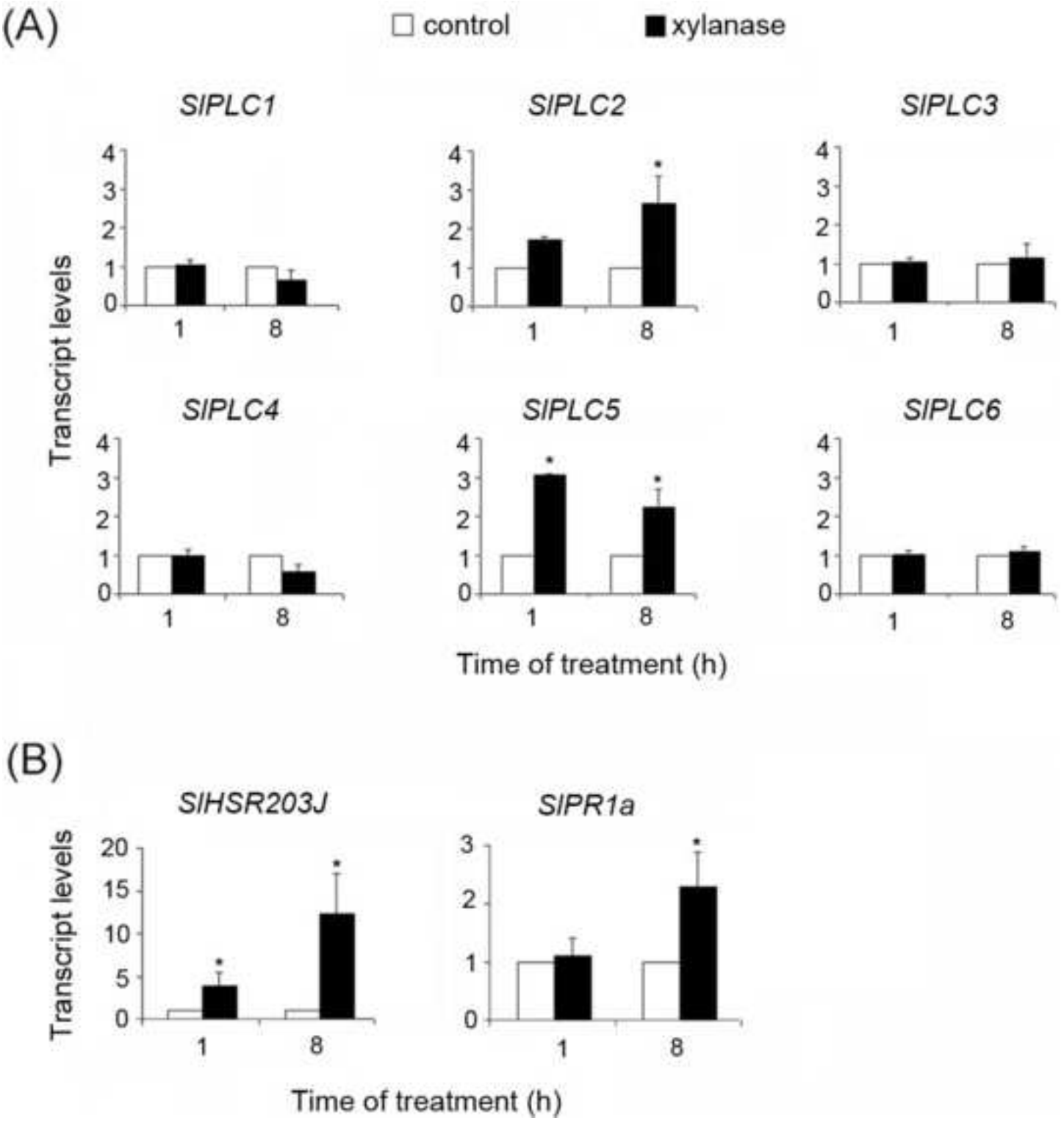
10
1
0.1
0.01
SlACT
SlPLC1
SlPLC2
SlPLC3
SlPLC4
SlPLC5
SlPLC6



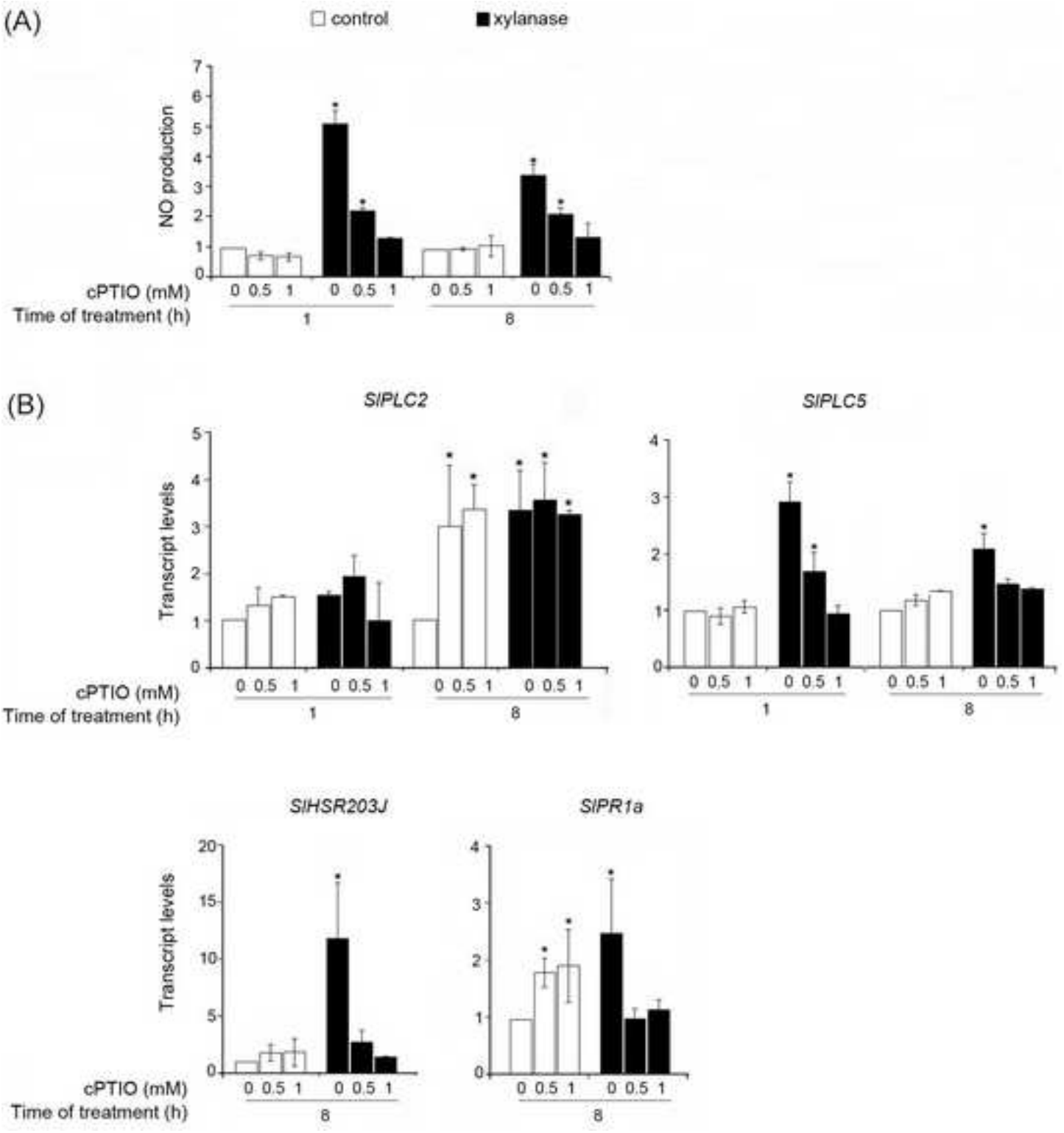
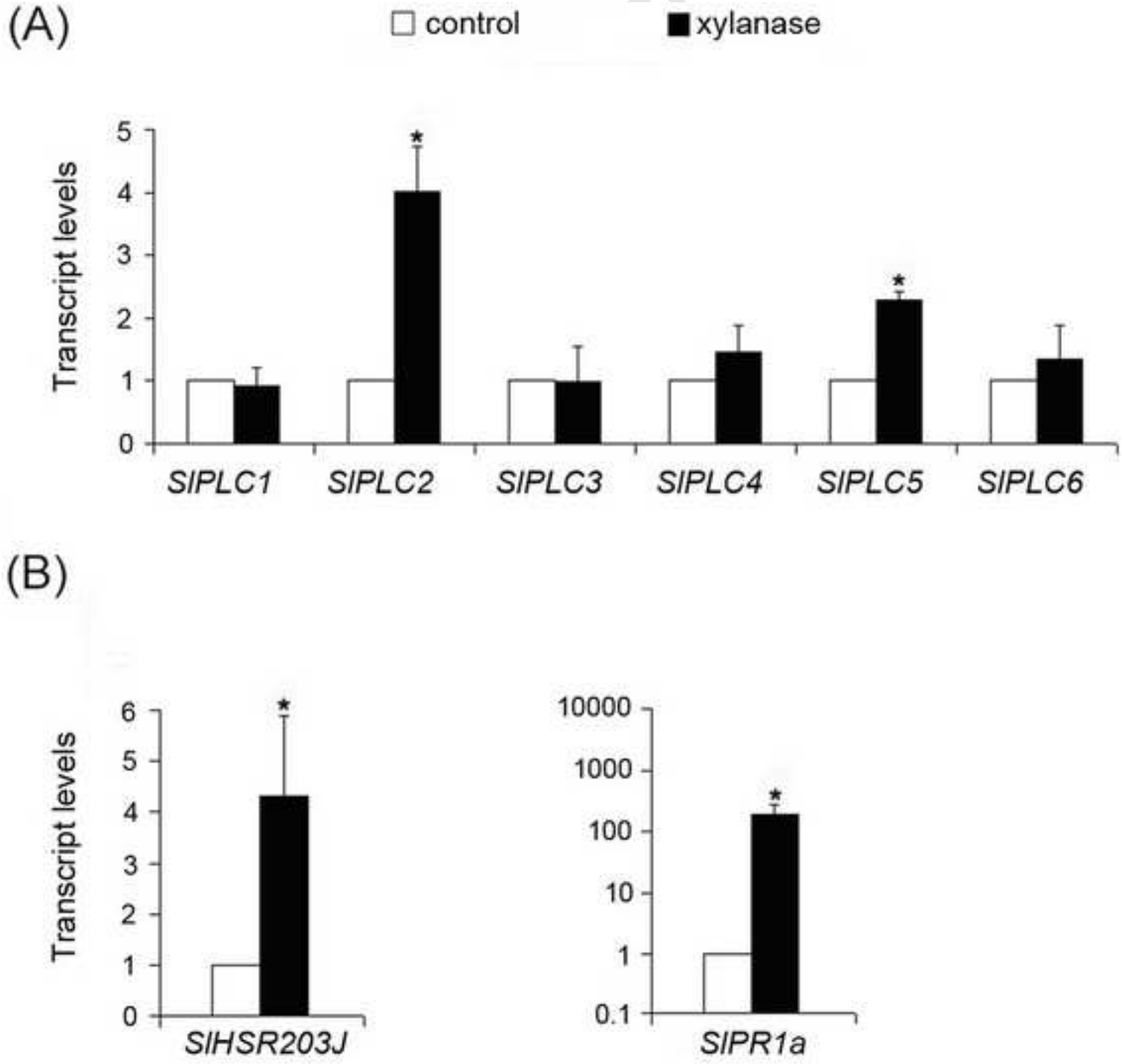


Figure 4


(A)
control
xylanase
Transcript levels
0
1
2
3
4
5
*
*
SlPLC1
SlPLC2
SlPLC3
SlPLC4
SlPLC5
SlPLC6
(B)
Transcript levels
0
1
2
3
4
5
6
*
SlHSR203J
0.1
1
10
100
1000
10000
*
SlPR1a