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Research article

In vitro differentiation of tracheary elements is induced by suppression of Arabidopsis phytoglobins

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ABSTRACT

Differentiation of tracheary elements (TEs) in vitro was affected by the expression level of the *Arabidopsis thaliana* Col-0 phytoglobins (Pgbs). Over-expression of *Pgb1* or *Pgb2* (35S:Pgb1 or 35S:Pgb2 lines) reduced the differentiation process while suppression of either Pgb (Pgb1-RNAi or *pgb2* lines) enhanced the production of TEs. The inductive effect of Pgb suppression on TE differentiation was linked to the reduced expression of the transcription factor *MYC2*. Suppression of this gene, observed under conditions of high NO levels or low *Pgb* expression, was sufficient to promote TE differentiation, while its over-expression abolished the promotive effect of Pgb suppression on the differentiation process. Cells in which *MYC2* was mutated accumulated ethylene which induced the expression of the homeodomain-leucine zipper (HD-Zip) III *ATHB8*. Production of ethylene was reduced in cells over-expressing *MYC2* in a WT or a *pgb* mutant background. While stabilizing procambial cell specification, ATHB8 in known to activate downstream components triggering programmed cell death (PCD) and modifications of cell wall components, required steps of the TE differentiation process. Collectively, we provide evidence that in addition to their recognised participation in stress responses, Pgbs may play a key role in the specification of cell fate during development.

1. Introduction

Together with xylem fiber and parenchyma cells, tracheary elements (TEs) are specialized cells found in xylem with the primary function to transport water and nutrients (Schuetz et al., 2013). This transport is driven by the negative pressure generated in the xylem tissue as a result of differences in water potential between soil and atmosphere (Sperry, 2004). To sustain this pressure while facilitating transport of xylem sap, TEs have undergone unique and characteristic evolutionary modifications. Tracheary element differentiation is delineated by the execution of programmed cell death (PCD), so that mature TEs are “hollow cadavers” in which the autolysis of the symplastic components by the suicide process creates an empty lumen to reduce resistance during sap conduction (reviewed by Ménard and Pesquet, 2015). To cope with the negative water pressure, TEs have lateral walls reinforced by the deposition of a thick secondary wall of heterogeneous composition including complex phenolic compounds, cellulose and hemicellulose polymers, and lignin (Ménard and Pesquet, 2015). Thickening of the secondary cell wall during TE differentiation follows a specific organization pattern described as spiral, reticulated and pitted (Schuetz et al., 2013).

Differentiation of TEs can be induced in vitro using several culture systems including *Zinnia elegans*, first described by the pioneering work of Fukuda and Komamine (1980). In this system, TE trans-differentiation is induced from single mesophyll cells in a semi-synchronous fashion. This system was subsequently optimized and utilized to examine the cellular, biochemical and molecular processes occurring during TE formation (Ménard and Pesquet, 2015). More recent studies have documented the hormone-induced TE differentiation from Arabidopsis parenchyma cells (Oda et al., 2005; Pesquet et al., 2010). The development of these in vitro systems, in conjunction with studies on xylem development *in vivo*, has contributed to the understanding on the

Abbreviations: AOA, aminooxyacetic acid; CESA4, cellulose synthase 4; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; ETH, ethephon; MC9, metacaspase 9; NO, nitric oxide; PCD, programmed cell death; Pgbs, phytoglobins; RNS3, ribonuclease 3; SNAP, *S*-Nitroso-N-Acetyl-D,L-Penicillamine; TEs, tracheary elements; VND6, vascular-related nac domain6; XCP1, cysteine protease 1

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molecular network regulating TE differentiation. Early molecular markers include the homeodomain-leucine zipper (HD-Zip)III gene *ATHB8*, specifying procambial cell fate (Donner et al., 2009), and *VASCULAR-RELATED NAC DOMAIN6* (*VND6*) and 7 (*VND7*), which through the regulation of direct down-stream targets induce the concomitant activation of PCD and cell wall biosynthesis (Ohashi-Ito et al., 2010; Zhong et al., 2008). Execution of these two processes in vitro is also mediated by hormone and signal molecules, with nitric oxide (NO) (Gabaldon et al., 2005) and ethylene (Pesquet and Tuominen, 2011) playing a central role.

Phytoglobins (Pgbs), plant hemoglobins (Hill et al., 2016) with similar characteristics to mammalian hemoglobins (Hill, 2012), are key regulators of PCD through the mediation of NO and ethylene. Through their ability to bind oxygen (Dordas, 2009; Hoy and Hargrove, 2008) and scavenge NO at low oxygen levels (Dordas et al., 2003, 2004), Pgbs contribute to protecting cells, tissues and organs from stress conditions (Perazzolli et al., 2004). Using maize somatic embryogenesis as a model system, Huang et al. (2014) demonstrated that suppression of *ZmPgb1.1* and *ZmPgb1.2* is sufficient to induce PCD in specific cells. Cells destined to die accumulated NO triggering the dismantling of the cell via metacaspase activity (Huang et al., 2014). As an extension of this work, Mira et al. (2016a, 2016b, 2016c) showed that over-expression of the same *ZmPgbs* alleviate cellular death in root apical cells subjected to low oxygen levels while their suppression aggravate the effects by triggering massive PCD along the root profile through an over-production of ethylene. A model emerging from both studies (Huang et al., 2014; Mira et al., 2016a, 2016b) suggests that Pgbs might act as key regulators ensuring plant cell survival under adverse conditions and that their suppression triggers the death program by elevating the levels of NO and ethylene. A possible intermediate in Pgb responses is the transcription factor MYC2, suppressed under conditions of reduced Pgb levels (or high NO) (Elhiti et al., 2013) and interfering with the synthesis/signalling of several hormones including ethylene (Kazan and Manners, 2013).

The observations that Pgbs are highly expressed in undifferentiated meristematic cells (reviewed by Stasolla and Hill, 2017) and that death of the same cells as a result of suppression of *Pgbs* is preceded by vacuolation, a sign of cellular differentiation (Mira et al., 2016a), suggests that the Pgb-mediated death program is a consequence of “terminal differentiation”. That is, suppression of *Pgbs* triggers the cellular differentiation pathway with cell death as the terminal event.

To establish a cause-effect relationship between Pgbs and cell differentiation and assess the involvement of NO and ethylene, in vitro TE trans-differentiation of Arabidopsis cells over-expressing or down-regulating two *Pgbs* (*Pgb1* and *Pgb2*, Hebelstrup et al., 2006) was examined to test if suppression of *Pgbs* encourages cell differentiation. We show that differentiation of TEs was delayed in cells over-expressing *Pgb1* and *Pgb2*, and encouraged in those cells where the expression of the two genes was reduced. This effect fits a model in which Pgbs lower the level of NO, a repressor of MYC2 which inhibits ethylene accumulation. Ethylene acts a positive regulator of TE formation by inducing the expression of genes required for procambial cell fate specification.

2. Materials and methods

2.1. Plant material and differentiation of tracheary elements (TEs) in vitro

Arabidopsis thaliana Col-0 in which *Phytoglobin 1* (*Pgb1*) was over-expressed (35S:Pgb1 line) or downregulated (Pgb1-RNAi line), as well as lines over-expressing *Phytoglobin 2* (*Pgb2*) (35S:Pgb2 line) or in which Pgb2 was mutated (*pgb2* line) were those characterized by Hebelstrup et al. (2006). The *myc2-1* mutant and the 35S:MYC2 lines were provided by Prof. Kazan (Dombrecht et al., 2007). Lines over-expressing *MYC2* in the *pgb2* mutant background (line 35S:MYC2/*pgb2*) were characterized by Elhiti et al. (2013).

Differentiation of TEs was conducted as described in Pesquet et al. (2010). Seeds were first sterilized in 70% ethanol containing 0.5% Triton X-100 for 15 min followed by 95% ethanol for 15 min, stratified on MS medium containing 3% sucrose and 100 $mg.L^{-1}$ myo-inositol for 24 h at 4 °C, and subsequently transferred to a growth cabinet (20–22 °C, 16 h light/8 h dark photoperiod). After one week, the roots were dissected and placed on callus induction medium [Gamborg's B5 medium supplemented with 2% sucrose, 0.5 $mg.L^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4 D), and 0.05 $mg.L^{-1}$ kinetin] for 4 weeks. Callus growth was encouraged on MS medium, containing 3% sucrose, and 1 $mg.L^{-1}$ 2,4-D) for 2 weeks. Calli were then transferred to liquid culture (MS medium with 3% sucrose, 1 $mg.L^{-1}$ 2,4-D, and 100 $mg.L^{-1}$ myo-inositol). All steps were conducted in the dark at 25 °C. For the induction of TEs the calli were washed three times in MS medium with 3% sucrose, and subsequently transferred in fresh liquid medium supplemented with 6 $mg.L^{-1}$ α-naphthalene acetic acid, 1 $mg.L^{-1}$ BAP, and 5 μM epibrassinolides.

2.2. Histological analyses

For histological examinations, cells (2 g FW in three biological replicates) were fixed in 2.5% glutaraldehyde and 1.6% paraformaldehyde in 0.05 M phosphate buffer (pH 6.9), followed by three washes with absolute ethanol, and then stained with toluidine blue (TBO) (Mira et al., 2016a).

Nitric oxide (NO) localization was performed using DAF-2DA (Mira et al., 2016a). Programmed cell death (PCD) was monitored with the In Situ Cell Death Detection Kit-Fluorescein (Roche), exactly as detailed by Mira et al. (2017a).

2.3. Chemical treatments

The NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) and the NO donor S-Nitroso-N-Acetyl-D,L-Penicillamine (SNAP) were applied at concentrations of 100 μM and 10 μM, respectively, to the TE induction media (Mira et al., 2016a, 2016c). The ethylene donor ethephon (ETH) and the ethylene biosynthetic inhibitor aminooxyacetic acid (AOA) were applied at 200 μM and 10 μM, respectively (Mira et al., 2015).

2.4. β-Glucuronidase assay

β-Glucuronidase (GUS) histochemical staining assay was performed using 2 g FW of cells as described by Sieburth and Meyerowitz (1997). A minimum of 20 samples per treatment were imaged.

2.5. DNA isolation and electrophoresis

To estimate DNA fragmentation, the samples (100 mg of cells in three biological replicates) were frozen and ground in liquid nitrogen. DNA extraction was performed using the Apop-ladder EX™ DNA fragmentation assay kit (Clontech Laboratories Inc.) according to the manufacturer's protocol. The samples were run on 2% agarose gels.

2.6. Ethylene measurements

Ethylene measurements were performed as previously described (Mira et al., 2016a). Briefly, 100–200 mg fresh weight of tissue was incubated in a sealed 3 mL syringe for 3 h in the dark at 22 °C. The gas (1 mL) accumulated in the headspace was analyzed with a Bruker 450-GC Gas chromatograph. Data analysis was carried out using the Bruker Compass Data analysis 3.0 software. All experiments were performed in triplicate.

2.7. Expression studies

RNA extraction was carried out using the TRI Reagent Solution

according to the manufacturer's protocol (Invitrogen). The total RNA was treated with DNase I recombinant, RNase-free (Roche) and the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for cDNA synthesis. Quantitative RT-PCR was performed as described in Mira et al. (2016a,b,c). All primers used for gene expression studies are listed in http://www.plantphysiol.org/cgi/content/full/pp.114.239335/DC1, Supplementary Table 1. The relative gene expression level was analyzed with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) using Elongation factor 1α4 as the reference gene. For this gene Ct values at each experimental time point are shown in Supplemetary Table 2. Experiments were conducted using three biological replicates.

2.8. Statistical analysis

Data were analyzed by one way ANOVA using the SPSS program (IBM Corp. Released, 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp.). Treatments means were compared by Duncan's test ($\alpha = 0.05$) to differentiate the significance of difference between various parameters. Linear regression analyses were performed using SigmaPlot 10 (Systat Software, Inc., San Jose, CA).

3. Results

3.1. Phytoglobins (Pgbs) influence TE differentiation

The function of Pgbs during cellular differentiation was investigated using an established in vitro system in which parenchyma cells are induced to differentiate into tracheary elements (TE) (Oda et al., 2005; Pesquet et al., 2010). In WT tissue, the expression of both Arabidopsis *Pgb1* and *Pgb2* decreased during the first 3 days of TE differentiation (Fig. 1A), and this also correlated with the reduced signal observed in the Pgb1:GUS and Pgb2:GUS reporter lines (Supplemental Fig. 1). As expected, the levels of *Pgb1* and *Pgb2* transcripts were highest in the respective 35S:Pgb1 and 35S:Pgb2 lines and lowest in the Pgb1-RNAi and *pgb2* lines (Fig. 1A). The over-expression of one Pgb also resulted in a marginal increase in the expression of the other Pgb gene (Fig. 1A).

Formation of TE was visible after 3 days in WT cells and, after 8 days in culture, about 15% of the same cells differentiated into TEs (Fig. 1B). Suppression of *Pgb1* [Pgb1-RNAi line] or knock out of *Pgb2* (*pgb2* line) significantly increased this percentage which reached values close to 35% at day 8 in culture. An opposite trend was observed in cells over-expressing the two *Pgb* genes [35S:Pgb1 and 35S:Pgb2 lines] where the percentage of TE formed did not exceed 10% (Fig. 1B). Spiral, reticulate and pitted TEs were observed in all the lines, irrespective of the level of the two *Pgbs* (Supplemental Fig. 2).

In agreement with the function of Pgbs as NO scavengers (Hebelstrup et al., 2006), cells suppressing either *Pgb1* or *Pgb2* stained more intensely for NO (Supplemental Fig. 3A), and manipulations in NO content (by SNAP, a NO releasing agent, and cPTIO, a NO scavenger) influenced the behaviour of the cells. In cells over-expressing *Pgbs* (depleted in NO), TE differentiation was restored by increasing NO (by SNAP), while in cells suppressing *Pgbs* (over-accumulating NO) TE differentiation was reduced by lowering NO (by cPTIO) (Supplemental Fig. 3B). Similar treatments were performed in WT cells where the combined supplementation of SNAP + cPTIO generated a percentage of TE differentiation intermediate between SNAP or cPTIO applied individually (Supplemental Fig. 3B), thus confirming the specificity of the two compounds in altering NO homeostasis.

3.2. Transcriptional modulation of xylem-specific genes by Pgbs

One of the earliest master regulators of xylem differentiation is the homeodomain-leucine zipper (HD-Zip) III ATHB8, required for the stabilization of procambial cell specification (Donner et al., 2009). The expression of this gene is needed to induce the NAC transcription

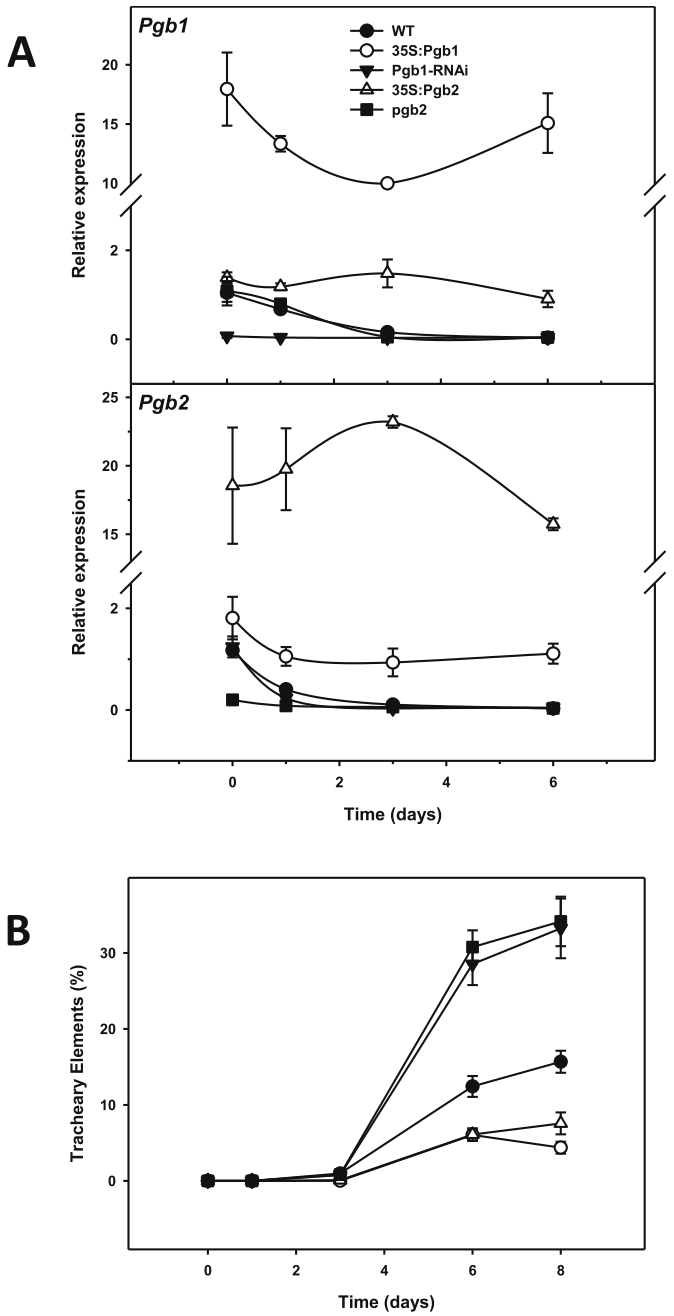

Fig. 1. Effect of *Pgbs* on the in vitro differentiation of TEs. **(A)** Relative transcript abundance of *Pgb1* and *Pgb2* during the differentiation process in WT cells, cells in which the level of *Pgb1* was over-expressed (35S:Pgb1) or down-regulated (Pgb1-RNAi), and cells in which *Pgb2* was over-expressed (35S:Pgb2) or mutated (*pgb2*). Values, means ± SE of at least three biological replicates are normalized to the WT values set at 1. **(B)** Percentage of tracheary elements formed in the WT and transgenic lines. Values are means ± SE of at least three biological replicates.

factors VND6 and 7, which trigger the differentiation process through modification of cell wall components and the execution of programmed cell death (PCD) (Kubo et al., 2005) (Supplemental Fig. 4). During TE differentiation, the expression of *ATHB8* increased from day 0 in cells suppressing *Pgbs*, while it remained low in WT cells and cells over-expressing either *Pgb* (Fig. 2). An incremental increase in *ATHB8* expression, albeit more moderate compared to that observed in the *Pgb* suppressing lines, was observed in the 35S:Pgb1 line during the second half of the culture period (Fig. 2). The transcript levels of *VND6* and *7* remained constant in the 35S:Pgb1 and 35S:Pgb2 lines, while the levels

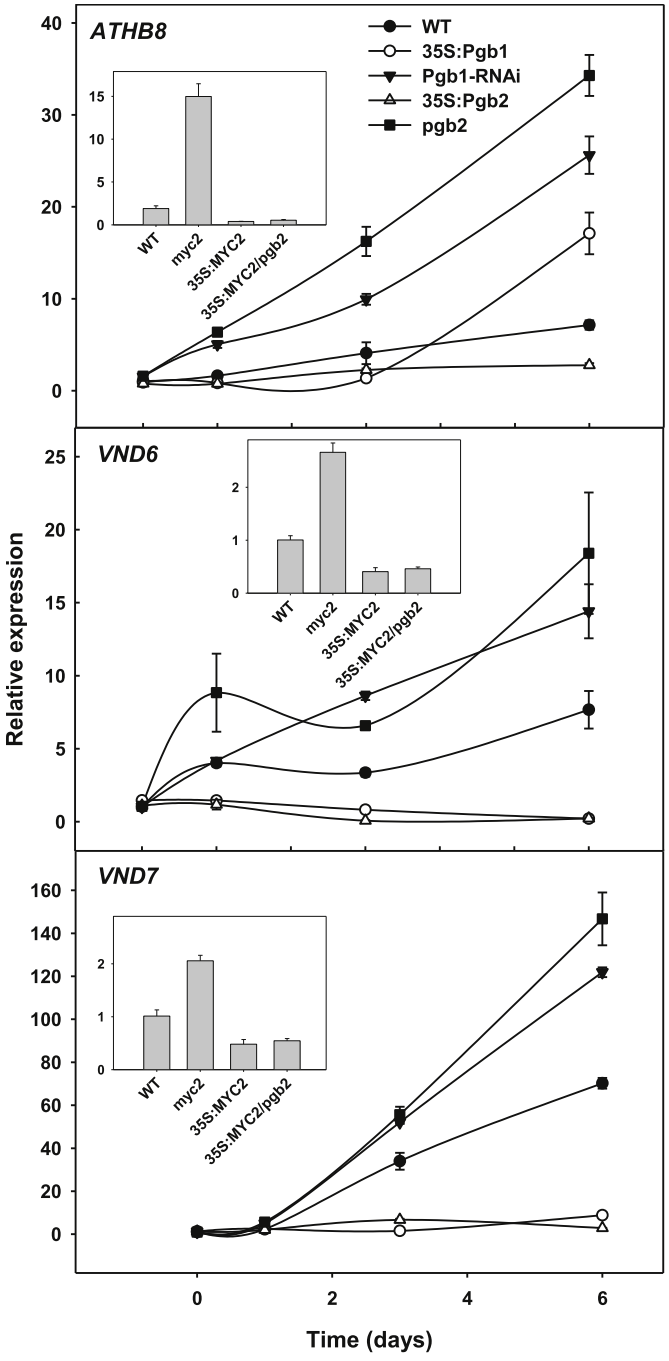

Fig. 2. Effect of Pgbs on the expression of early transcription factors of TE differentiation. Relative transcript abundance of *ATHB8*, *VND6*, and *VND7* during the differentiation process in WT cells, cells in which the level of *Pgb1* was over-expressed (35S:Pgb1) or down-regulated (Pgb1-RNAi), and cells in which *Pgb2* was over-expressed (35S:Pgb2) or mutated (*pgb2*). Values, means ± SE of at least three biological replicates, are normalized to the WT values set at 1. Insets show the expression of the same genes at day 1 in WT cells, cells with mutated *MYC2* (*myc2*), and cells over-expressing *MYC2* in a WT (35S:MYC2) or *pgb2* (35S:MYC2/*pgb2*) background.

increased in the other cells, especially in those suppressing the two *Pgbs* (Pgb1-RNAi and *pgb2* lines). This increase was observed after day 0 for *VND6* and after day 1 for *VND7* (Fig. 2).

Downstream components of VND6 and 7 signalling are executors of the death program, comprising the degradative enzymes xylem cysteine protease 1 (XCP1), ribonuclease 3 (RNS3) and metacaspase 9 (MC9), as well as regulators of cell wall, such as the transcription factors MYB 83

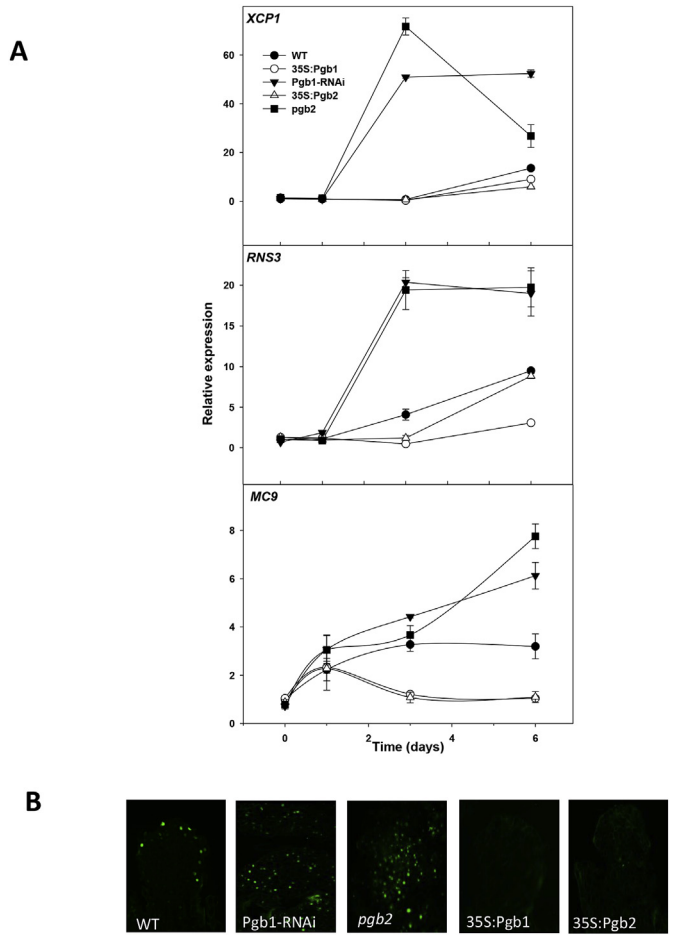

Fig. 3. Effect of Pgbs on the expression of genes regulating programmed cell death (PCD) during TE differentiation. **(A)** Relative transcript abundance of *XCP1*, *RNS3*, and *MC9* during the differentiation process in the WT cells, cells in which the level of *Pgb1* was over-expressed (35S:Pgb1) or down-regulated (Pgb1-RNAi), and cells in which *Pgb2* was over-expressed (35S:Pgb2) or mutated (*pgb2*). Values, means ± SE of at least three biological replicates, are normalized to the WT values set at 1. **(B)** TUNEL staining showing nuclei of cells undergoing PCD in WT and transgenic cells after 3 days in culture.

and 103, implicated in secondary wall biogenesis, and the cellulose biosynthetic enzymes cellulose synthase 4 (CESA4) and 8 (CESA8) (Ohashi-Ito et al., 2010) (Supplemental Fig. 4). The expression of *XCP1* and *RNS3* rose markedly in Pgb1-RNAi and *pgb2* cells from day 1 to day 3, while it remained low in the other cells, especially those over-expressing the two *Pgbs* (35S:Pgb1 and 35S:Pgb2 lines) (Fig. 3A). A similar profile was also observed for *MC9* especially during the second half of the culture period (Fig. 3A). The overall transcriptional induction of the executors of the death program under condition of low *Pgb* levels was also confirmed by extensive TUNEL staining (Fig. 3B) and DNA fragmentation (Supplemental Fig. 5) performed at day 3. Both are indicators of PCD.

Among the cell wall regulators, the transcription factors *MYB83* and *103* exhibited similar expression patterns with an induction in *Pgb* suppressing cells especially during the last 3 days of TE differentiation. A more moderate increase in expression of the same genes was observed in the other cell types (Fig. 4). Of the two cellulose synthase genes *CESA4* and *8*, a rapid increment in transcript level was measured between day 3 and 6 for *CESA4* in Pgb1-RNAi cells and for *CESA8* in *pgb2* cells. Relative to WT, the expression of both genes was reduced in lines suppressing *Pgbs* during the last days in culture (Fig. 5).

To assess the participation of NO in the transcriptional induction of TEs, the expression of *ATHB8*, the most upstream component of the

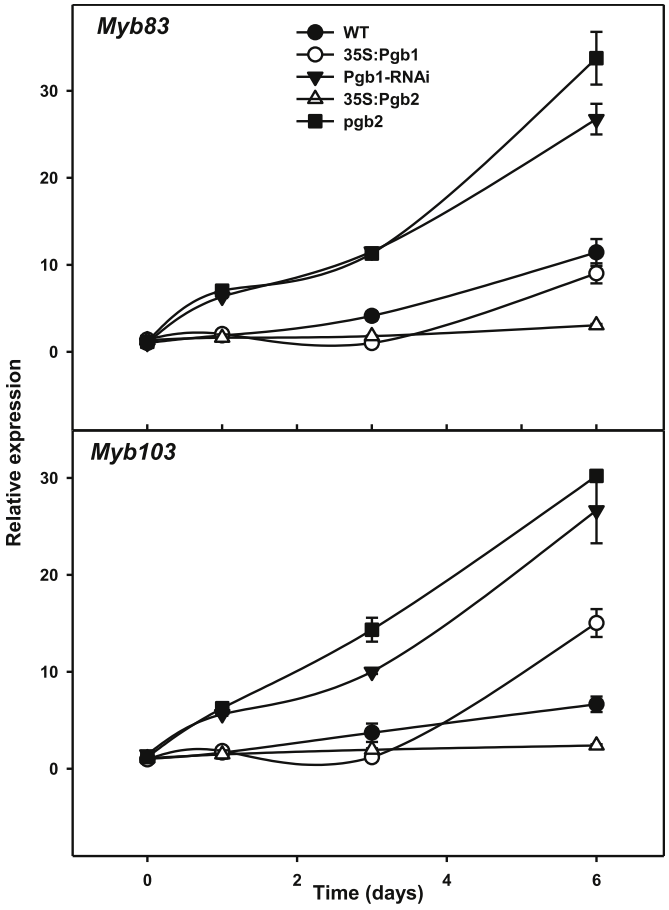

Fig. 4. Effect of Pgbs on the expression of selected *MYB* transcription factors linked to TE differentiation. Relative transcript abundance of *MYB83* and *103* during the differentiation process in WT cells, cells in which the level of *Pgb1* was over-expressed (35S:Pgb1) or down-regulated (Pgb1-RNAi), and cells in which *Pgb2* was over-expressed (35S:Pgb2) or mutated (pgb2). Values, means ± SE of at least three biological replicates, are normalized to the WT values set at 1.

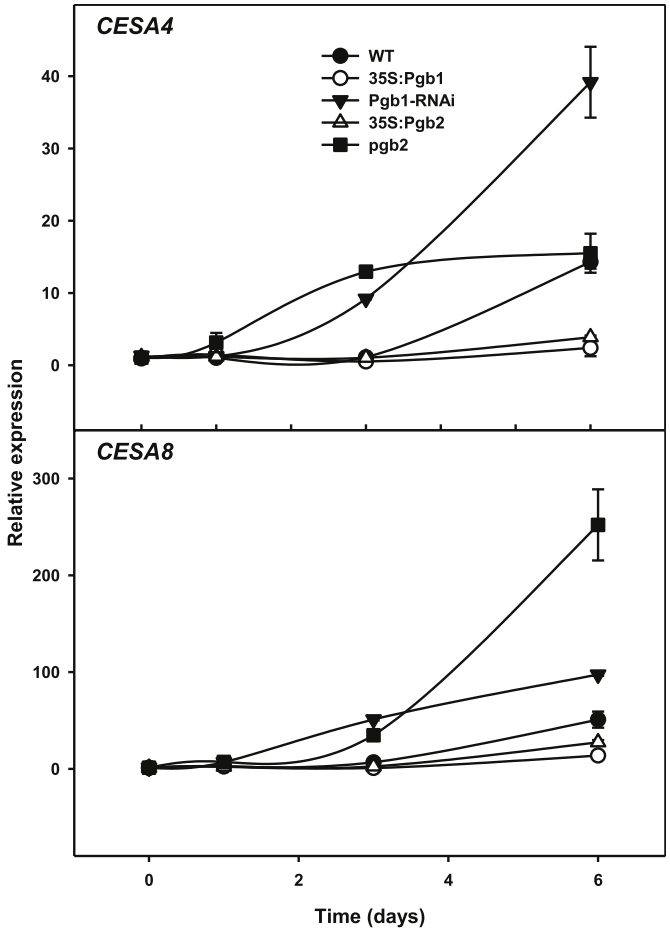

Fig. 5. Effect of Pgbs on the expression of selected cellulose synthase genes involved in TE differentiation. Relative transcript abundance of *CESA4* and *8* during the differentiation process in WT cells, cells in which the level of *Pgb1* was over-expressed (35S:Pgb1) or down-regulated (Pgb1-RNAi), and cells in which *Pgb2* was over-expressed (35S:Pgb2) or mutated (*pgb2*). Values, means ± SE of at least three biological replicates, are normalized to the WT values set at 1.

transcriptional cascade (Supplemental Fig. 4) was measured at day 1 in culture, following applications of SNAP or cPTIO. In the two lines down-regulating *Pgbs*, accumulating NO (Supplemental Fig. 3A) and inducing *ATHB8* at day 1 (Fig. 2), applications of cPTIO was sufficient to reduce the expression of the same gene (Supplemental Fig. 6). An increase in NO by SNAP resulted in an induction of *ATHB8* in the two *Pgb* up-regulating lines depleted in NO (Supplemental Fig. 3A). The ability of cPTIO and SNAP to modulate *ATHB8* expression was also confirmed in WT cells (Supplemental Fig. 6).

Collectively, these results suggest Pgbs influence PCD and in vitro xylogenesis with *Pgb* suppression inducing the formation of TEs and *Pgb* over-expression repressing their differentiation. These effects are mediated by NO through the ability of Pgbs to scavenge NO.

3.3. The transcription factor MYC2 modulates the Pgb response

The transcription factor MYC2 fine-tunes several hormonal responses (Dombrecht et al., 2007). In Arabidopsis the NO inhibition of MYC2 mediates the Pgb regulation of somatic embryogenesis (Elhiti et al., 2013). The expression levels of *MYC2* during TE differentiation is highest in the lines over-expressing *Pgbs* (35:Pgb1 and 35S:Pgb2 lines), especially during the initial phases of the culture period (Fig. 6A). Relative to WT, lines suppressing *Pgbs* (Pgb1-RNAi and *pgb2* lines) exhibited the lowest levels of *MYC2* transcripts. To better assess the involvement of MYC2 during xylogenesis and its link with Pgbs, differentiation of TE was induced in a *myc2* mutant line, a line over-expressing *MYC2* (35S:MYC2), and a line overexpressing *MYC2* in a *pgb2* mutant background (35S:MYC2/*pgb2*) which was characterized in previous studies (Elhiti et al., 2013). Relative to WT, differentiation of TEs was promoted in *myc2* cells (Fig. 6B).

Over-expressing *MYC2* independently of the genetic background (35S:MYC2 and 35S:MYC2/pgb2) slightly reduced the percentage of TEs (Fig. 6B). The observations that *MYC2* expression can be influenced by altering NO pharmacologically, with high levels of NO (by SNAP) suppressing the expression and low levels (by cPTIO) inducing the expression (Supplemental Fig. 7), and the MYC2 effect on TE differentiation is independent of NO modulations, i.e. SNAP and cPTIO treatments do not alter TE formation in the *MYC2* transgenic lines, (Supplemental Fig. 3B), supports a model in which NO acts upstream of MYC2 where it inhibits its expression and action on TE differentiation.

The promotive effect of *MYC2* suppression on TE differentiation was further examined at a transcriptional level. The three key regulators of TE differentiation, ATHB8, VND6 and 7 were induced in the *myc2* mutant and repressed slightly in cells over-expressing *MYC2* in a WT background (35S:MYC2) or in the *pgb2* background (35S:MYC2/*pgb2*) (Fig. 2).

Collectively, these data indicate that the Pgb regulation of TE differentiation is mediated by *MYC2* through NO, with elevated levels of NO, as a result of Pgb suppression, inhibiting *MYC2* and promoting the

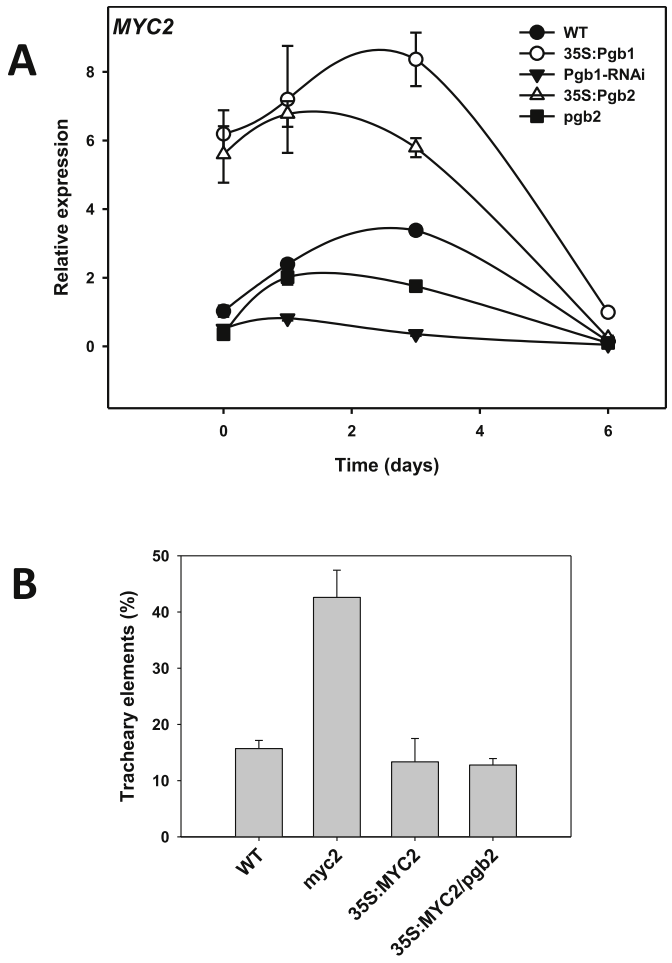

Fig. 6. Expression of *MYC2* influences TE differentiation. **(A)** Expression of *MYC2* during the differentiation process in WT cells, cells in which the level of *Pgb1* was over-expressed (35S:Pgb1) or down-regulated (Pgb1-RNAi), and cells in which *Pgb2* was over-expressed (35S:Pgb2) or mutated (*pgb2*). Values, means ± SE of at least three biological replicates are normalized to the WT values set at 1. **(B)** Percentage of tracheary elements in WT cells, cells with mutated *MYC2* (*myc2*), and cells over-expressing *MYC2* in a WT (35S:MYC2) or *pgb2* (35S:MYC2/*pgb2*) background.

differentiation of TEs.

3.4. Ethylene acts downstream of the Pgb and MYC2 regulation of TE differentiation

Ethylene is an inductive factor of TE differentiation (Pesquet and Tuominen, 2011), and plays an integral role in Pgb responses to abiotic stress (Mira et al., 2017b). At day 1 during the TE differentiation process, ethylene accumulated preferentially in those situations where NO levels increased as a result of *Pgb* suppression (Pgb1-RNAi and *pgb2* lines) or where *MYC2* was repressed (*myc2* line) (Fig. 7). Conditions depleting NO (35S:Pgb1 and 35S:Pgb2 lines) or elevating MYC2 in a WT or *pgb2* background (35S:MY2 and 35S:MYC2/*pgb2* lines) diminished ethylene content (Fig. 7). The requirement of NO for ethylene accumulation was also demonstrated pharmacologically in WT cells (Supplemental Fig. 8). To further establish the link between NO, ethylene and MYC2, the content of ethylene was manipulated under varying levels of NO and *MYC2*. An experimental reduction of ethylene (by aminooxyacetic acid, AOA) under conditions of high NO (Pgb1-RNAi and *pgb2* lines) or low *MYC2* expression (*myc2* line) was sufficient to reverse the inductive effect of NO or *MYC2* repression on TE differentiation (Supplemental Fig. 3B). Similarly, a rise of ethylene (by

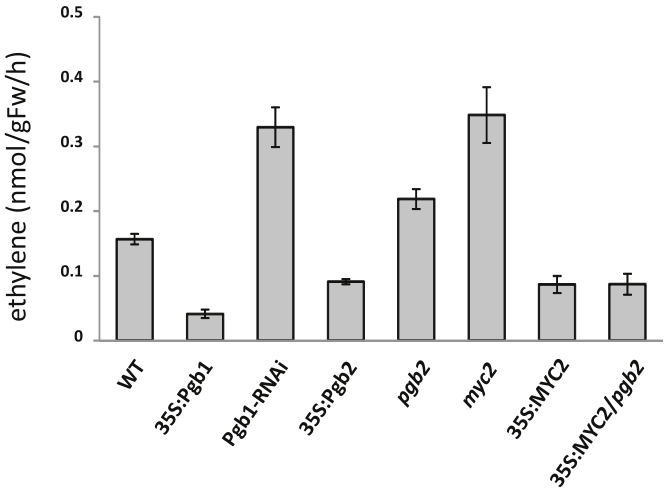

Fig. 7. Ethylene levels at day 1 in WT cells, cells in which the level of *Pgb1* was over-expressed (35S:Pgb1) or down-regulated (Pgb1-RNAi), cells in which *Pgb2* was over-expressed (35S:Pgb2) or mutated (*pgb2*), cells with mutated *MYC2* (*myc2*), and cells over-expressing *MYC2* in a WT (35S:MYC2) or *pgb2* (35S:MYC2/*pgb2*) background. Values are means ± SE of at least three biological replicates.

ethephon, ETH) in a low NO environment (WT, 35S:Pgb1 and 35S:Pgb2 lines) or elevated MYC2 levels (35S:MYC2 and 35S:MYC2/*pgb2* line) induced the formation of TEs (Supplemental Fig. 3B).

The effects of ethylene manipulation on TE differentiation were most likely due to changes in the transcript level of the master transcription factor *ATHB8*. The expression level of this gene at day 1 increased under conditions of elevated ethylene by ETH, but decreased following a reduction of ethylene by AOA (Supplemental Fig. 6). The observation that in WT cells applications of AOA reversed the effect of SNAP, and applications of ETH that of cPTIO, suggests that ethylene acts downstream of NO in TE differentiation.

4. Discussion

Several pieces of evidence demonstrate an unequivocal link between formation of TEs and levels of *Pgbs*, with a suppression of *Pgbs* promoting the differentiation process. First, the full differentiation of TEs visible after 8 days in culture (Supplemental Fig. 2) is accompanied by a decline in *Pgb* expression (Fig. 1A and Supplemental Fig. 1). Second, the differentiation process is encouraged in cells suppressing either *Pgb1* (Pgb1-RNAi line) or *Pgb2* (*pgb2* line), and compromised when the two *Pgbs* are over-expressed (35S:Pgb1 or 35S:Pgb2 lines) (Fig. 1B). In agreement with the documented NO scavenging properties of Pgbs (Hebelstrup et al., 2006), the effect of Pgbs on TE differentiation is mediated by NO. Nitric oxide accumulates preferentially in *Pgb* down-regulating cells (Supplemental Fig. 3A), and pharmacological alterations of NO levels (increasing NO in lines over-expressing *Pgbs* or decreasing NO in those suppressing *Pgbs*) revert the percentage of TE differentiation close to WT values (Supplemental Fig. 3B). The influence of NO alterations on TE differentiation was also confirmed by TUNEL analyses (Supplemental Fig. 9).

The TE regulation by Pgbs, and its mediation by NO, appears to be executed through the transcriptional induction of master regulators of xylogenesis, including *ATHB8* which is induced very early under those conditions suppressing Pgbs (Fig. 2) or increasing NO (Supplemental Fig. 6). Expression of this gene is required for procambial cell specification and fate acquisition, and coordination of procambium formation *in vivo* (Donner et al., 2009), as it activates the vascular-related NAC domain transcription factors *VND6* and *7* which are able to induce the trans-differentiation of metaxylem elements (Kubo et al., 2005). Abnormalities in vessel element formation, not noted in our work, were

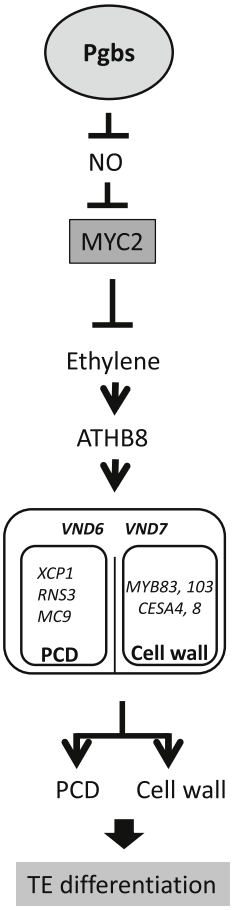

Fig. 8. Proposed mode of action of Pgbs during the differentiation of TEs. By scavenging NO, Pgbs modulate the expression of *MYC2*, a repressor of ethylene inducing the expression of the transcription factor ATHB8. Induction of this transcription factor triggers downstream signalling components involved in programmed cell death (PCD) (XCP1, RNS3, and MC9) and cell wall modification (MYB83, 103, and CESA 4, 8) required for the differentiation of parenchyma cells into TEs.

observed following suppression of either gene (Kubo et al., 2005; Yamaguchi et al., 2008). Both VND6 and 7 directly regulate genes triggering PCD (*XCP1*, *RNS3*, and *MC9*) and cell wall modification (*MYB83* and *103*, and *CESA 4*, and *8*) (Zhong et al., 2008) (Supplemental Fig. 4). The expression of all these genes is induced in cells down-regulating *Pgbs* and repressed in those where the levels of *Pgbs* are up-regulated (Figs. 3–5).

From data presented here, it is suggested that a central player of the Pgb regulation of TE trans-differentiation is MYC2, a bHLH domain-containing transcription factor (Dombrecht et al., 2007) located at the interphase of several hormonal pathways participating in abiotic stress (Guerineau et al., 2003) and developmental (Maurya et al., 2015) responses. The expression of *MYC2* is lowest in cells suppressing *Pgbs* and highest in those where the two *Pgbs* are up-regulated (Fig. 6A). The Pgb modulation of MYC2 is most likely exercised through NO, as a depletion of NO (by cPTIO) reverts the *MYC2* suppression observed in the Pgb1-RNAi and *pgb2* lines, while an increment of NO (by SNAP) reduces *MYC2* expression in the 35S:Pgb1 and 35S:Pgb2 lines (Supplemental Fig. 7). This regulation pattern is consistent with that observed during Arabidopsis somatic embryogenesis (Elhiti et al., 2013; Mira et al., 2016c). Suppression of *MYC2* is sufficient to transcriptionally induce *ATHB8, VND6*, and *7* (Fig. 2), and promote differentiation of TEs (Fig. 6B). These events are precluded under conditions when *MYC2* is constitutively expressed in a WT (35S:MYC2) or pgb2 (35S:MYC2/*pgb2*) background. The behaviour of the 35S:MYC2/*pgb2* cells clearly suggests a MYC2 mediation in the Pgb2 control of these events.

Several studies have demonstrated the dependence of TE formation on ethylene. Ethylene synthesis and accumulation have a stimulatory effect on the rate of TE differentiation (Pesquet and Tuominen, 2011). Consistent with the previously documented elevation of ethylene levels in cells suppressing *Pgbs* (Mira et al., 2017b), ethylene accumulates preferentially when either *Pgb* (Pgb1-RNAi and *pgb2* lines) or MYC2 (*myc2* line) is repressed while it decreases slightly under those conditions elevating *Pgb* (35S:Pgb1 and 35S:Pgb2 line) or *MYC2* (35S:MYC2 and 35S:MYC2/*pgb2* lines) (Fig. 7). Furthermore, the fact that an experimental rise in ethylene by ETH promotes TE formation in the lines over-expressing *Pgbs* or *MYC2* (conditions inhibiting differentiation), and that a depletion of ethylene by AOA reduces the number of TEs in lines suppressing *Pgbs* or *MYC2* (conditions favoring differentiation) suggests ethylene as a downstream component of Pgb action. While the stimulatory role played by ethylene in the trans-differentiation of TEs is not well defined, we suggest it might affect the transcription of early master transcription factors, as shown by the early induction of *ATHB8* with ETH and its repression with AOA (Supplemental Fig. 6).

5. Conclusions

Based on our results we proposed a model linking Pgbs and MYC2 to previously characterized components of TE differentiation (Fig. 8). In this model Pgbs lower the level of NO, a repressor of MYC2 which inhibits ethylene accumulation. Ethylene acts a positive regulator of TE formation inducing the expression of *ATHB8*, required to activate the transcriptional cascade culminating in PCD and modifications of cell wall components which are key steps of the trans-differentiation process (Fig. 8). It is speculated that this model, in which Pgbs act as inhibitors of cell differentiation, might also operate *in vivo* systems. Phytoglobins are in fact present in meristematic (undifferentiated) domains (Stasolla and Hill, 2017) and their suppression triggers morphological changes typical of differentiation and PCD (Mira et al., 2017a). Thus, besides their well-established involvement in stress responses, Pgbs likely govern broader aspects of growth and development involving cell fate acquisition.

Declarations of interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Mohamed M. Mira: Formal analysis. **Katarzyna Ciacka:** Investigation. **Robert D. Hill:** Writing-original draft. **Claudio Stasolla:** Writing-original draft, Supervision, Writing – review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2018.11.036.

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