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ARTICLE

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 Ethephon (ETH), as well as by the increased embryo production obtained with the two ethylene insensitive mutant lines (*ein*2-1 *and ein*3-1). 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.

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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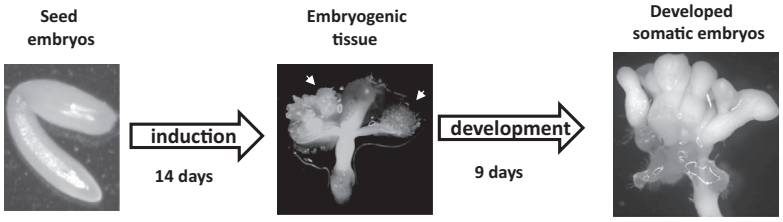

Figure 1 The *Arabidopsis* somatic embryogenic system. Seed embryos are dissected and placed on an auxin-containing induction medium for 14 days. During this time the embryogenic tissue (arrows) forms from the cotyledons of the explants. Transfer of the tissue on an auxin-free development medium for 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 *PIN1*, 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	TCGACTTGTCATTAGAAAGAAAGAGATAA	AT4G05320	UBIQUITIN 10
$ACCO_X$-F	CAAACTCTCTCGGTACACAATGA	AT2G19590	ACC OXIDASE
$ACCO_X$-R	GGATGAATGCGAGGCCAATA	AT2G19590	ACC OXIDASE
$ACCS_{YN}$-F	GCGCTTTGGCGAGTTATTATC	AT3G61510	ACC SYNTHASE
$ACCS_{YN}$-R	GGAGTGTGTCTTCGTCCATATT	AT3G61510	ACC SYNTHASE
ERF1-F	CCGCTCCGTGAAGTTAGATAAT	AT3G23240	ETHYLENE RESPONSE FACTOR 1
ERF1-R	TCTTTCACCAAGTCCCACTATTT	AT3G23240	ETHYLENE RESPONSE FACTOR 1
ERF10-F	CGAGTTTGTCCTGACCAGTTT	AT1G03800	ETHYLENE RESPONSE FACTOR 10
ERF10-R	GGTTCCATTCGCAGCTTACA	AT1G03800	ETHYLENE RESPONSE FACTOR 10
ASA1-F	ACAAGGATGCTAACAAACGGCGTG	AT1G19920	ATP SULFURYLASE ARABIDOPSIS 1
ASA1-R	TCTGGCACTCACAGTGTTCGTCTT	AT1G19920	ATP SULFURYLASE ARABIDOPSIS 1
Yuc4-F	CTAACGGATGGAAAGGAGAGAAG	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	ATGCTCGCGAGACTTCTTCAAGGT	AT4G39950	CYTOCHROME P450, FAMILY 79, SUBFAMILY B, POLYPEPTIDE 2
CYP79B2-R	AGATGCTCCGGCAATCTAAGGTCA	AT4G39950	CYTOCHROME P450, FAMILY 79, SUBFAMILY B, POLYPEPTIDE 2