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Ethylene is integrated into the nitric oxide regulation of *Arabidopsis* somatic embryogenesis



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Abstract The study confirms the role of the two *Arabidopsis* hemoglobin genes (*Glb1* and *Glb2*) during somatic embryogenesis and proposes the involvement of ethylene in the regulation of embryo development. Suppression of both *Glb1* and *Glb2* results in accumulation of nitric oxide (NO) and a different embryogenic response. Compared to WT tissue, down-regulation of *Glb1* (*Glb1* RNAi line) compromises the embryogenic process, while repression of *Glb2* (*Glb2*–/– line) increases the number of embryos. These differences were ascribed to the differential accumulation of NO in the two lines, as *Glb1* is a more effective NO scavenger compared to *Glb2*. A high elevation of NO level [achieved pharmacologically using the NO donor sodium nitroprusside (SNP), or genetically using the *Glb1* suppressing line], activated the two ethylene biosynthetic genes 1-aminocyclopropane-1-carboxylate synthase (*ACC synthase*) and 1-aminocyclopropane-1-carboxylate oxidase (*ACC oxidase*). Ethylene accumulation repressed embryogenesis, as shown by the decreased embryo number observed in tissue treated with the ethylene releasing agent Ethepon (ETH), as well as by the increased embryo production obtained with the two ethylene insensitive mutant lines (*ein2-1* and *ein3-1*). A repression in ethylene level increased the expression of many auxin biosynthetic genes and favored the accumulation of the auxin indole-acetic acid (IAA) at the sites of the explants where embryogenic tissue will form. Collectively these data reveal that high levels of NO, generated by the *Glb1* suppressing line, but not by the *Glb2* suppressing line, might increase the level of ethylene, which represses the production of auxin. Auxin is the inductive signal required for the formation of the embryogenic tissue.

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1. Introduction

Plant hemoglobins (Hb) were discovered in the early 20th century [1], and are mainly involved in oxygen transport and nitric oxide (NO) scavenging. Hemoglobins can be classified into 3 main classes; class 1 includes non-symbiotic Hbs, class 2 symbiotic Hbs, and class 3 truncated Hbs. In *Arabidopsis*, two Hb

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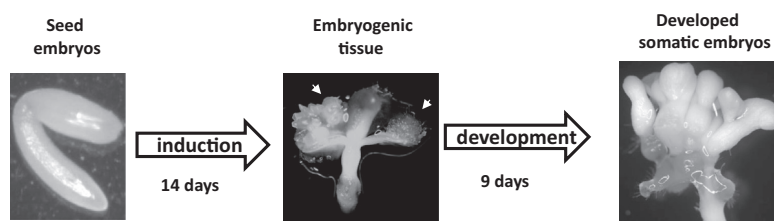


Figure 1 The *Arabidopsis* somatic embryogenic system. Seed embryos are dissected and placed on an auxin-containing induction medium for 14 days. During this time the embryogenic tissue (arrows) forms from the cotyledons of the explants. Transfer of the tissue on an auxin-free development medium for 9 days results in the formation of fully mature somatic embryos.

genes have been characterized: *Glb1* (class 1) and *Glb2* (class 2) which are encoded by single genes [12,51,54]. Classes 1 and 2 are similar in structure to animal myoglobins and human globins [51], while class 3 globins are closer to truncated globins from prokaryotes [54]. Class 1 Hbs have high affinity for oxygen comparing to class 2 Hb, while class 3 Hbs have the weakest oxygen binding ability [12].

Hemoglobins are expressed in many organisms including bacteria, fungi and plants [26,53], where they participate in many tasks, such as oxygen transport and NO scavenging [12,15,20]. Also, there are several recent studies showing that modulation of class 1 Hb levels may affect development and morphogenetic processes in plants [15,14]. Plant Hbs are involved in dormancy breakage by modulating NO and ethylene that control abscisic acid (ABA) metabolism and signaling pathways [2]. Recently, it was reported that Hbs play also an effective role in somatic embryogenesis through auxin modulation [10].

Somatic embryogenesis is a process where somatic embryos, similar in morphology and structure to seed embryos, are produced by somatic cells in culture [60,22,5].

Somatic embryogenesis was first described almost 50 years ago by Steward et al. [48] who were able to produce viable embryos from isolated carrot cells. This system was recognized as a model to study the regulatory mechanisms underlying early events in plant embryogenesis [60,5].

Auxin biosynthesis and distribution are critical for plant embryogenesis [7,49,3]. Quadruple mutations of *YUCs*, key enzymes in auxin biosynthesis, impair distribution of auxin resulting in severe developmental defects such as the absence of hypocotyls or root meristem. These studies indicate that depletion in auxin synthesis and/or transport compromises embryogenesis [7]. During the induction phase of *Arabidopsis* somatic embryogenesis, auxin polar transport, mediated by *PINI*, is essential for the establishment of auxin gradients and the formation of somatic embryos [3].

Evidence indicates that non-symbiotic Hbs influence and modify the auxin signaling and subsequently somatic embryogenesis by modulating the endogenous NO levels [10]. Nitric oxide is tightly linked to many hormones such as auxin and ethylene [27,59,32,34]. The two *Arabidopsis* Hbs: *Glb1* and *Glb2* have been shown to scavenge NO. Compared to *Glb2*,

Table 1 Primers used for the quantitative (q)RT-PCR results.

Gene	Sequence	Acc. No.	Name
UBQ10-F	AACTTTGGTGGTTTGTGTTTTGG	AT4G05320	UBIQUITIN 10
UBQ10-R	TCGACTTGTTCATTAGAAAGAAAAGAGATAA	AT4G05320	UBIQUITIN 10
ACCO _x -F	CAAACCTCTCTCGGTACACAATGA	AT2G19590	ACC OXIDASE
ACCO _x -R	GGATGAATGCGAGGCCAATA	AT2G19590	ACC OXIDASE
ACCS _{YN} -F	GCGCTTTGGCGAGTTATTATC	AT3G61510	ACC SYNTHASE
ACCS _{YN} -R	GGAGTGTGTCTTCGTCATATT	AT3G61510	ACC SYNTHASE
ERF1-F	CCGCTCCGTGAAGTTAGATAAT	AT3G23240	ETHYLENE RESPONSE FACTOR 1
ERF1-R	TCTTTCACCAAGTCCCCTATT	AT3G23240	ETHYLENE RESPONSE FACTOR 1
ERF10-F	CGAGTTTGTCTGACCAGTTT	AT1G03800	ETHYLENE RESPONSE FACTOR 10
ERF10-R	GGTTCATTTCGACGTTACA	AT1G03800	ETHYLENE RESPONSE FACTOR 10
ASA1-F	ACAAGGATGCTAACAAACGGCGTG	AT1G19920	ATP SULFURYLASE ARABIDOPSIS 1
ASA1-R	TCTGGCACTCACAGTGTTCGTCTT	AT1G19920	ATP SULFURYLASE ARABIDOPSIS 1
Yuc4-F	CTAACGGATGAAAAGGAGAGAAG	AT4G32540	YUCCA 4
Yuc4-R	GCGATCTTAACGGCGTCATA	AT4G32540	YUCCA 4
AMI1-F	ATCTCGTCGGTGAAGCCAGAGTTT	AT1G08980	AMIDASE 1
AMI1-R	CCGAGCAAAGTTGAAAGAGCCGTT	AT1G08980	AMIDASE 1
IGPS-F	TCTTGGAGGAGATCACATGG	AT2G04400	INDOLE-3-GLYCEROL PHOSPHATE SYNTHASE
IGPS-R	GGAGGAGCATCCTCTACAGC	AT2G04400	INDOLE-3-GLYCEROL PHOSPHATE SYNTHASE
PAI3-F	ACACAACACCTTTCAAACCCGTGG	AT1G29410	PHOSPHORIBOSYLANTHRANILATE ISOMERASE 3
PAI3-R	CAAAGCACTGCACTGAGCCATGAT	AT1G29410	PHOSPHORIBOSYLANTHRANILATE ISOMERASE 3
CYP79B2-F	ATGCTCGGAGACTTCTTCAAGGT	AT4G39950	CYTOCHROME P450, FAMILY 79, SUBFAMILY B, POLYPEPTIDE 2
CYP79B2-R	AGATGCTCCGGCAATCTAAGGTCA	AT4G39950	CYTOCHROME P450, FAMILY 79, SUBFAMILY B, POLYPEPTIDE 2

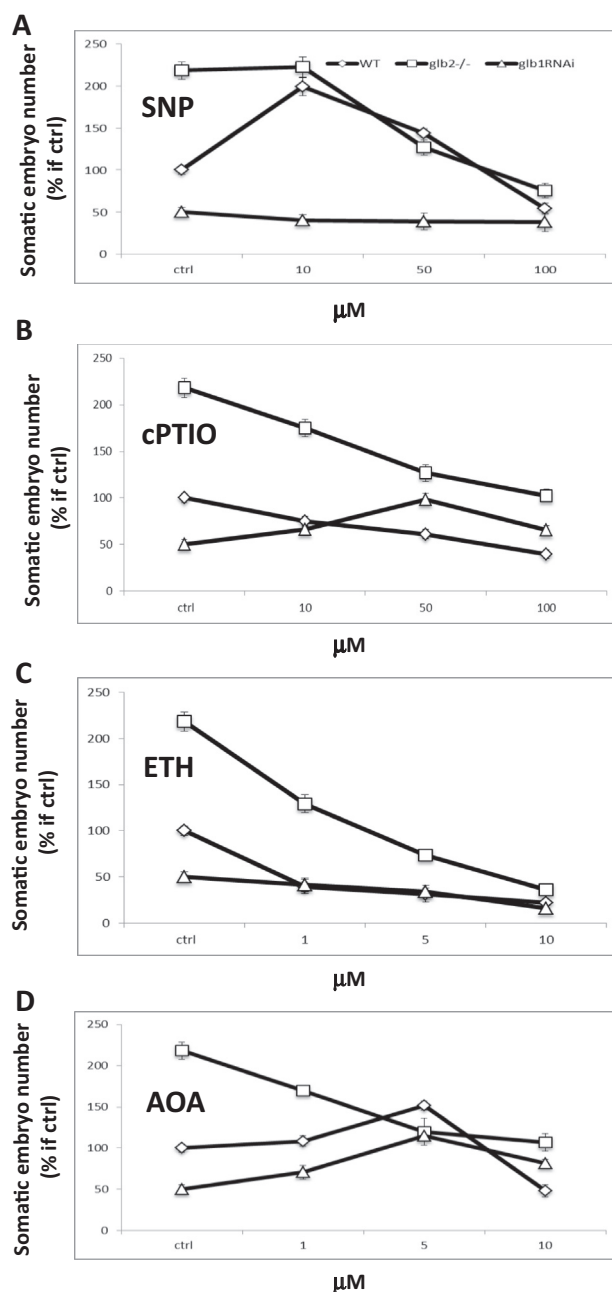


Figure 2 Number of somatic embryos [expressed as a percentage of WT control (ctrl)] collected from the WT, *Glb1 RNAi* and *Glb2*^{-/-} mutant lines treated with increasing levels of NO donor sodium nitroprusside (SNP) (A), the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (cPTIO) (B), the ethylene releasing agent Ethephon (ETH) (C), and the ethylene biosynthetic inhibitor O-(carboxymethyl)hydroxylamine hemihydrochloride (AOA) (D). Compounds were added in the induction medium. Values are means \pm SE of at least three biological replicates.

Glb1 is a more efficient NO scavenger, as shown by the high levels of NO accumulating in *Glb1*-suppressing tissue [18].

Ethylene regulates a number of developmental and physiological processes such as seed germination, root hair development, root nodulation, abscission, flower senescence and fruit ripening [31,58,57,36]. Production of this plant growth

regulator is tightly regulated by internal signals during development and in response to environmental stimuli triggered by biotic and abiotic stresses. Ethylene synthesis is also induced during the excision of plant explants utilized for the induction of somatic embryogenesis [23,6,28]. Hence, the role of this hormone has been examined in various culture systems. The regulation of ethylene biosynthesis and ethylene signaling influences the efficiency of *de novo* organogenesis. Ethylene was in fact shown to modulate the formation and development of somatic embryos [3,35,37]. The inhibition of ethylene production by AgNO_3 or CoCl_2 induces somatic embryogenesis in coffee [25] and *Arabidopsis* [3]. In line with these observations, Chen and Chang [7] observed that low levels of 1-aminocyclopropane-1-carboxylic acid (*ACC*), the precursor of ethylene, compromise the formation of embryos in *Oncidium* leaf cultures, whereas high levels elevated the frequency of somatic embryos.

Auxin and ethylene interaction is at the center of many developmental processes [11,33,29,40,46,47,13]. Exogenous auxin induced ethylene biosynthesis in root tips by activating several genes including those encoding 1-aminocyclopropane-1-carboxylate synthase (*ACC* synthase) [52]. Ethylene inhibited root growth by activating *WEAK ETHYLENE INSENSITIVE 2*, *7*, *I2* and *I7* [47], genes encoding subunits of a rate-limiting enzyme in the biosynthesis of tryptophan, the precursor of auxin [42]. Genetic studies in *Arabidopsis* roots demonstrated that ethylene positively regulates auxin biosynthesis in the apex, and enhances the auxin response in the elongation zone resulting in changes in cell expansion [47,44,50].

The present study tries to define the relationship between *Glbs*, NO and ethylene in the regulation of somatic embryogenesis by using the *Arabidopsis* system. *Arabidopsis* somatic embryos are generated by a two-step process: an induction step where embryogenic tissue forms from the zygotic embryos used as explants, and a developmental phase required for the development and growth of the embryos in a medium devoid of growth regulators (Fig. 1) [10,4]. Our results confirm that suppression of the *Arabidopsis* class 1 Hb (*Glb1*) and class 2 Hb (*Glb2*) has different effects on the formation of somatic embryos, and places ethylene as a possible downstream component of the *Glb*-regulation of embryogenesis.

2. Materials and methods

2.1. Plant materials

Two *Arabidopsis* (Columbia) lines suppressing *Glb1* or *Glb2* were donated by Dr. Hebelstrup [16]. They included the *Glb1 RNAi* line where *Glb1* (class 1 Hb) was partially suppressed by RNAi mediated mechanisms, and the *Glb2*^{-/-} knock out line where *Glb2* (class 2 HB) was completely repressed. The *pASAI::GUS* reporter line (CS16701) and the *ein2-1* and *ein3-1* mutant lines were obtained from the *Arabidopsis* Biological Resource Center (ABRC). The following lines were received as gifts: the *pYUC4::GUS* line [8], the *pPDF1.2::GUS* line [24] and the *pEBS::GUS* [30].

2.2. Growth conditions and induction of somatic embryogenesis

Arabidopsis seeds were sterilized in a solution containing 70% ethanol and 0.5% Triton X-100 for 5 min, followed by a

wash in 90% ethanol for 10 min. The sterilized seeds were germinated on half-strength MS medium [39] in a tissue culture cabinet (20–22 °C, 16 h light/8 h dark photoperiod). Following the procedure described by Bassuner et al. [4], developing siliques were harvested from the plants and the zygotic embryos were excised and used as explants for somatic embryogenesis. The explants were initially placed on the 2,4-dichlorophenoxyacetic acid (2,4-D) containing induction medium for 14 days and then transferred onto a development medium devoid of growth regulators. Fully developed embryos were counted 9 days after transfer onto the development medium (Fig. 1).

2.3. Chemical treatments

Modulations of NO levels were performed using the NO donor sodium nitroprusside (SNP) or the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (cPTIO), at the concentrations reported by Elhiti et al. [10]. The ethylene donor Ethephon (ETH) and the ethylene biosynthetic blocker O-(carboxymethyl)hydroxylamine hemihydrochloride (AOA) were also applied at the concentrations indicated in the text. Both compounds were dissolved in water. All treatments were performed during the 14 days on induction medium.

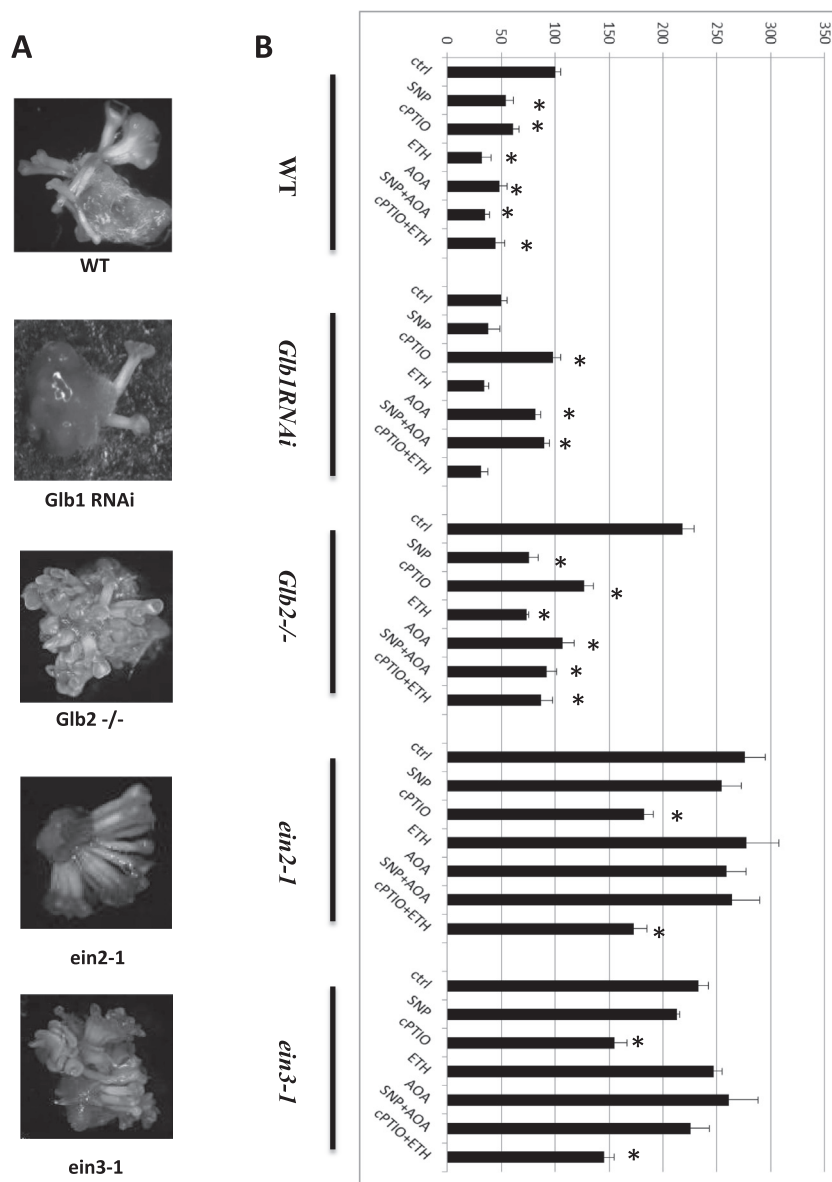


Figure 3 (A) Micrographs showing embryo production after 9 days on development medium in the WT, *Glb1 RNAi*, *Glb2-/-* mutant, and the two ethylene insensitive mutant lines *ein2*, *ein3*. (B) Number of embryos expressed as a percentage of WT control (ctrl) in the different lines subjected to different treatments in the induction medium: SNP (100 μ M), ETH (10 μ M), cPTIO (50 μ M), or AOA (10 μ M). Values are means \pm SE of at least three biological replicates. * indicates statistically significant differences ($P < 0.005$) from the WT (ctrl) value.

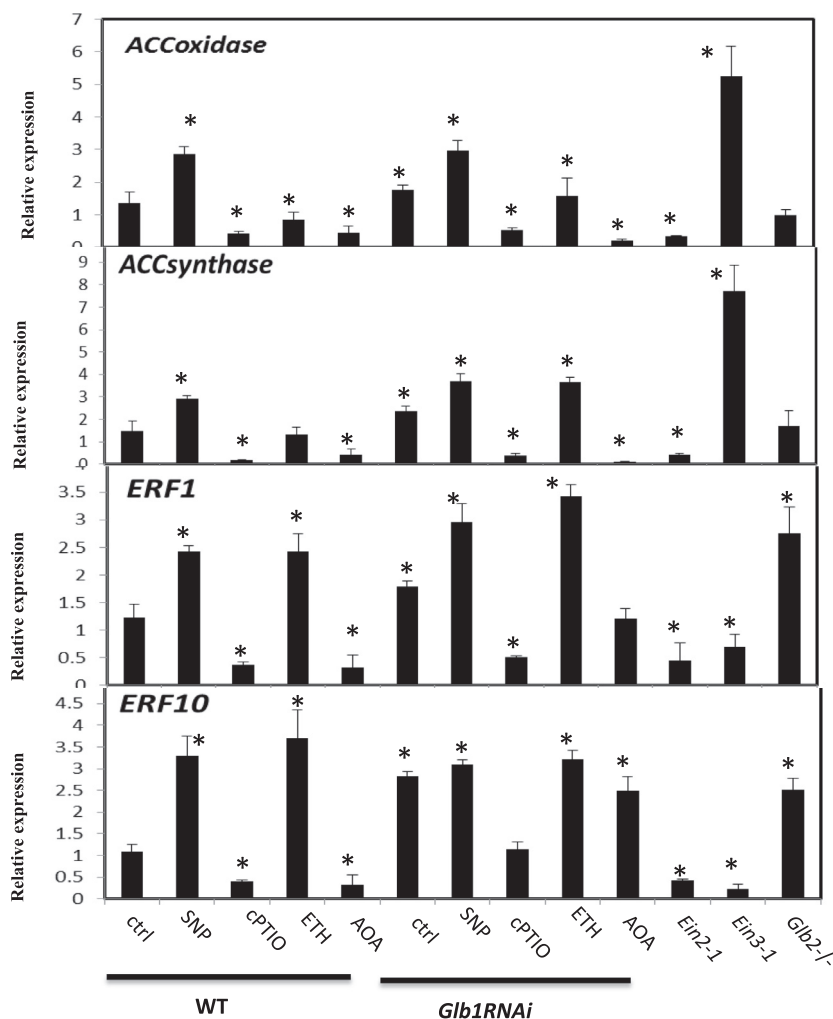


Figure 4 Expression level of the two ethylene biosynthetic aminocyclopropane-1-carboxylic acid oxidase (ACC oxidase) and aminocyclopropane-1-carboxylic acid synthase (ACC synthase), and the ethylene responsive factor 1 (ERF1), 10 (ERF10). Treatments were conducted as described in Fig. 3. Values are means \pm SE of at least three biological replicates. * indicates statistically significant differences ($P < 0.005$) from the WT (ctrl) value set at 1.

2.4. Total RNA isolation and quantitative real-time PCR analysis

TRIzol reagent (Invitrogen) was used to extract the total RNA from tissue harvested 7 days on induction medium. The extracted RNA was first treated with DNase I RNase-free (Promega) and these used for cDNA synthesis using the cDNA Reverse Transcription Kit (Applied Biosystems).

Quantitative real-time PCR was performed as described by Elhiti et al. [9]. All primers are listed in Table. 1. The relative expressions of the target genes were calculated with the $2^{-\Delta\Delta Ct}$ method using UBQ10 (AT4G05320) as a reference [19].

2.5. β -GUS assays

GUS staining was carried out exactly as described by Sieburth and Meyerowitz [45]. A minimum of 20 samples were used per treatment. A dissecting microscope equipped with a Leica DC500 digital camera was used for capturing the images.

2.6. IAA immunolocalization

IAA localization was performed exactly as described by Elhiti et al. [10]. Material at day 7 on induction medium was first pre-fixed in freshly prepared 4% aqueous 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride at 4 °C for 2 h, and then post-fixed in FAA (10% formalin, 5% acetic acid, and 50% ethanol) overnight at 4 °C. The fixed tissue was dehydrated in ethanol series, embedded in paraplast, sectioned (10 μ M), and deparaffinized in xylene. The sections were incubated in blocking solution [1 \times phosphate-buffered saline (PBS) solution pH 7, 0.1% Tween 20, 1.5% glycine, and 5% bovine serum albumin (BSA)] at room temperature for 1 h. 150 μ l of monoclonal primary IAA-antibodies (1 mg/ml, Sigma) diluted 1:200 in 10 mM PBS containing 0.8% BSA were applied to the sections and incubated in a high humidity chamber for 4 h at room temperature. The slides were washed first in 10 mM PBS containing 0.88 g/L NaCl, 0.1% Tween 20, and 0.8% BSA for 5 min, and then in 10 mM PBS with 0.8% BSA for

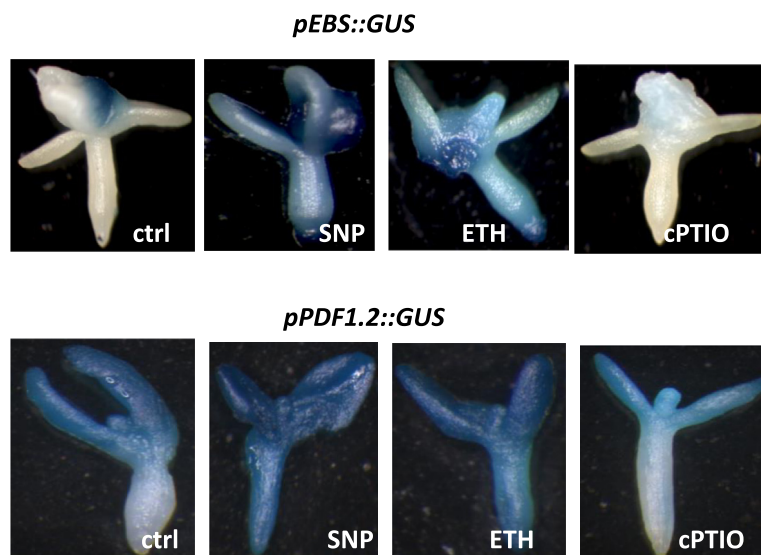


Figure 5 GUS localization in the pEBS::GUS and pPDF1.2::GUS reported lines. Embryos were cultured for 7 days on induction medium before being harvested and stained for GUS. Concentrations of the treatments were identical to those reported in Fig. 3.

5 min in order to remove any excess of Tween 20. The slides were incubated in 200 μ l secondary antibodies [anti-mouse IgG alkaline phosphatase conjugate (1 mg/ml), Promega, USA] overnight in a high humidity chamber, washed 2 times in 1 \times PBS containing 0.88 g/L NaCl, 0.1% Tween 20, and 0.8% BSA for 10 min, and then incubated in water for 15 min to remove the excess of secondary antibodies. Samples were stained using 250 μ l Western blue (Promega) for 40 min.

2.7. Statistical analysis

Tukey's post hoc test for multiple variances was used to compare differences among samples. All experiments were carried out using three biological replicates.

3. Results

3.1. Embryo number is differentially affected by manipulations in NO and ethylene levels

Arabidopsis somatic embryogenesis is a two-step process including an induction phase where 2,4-D is used to stimulate the formation of the embryogenic tissue (arrows in Fig. 1) from the cotyledons of the explant, and a development phase required for the growth of the embryos in an environment devoid of growth regulators. The number of WT embryos harvested after 9 days on development medium was increased by applications of the NO donor SNP at a concentration of 10 μ M. Higher concentrations of the donor repressed embryogenesis in both the WT and the *Glb2*^{-/-} mutant line. Inclusions of SNP had no effects on the embryogenic output of the *Glb1 RNAi* line which was consistently lower than that of the other lines (Fig. 2A).

Scavenging of NO by (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide) (cPTIO) reduced the embryo number in both WT and *Glb2*^{-/-} knock out line and this effect was concentration dependent. The embryo number in

the *Glb1 RNAi* line increased following applications of cPTIO reaching a maximum value at a concentration of 50 μ M, before declining (Fig. 2B).

Inclusions of the ethylene donor Ethephon (ETH) repressed embryogenesis in the WT and *Glb2*^{-/-} mutant line, and this repression increased with elevated levels of the compound. Ethephon had no significant effects on the number of embryos produced by the *Glb1 RNAi* line (Fig. 2C).

The number of embryos in the *Glb2*^{-/-} mutant line declined significantly following applications of the ethylene biosynthetic inhibitor O-(carboxymethyl)hydroxylamine hemihydrochloride (AOA). This was in contrast to the WT and *Glb1 RNAi* lines, which showed an increase in embryogenesis up to a concentration of 5 μ M (AOA). Higher levels of the inhibitor decreased the embryo number in these two cell lines (Fig. 2D).

To further confirm the involvement of ethylene in somatic embryogenesis two ethylene insensitive lines: ethylene-insensitive 2 (*ein2-1*) and 3 (*ein3-1*) were used. Compared to WT, embryo number was significantly increased in these lines, and an increase in NO levels by SNP had no significant effect (Fig. 3).

3.2. Nitric oxide (NO) alters the expression of ethylene biosynthetic genes

Ethylene synthesis was analyzed as a transcriptional level by measuring the expression of the two ethylene biosynthetic genes: *1-amino-cyclopropane-1-carboxylate synthase (ACC synthase)* which synthesizes the ethylene precursor 1-aminoacyl cyclopropane-1-carboxylic acid (ACC) from S-adenosyl-L-methionine, and *1-amino-cyclopropane-1-carboxylate oxidase (ACC oxidase)* which oxidizes ACC to form ethylene. At day 7 on the induction medium, the expression of *ACC oxidase* and *ACO synthase* was increased in NO enriched environments, i.e. (SNP-treated WT line and *Glb1 RNAi* line) (Fig. 4). Consistent with these results, a reduction in NO level by cPTIO in both WT and *Glb1 RNAi* line reduced the

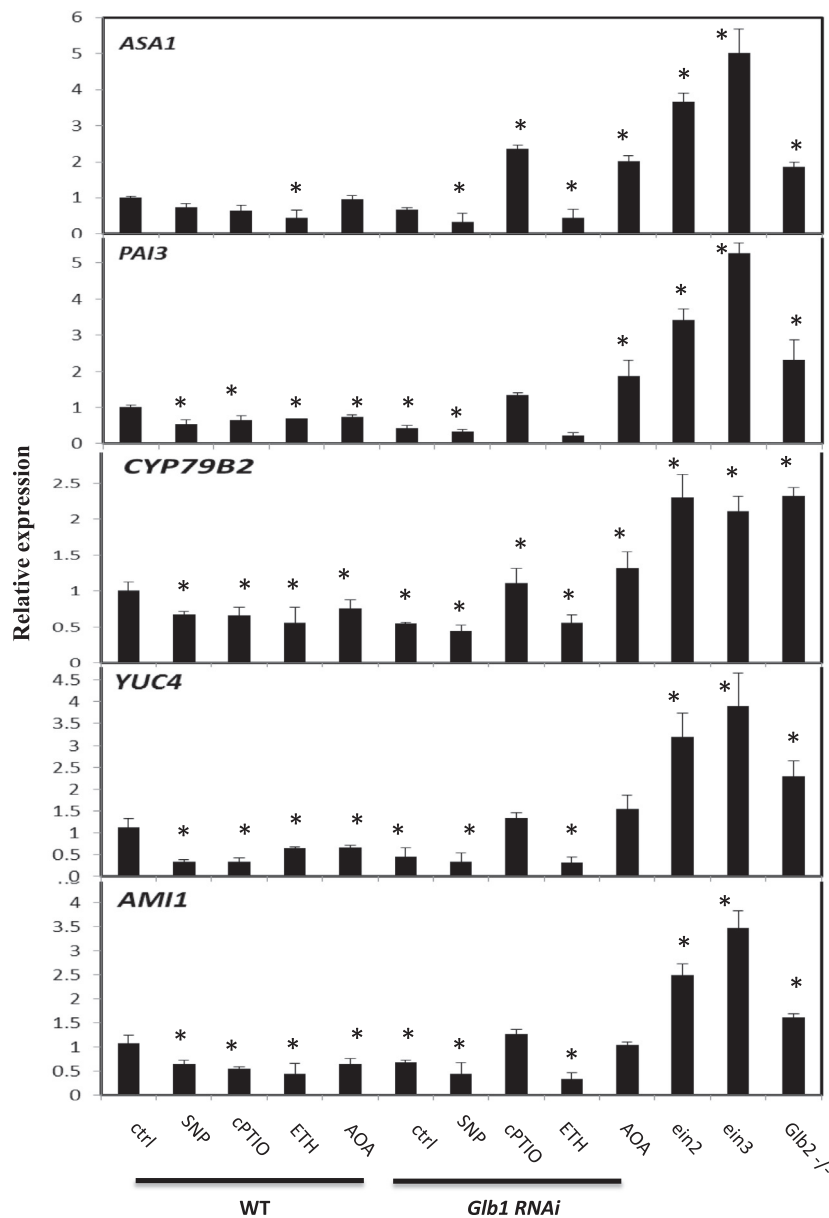


Figure 6 Expression level by quantitative (q) RT-PCR of the auxin biosynthetic genes anthranilate synthase α -subunit (*ASA1*), anthranilate isomerase (*PAI3*), amidase1 (*AMI1*), cytochrome P450 CYP79B2 (*CYP79B2*) and YUCCA4 (*YUC4*). Values are means \pm SE of at least three biological replicates and normalized to WT (control, ctrl) set at 1. * indicates statistically significant differences ($P < 0.005$) from the WT (control, ctrl) value.

expression of both genes (Fig. 4). The changes in expression levels of the ethylene responsive factor 1 (*ERF1*) and 10 (*ERF10*) also suggest that NO and ethylene affect the overall mechanisms of ethylene response (Fig. 4).

The effect of NO on ethylene level was further confirmed using two reporter lines (pEBS::GUS) (an ethylene reporter construct in which the GUS reporter gene is driven by a synthetic EIN3-responsive promoter) and (pPDF1.2::GUS) (in which the GUS reporter gene is driven by ethylene responsive promoter PDF1.2) (Fig. 5). In both lines the GUS signal increased with high levels of NO (by SNP) and ethylene (by ETH), while it decreased following depletion of NO (by cPTIO).

3.3. Ethylene response affects the expression of IAA biosynthesis genes

Auxin is the main inductive signal promoting the re-differentiation of the somatic cells and formation of embryogenic tissues [43]. Previous studies showed that suppression of *Glb2* increased IAA production at the site of embryo formation [10]. To further expand whether IAA biosynthesis was regulated through an ethylene response, the expression of several key genes involved in IAA biosynthesis was measured at day 7 on induction medium. The expression of anthranilate synthase α -subunit (*ASA1*), anthranilate isomerase (*PAI3*),

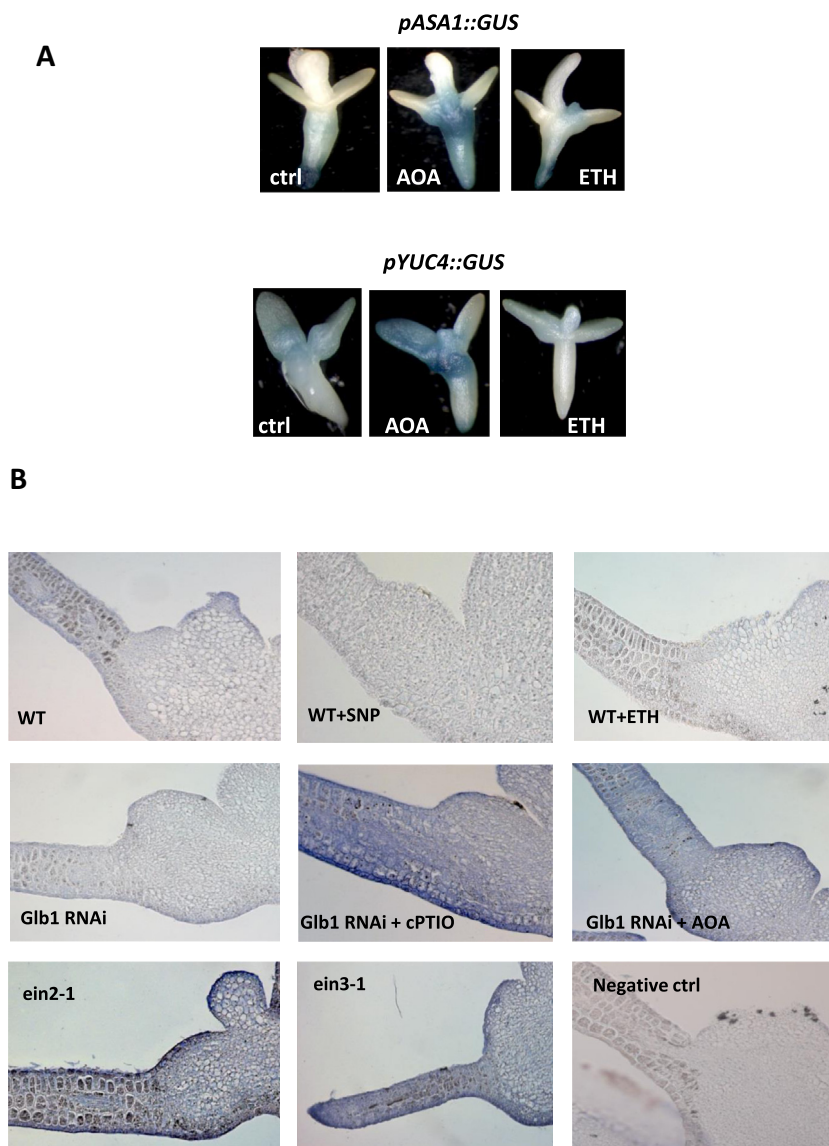


Figure 7 (A) GUS localization in the *pASA1::GUS* and *pYUC4::GUS* reported lines. Embryos were cultured for 7 days on induction medium before being harvested and stained for GUS. Concentrations of the treatments were identical to those reported in Fig. 3. (B) Immunolocalization of IAA along the cotyledons of the zygotic explants. Tissue was collected at 7 days on induction medium. Concentrations of the treatments were identical to those reported in Fig. 3.

cytochrome P450 *CYP79B2* (*CYP79B2*), *YUCCA4* (*YUC4*), and amidase1 (*AMI1*) increased in the ethylene mutants *ein2* and *3* (Fig. 6). Their expression was generally lower in the *Glb1 RNAi* line compared to the WT and the *Glb2*^{-/-} mutant lines. Furthermore, in the *Glb1 RNAi* line treatments depleting NO (by cPTIO) and ethylene (by AOA) increased the expression level of all the genes analyzed.

These expression patterns were confirmed using 2 different promoter lines (*pASA1::GUS*) and (*pYUC4::GUS*). In both lines GUS signal was increased when ethylene level was depleted with AOA, and decreased when ethylene level was augmented with ETH (Fig. 7A).

Immunolocalization of the auxin IAA in the embryogenic tissue arising from the cotyledons of the zygotic explants was also performed (Fig. 7B). Wild type (WT) tissue treated with SNP or ETH, as well as *Glb1 RNAi* tissue had low IAA signals.

A strong IAA signal was detected in tissues with depleted NO level (*Glb1 RNAi* tissue treated with cPTIO) or ethylene (*Glb1 RNAi* tissue treated with AOA), and in the ethylene mutants (*ein2-1* and *ein3-1*).

4. Discussion

Nitric oxide (NO) plays a vital role in different developmental processes ranging from leaf expansion, root growth and senescence [55,56]. Besides being involved in wounding and pathogen interactions [38], NO has proven to contribute to ethylene biosynthesis [17]. However, some studies found that NO represses ethylene biosynthesis [41]. The level of cellular NO is mediated by several factors, including the presence of Hbs, which are effective regulators of NO. As reviewed by Hill [18], both the *Arabidopsis* *Glb1* and *Glb2* are able to reduce

NO levels, although Glb1 is a stronger scavenger due to its higher affinity to NO. Therefore, tissue suppressing Glb1 accumulates more NO than tissue suppressing Glb2 [18]. Small differences in NO levels might have divergent effects on embryogenesis, with high levels decreasing embryo production and intermediate levels improving the process.

The relationship between NO and ethylene during somatic embryogenesis has not been studied before. Here we show that excessive NO levels, produced pharmacologically using SNP or genetically using the *Glb1 RNAi* line, reduce somatic embryo number, possibly by elevating the level of ethylene, and this response might be integrated in the Hb-regulation of embryogenesis.

Effects of exogenous application of ethylene during somatic embryogenesis are contradictory. While ethylene improves production of embryogenic callus in spinach [21], it represses the embryogenic competence in *Arabidopsis* [3]. Applications of the ethylene blocker (O-(carboxymethyl) hydroxylamine hemihydrochloride) (AOA) enhance the embryo production in the *Glb1* down regulating line (*Glb1 RNAi* line) (Fig. 3) characterized by the highest NO level [18], but not in the *Glb2*^{-/-} mutant line which has intermediate NO levels between the WT and the *Glb1 RNAi* values. These effects were independent from increasing the levels of NO by SNP, thus suggesting that NO is upstream of the ethylene response. An

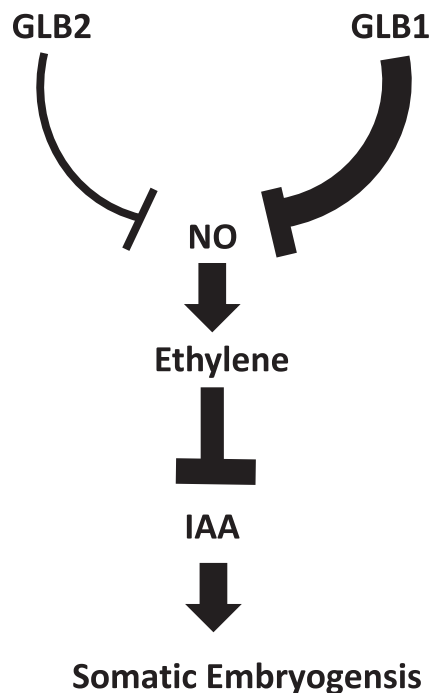


Figure 8 Proposed model regulating *Arabidopsis* somatic embryogenesis. Glb1 is a more effective NO scavenger of Glb2, therefore, their suppression would result in a differential accumulation of NO. The *Glb1 RNAi* line accumulates more NO than the *Glb2*^{-/-} line. Accumulation of NO activates the transcription of the two ethylene biosynthetic genes (*ACC synthase* and *ACC oxidase*) and would possibly increase ethylene level. Ethylene transcriptionally down-regulates many IAA biosynthetic genes and reduces the IAA signal on the zygotic explants. Since IAA is the inductive signal for somatic embryogenesis, a depletion in IAA reduces the ability of the *Glb1 RNAi* line to produce embryos.

experimental increase of ethylene by ETH repressed embryogenesis in both the *Glb1* down regulating line and the *Glb2*^{-/-} mutant line (Fig. 3). Compared to WT tissue, embryogenesis was significantly increased in lines insensitive to ethylene: *ein2-1* and *ein3-1*. Embryo production in these lines remained higher than WT even following manipulation in NO (by SNP and cPTIO) (Fig. 3).

In a high NO environment, such is the case of SNP-treated tissue or tissue suppressing *Glb1*, the expression level of the two ethylene biosynthetic genes ACC oxidase and ACC synthase is up-regulated (Fig. 4). Furthermore, this up-regulation can be reversed if NO level is experimentally reduced by cPTIO. This trend was observed in both WT and GLB1 line. The expression of both genes was reduced in the *ein2-1* line, but increased in the *ein3-1* line. No significant differences in the expression levels of both genes were measured in the *Glb2*^{-/-} mutant line (Fig. 4), which accumulated less NO compared to the *Glb1 RNAi* line. Generally similar expression profile patterns were also observed for the ethylene responsive factor 1 (ERF1) and 10 (ERF10), thus suggesting that NO and ethylene affect the overall mechanisms of ethylene response (Fig. 4).

Compared to WT, the expression of these two genes is not altered in the *Glb2*^{-/-} mutant line, thus suggesting that both ACC oxidase and ACC synthase are only responsive to high levels of NO. Activation of the two biosynthetic genes most likely results in increased ethylene synthesis, which in *Arabidopsis* compromises somatic embryogenesis. This is demonstrated both pharmacologically, by the fact that application of the ethylene releasing agent ETH reduces the number of embryos in the WT line (Fig. 3), and genetically, using the two ethylene insensitive mutants *ein2-1* and *ein3-1*. Production of somatic embryos in these two mutants is highly favored (Fig. 3). Furthermore, ethylene appears to be downstream of NO as an increase in NO production by SNP in the *ein2-1* and *ein3-1* has no effects on the embryogenic output of the lines. This notion is also supported by the observation that the two ethylene responsive promoters pEBS and pDF1.2 are activated in a high NO environment (SNP) and repressed in a NO depleted environment (cPTIO) (Fig. 5).

The elevated levels of ethylene triggered by excessive NO might be linked to the repression of auxin, the inductive signal triggering the de-differentiation of the cotyledon cells and the production of embryogenic tissues [43]. The expression of all IAA biosynthetic genes investigated: *ASA1*, *PAI3*, *AMI1*, *CYP79B2*, and *YUC4* was suppressed when ethylene level was increased by ETH but increased in the ethylene insensitive mutants *ein2-1* and *ein3-1* (Fig. 6). The effect of ethylene was particularly apparent on *ASA1* and *YUC4*, the expression of which was induced when ethylene biosynthesis was blocked by AOA (Fig. 7A). Immunolocalization studies of IAA further confirm that IAA localizes within the cotyledons of the zygotic explants producing the embryogenesis tissue, and that this localization is affected by levels of NO and ethylene consistent with a model in which NO and ethylene regulate IAA accumulation through ethylene (Fig. 7B).

Collectively, these data suggest that ethylene is integrated in the *Glb1* and NO regulation of *in vitro* embryogenesis. In the proposed model (Fig. 8), the excessive level of NO generated in the *Glb1* down regulating line (or by pharmacological treatments) induces the expression level of the ethylene biosynthetic genes *ACC synthase* and *ACC oxidase*, and possibly increases

the endogenous ethylene content. Accumulation of ethylene down regulates several auxin biosynthetic genes leading to the depletion of endogenous auxin, the inductive signal for the formation of the embryogenic tissues. Depletion of IAA reduces somatic embryo production in the line suppressing *Glb1*. These events compromising the embryogenic output, do not occur in the *Glb2*^{-/-} mutant line as *Glb2* is not an effective NO scavenger as *Glb1*. As a result, the level of NO accumulated as a result of *Glb2* suppression is lower and possibly not sufficient to activate ethylene biosynthesis (Fig. 8).

Future studies will be undertaken to investigate the precise mode of action of ethylene response in the modulation of auxin biosynthesis, and possibly in the identification of transcription factors directly influencing the expression of genes encoding auxin biosynthetic enzymes.

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