

## Ectopic expression of *LEAFY COTYLEDON1-LIKE* gene and localized auxin accumulation mark embryogenic competence in epiphyllous plants of *Helianthus annuus* × *H. tuberosus*

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Received: 28 July 2008 Returned for revision: 1 October 2008 Accepted: 27 November 2008 Published electronically: 16 January 2009

- **Background and Aims** The clone EMB-2 of the interspecific hybrid *Helianthus annuus* × *H. tuberosus* provides an interesting system to study molecular and physiological aspects of somatic embryogenesis. Namely, in addition to non-epiphyllous (NEP) leaves that expand normally, EMB-2 produces epiphyllous (EP) leaves bearing embryos on the adaxial surface. This clone was used to investigate if the ectopic expression of *H. annuus* *LEAFY COTYLEDON1-LIKE* (*Ha-LIL*) gene and auxin activity are correlated with the establishment of embryogenic competence.
- **Methods** *Ha-LIL* expression was evaluated by semi-quantitative RT-PCR and *in situ* hybridization. The endogenous level and spatial distribution of free indole-3-acetic acid (IAA) were estimated by a capillary gas chromatography–mass spectrometry–selected ion monitoring method and an immuno-cytochemical approach.
- **Key Results** Ectopic expression of *Ha-LIL* was detected in specific cell domains of the adaxial epidermis of EP leaves prior to the development of ectopic embryos. *Ha-LIL* was expressed rapidly when NEP leaves were induced to regenerate somatic embryos by *in vitro* culture. Differences in auxin distribution pattern rather than in absolute level were observed between EP and A-2 leaves. More precisely, a strong IAA immuno-signal was detected in single cells or in small groups of cells along the epidermis of EP leaves and accompanied the early stages of embryo development. Changes in auxin level and distribution were observed in NEP leaves induced to regenerate by *in vitro* culture. Exogenous auxin treatments lightly influenced *Ha-LIL* transcript levels in spite of an enhancement of the regeneration frequency.
- **Conclusions** In EP leaves, *Ha-LIL* activity marks the putative founder cells of ectopic embryos. Although the ectopic expression of *Ha-LIL* seems to be not directly mediated by auxin levels *per se*, it was demonstrated that localized *Ha-LIL* expression and IAA accumulation in leaf epidermis domains represent early events of somatic embryogenesis displayed by the epiphyllous EMB-2 clone.

**Key words:** *Helianthus annuus* × *H. tuberosus*, auxin, epiphyllous, *LEAFY COTYLEDON1-LIKE* gene, somatic embryogenesis.

### INTRODUCTION

In higher plants, embryogenesis has been largely characterized at the morphological and physiological levels (West and Harada, 1993; Jürgens, 2001; Laux *et al.*, 2004). Also the genetic network that controls embryogenic processes is becoming better understood because of the identification of several genes that play regulatory roles either in specific embryogenesis phases (reviewed in Jenik *et al.*, 2007) or during the whole process (reviewed in Park and Harada, 2008). For example, the class of *LEAFY COTYLEDON* (*LEC*) genes, such as *LEC1*, *LEC1-LIKE* (*LIL*), *LEC2* and *FUSCA3* (*FUS3*) all encoding regulatory proteins involved in embryogenesis (Laurssen *et al.*, 1998; Lotan *et al.*, 1998; Stone *et al.*, 2001; Kwong *et al.*, 2003).

Mutational analyses showed that the *LEC* genes function early in embryogenesis to maintain suspensor cell fate and

specify cotyledon identity (Meinke, 1992; Meinke *et al.*, 1994; West *et al.*, 1994). Late in embryogenesis, the *LEC* genes are required for initiating and/or maintaining maturation phase and repressing precocious germination (Parcy *et al.*, 1997; Lotan *et al.*, 1998; Stone *et al.*, 2001). There is also evidence of a mutual interaction among different *LEC* genes, during embryo development (Parcy *et al.*, 1997; Kagaya *et al.*, 2005; Santos Mendoza *et al.*, 2005; To *et al.*, 2006). An upstream gene, *PICKLE* (*PKL*), represses *LEC* gene expression outside of the embryo (Ogas *et al.*, 1999; Dean Rider *et al.*, 2003). In addition, members of the *VIVIPAROUS1* (*VP1*)/*ABSCISIC ACID INSENSITIVE* (*ABI3*)-*LIKE* (*VAL*) family of B3 domain transcription factors function as global repressors of the *LEC1*/B3 transcription factors network in germinating seedlings (Suzuki *et al.*, 2007).

Plant embryogenesis is not always strictly dependent on fertilization because a variety of dicot and monocot species

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reproduce asexually by apomixis (Koltunow and Grossniklaus, 2003). Moreover, gametophytic and somatic cells can be induced *in vitro* to undergo embryogenic development. Embryos can also develop ectopically from single cells of stem or leaf epidermis (Konar *et al.*, 1972; Dickinson, 1978), a process that can occur naturally in epiphyllous species (Dickinson, 1978), as well as in somaclonal variants (Konar *et al.*, 1972; Fambrini *et al.*, 2000).

Although sexual, apomictic and *in vitro* embryos often arise from different starting tissues and are activated by different signals, it is likely that at a very early stage all three depend on the same signalling pathway (Mordhorst *et al.*, 1997; Lotan *et al.*, 1998; Koltunow and Grossniklaus, 2003; Tucker *et al.*, 2003; Xu *et al.*, 2006). This is consistent with the initiation of somatic embryos from vegetative cells mis-expressing embryo-specific genes *LEC1* (Lotan *et al.*, 1998), *LEC2* (Stone *et al.*, 2001), *BABY BOOM* (Boutilier *et al.*, 2002), *Ha-LIL* (Fambrini *et al.*, 2006b) and *PGA37/MYB118* (Wang *et al.*, 2008). Further support is provided by the involvement of *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK)* genes in both zygotic and somatic embryo initiation (Hecht *et al.*, 2001) as well as in the development of embryo sacs from nucellar tissue in apomictic species (Albertini *et al.*, 2005). The conclusion from these studies is that zygotic, apomictic and *in vitro* embryogenesis are morphogenetic end points of closely related pathways. However, relatively little is known about the early inductive events underlying the transition of a somatic cell into an embryogenic mode of development.

It is generally believed that *in vitro* embryogenesis is mediated by a signalling cascade triggered by auxin and/or stressful conditions (Dodeman *et al.*, 1997; Mordhorst *et al.*, 1997; Ikeda *et al.*, 2006). In particular, in *Helianthus annuus* the transient but non-uniform accumulation of endogenous auxin is among the first signals leading to the induction of somatic embryogenesis from immature zygotic embryos (Thomas *et al.*, 2002). On the other hand, *LEC* genes directly interact with hormone-response genes (Brocard-Gifford *et al.*, 2003; Gazzarrini *et al.*, 2004; Kagaya *et al.*, 2005; Braybrook *et al.*, 2006; Stone *et al.*, 2008). For example, the penetrance of the *turnip (tnp)* mutant phenotype of *Arabidopsis*, a gain-of-function mutant of *LEC1*, is strongly enhanced or antagonized in the presence of exogenous auxin and cytokinins, respectively (Casson and Lindsey, 2006).

In order to add further insights into molecular and physiological aspects of somatic embryogenesis, in the present study an epiphyllous clone (EMB-2) derived by *in vitro* tissue culture of the interspecific hybrid *Helianthus annuus* × *H. tuberosus* was used. This clone produces epiphyllous (EP) leaves, both *in vitro* and *in vivo*, that exhibit prominent ectopic embryo- and shoot-like structures (Fambrini *et al.*, 2000; Chiappetta *et al.*, 2006). EP leaves are produced alongside normal non-epiphyllous (NEP) leaves. In previous work, we demonstrated that *Ha-LIL*, a *LIL* gene of *H. annuus*, was highly expressed in epiphyllous embryos of the EMB-2 clone. In the present study, the extent to which auxin and *Ha-LIL* expression were integrated in the initial stages of EMB-2 epiphyllous embryo development was investigated. To this end, both endogenous amount and spatial distribution of indole-3-acetic acid (IAA) were

determined in EP and NEP leaves of EMB-2, as well in control leaves of a non-epiphyllous interspecific hybrid (A-2). Spatial expression of *Ha-LIL* was also monitored during the early stages of ectopic embryo development. Finally, the relationship between *Ha-LIL* and endogenous IAA together with spatial distribution of each were monitored in NEP leaves of EMB-2 cultured *in vitro* on different media.

## MATERIALS AND METHODS

### *Plant material and growth conditions*

The somaclone EMB-2 was previously induced *in vitro* from leaf explants of the tetraploid ( $2n = 4x = 68$ ) interspecific hybrid *Helianthus annuus* × *H. tuberosus* (A-2 clone; Fambrini *et al.*, 2000, 2006b). To obtain clones under sterile conditions, both A-2 and EMB-2 plants were multiplied by culturing single-nodes on solidified ( $8 \text{ g L}^{-1}$  Bactoagar; Oxoid, Basingstoke, UK) MS basal medium (Murashige and Skoog, 1962) without growth regulators in 150-mL Erlenmeyer flasks (Fambrini *et al.*, 2000). The cultures were incubated in a growth chamber at  $25 \pm 1^\circ \text{C}$  in a 16-h daily photoperiod. Irradiance intensity during the light period was  $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ , by means of cool-light fluorescent lamps (Philips TLD 36W/33; Philips, Eindhoven, The Netherlands). EMB-2 plants were also grown in a growth chamber in pots containing a mixture of vermiculite, peat and soil at  $23 \pm 1^\circ \text{C}$  under a 16-h daily photoperiod (Fambrini *et al.*, 2000). Irradiation was  $200 \mu\text{mol m}^{-2} \text{s}^{-1}$  provided by mercury vapour lamps (Osram HQI-TS 250 W/NDN, Wembley, UK).

### *Histological analysis*

Leaves from *in vitro* and *in vivo* grown EMB-2 plants were collected and fixed for 24 h in FAA (formalin/glacial acetic acid/ethanol/distilled water, 10 : 5 : 50 : 35, v/v) at room temperature before being transferred into 70 % ethanol (Fambrini *et al.*, 2006a). Water was removed by a graded ethanol series while the dehydrated material was cleared in xylene according to Ruzin (1999). Paraffin-embedded tissues were sectioned ( $10 \mu\text{m}$  thick) using a rotary microtome (Reichert, Vienna, Austria). The serial sections obtained were then stained in Delafield's haematoxylin (BDH Chemicals Ltd, Poole, UK).

### *IAA analysis*

Leaves of control (A-2) and epiphyllous clone (EMB-2) were collected from different *in vitro* grown plantlets ( $n = 3$ ). For EMB-2 plants, EP leaves were separated from NEP ones. Freeze-dried samples ( $300 \text{ mg f. wt}$ ) were extracted in 65 % isopropanol (v/v) with 0.02 M imidazole buffer at pH 7 to which [ $^3\text{H}$ ]IAA as radiotracer and [ $^{13}\text{C}_6$ ]IAA (kindly provided by J. D. Cohen, Department of Horticultural Science, Saint Paul, MN, USA), as an internal standard for quantitative mass-spectral analysis, were added. After overnight isotope equilibration, free IAA was measured according to Baraldi *et al.* (1995). Quantitative IAA analysis was done by a capillary gas chromatography–mass spectrometry–selected ion monitoring using a Hewlett Packard 5890–5970 System equipped with a 30-m HP 1 capillary column (i.d. 0.25 mm; film thickness

0.25  $\mu\text{m}$ ), carrier gas He (1 mL  $\text{min}^{-1}$ ), injector at 280 °C, oven temperature increased from 50 °C to 110 °C at 30 °C  $\text{min}^{-1}$ , then 6 °C  $\text{min}^{-1}$  to 280 °C, source temperature 270 °C, ionizing voltage 70 eV. Ions monitored were  $m/z$  130 and 136 for the base peak (quinolinium ion) and 189 and 195 for the molecular ion of the methyl-IAA and methyl- $^{13}\text{C}_6$ IAA, respectively (Rapparini *et al.*, 2002). The ratios of 130 : 136 and 189 : 195 were used to calculate endogenous levels of IAA. The data are presented as means ( $\pm$  s.d.) of three independent experiments with three replicates.

#### IAA immuno-cytolabelling

Control (A-2) and epiphyllous clone (EMB-2) leaves were collected from *in vitro* grown plantlets ( $n = 3$ ). For EMB-2 plants, EP leaves were separated from NEP ones. Excised tissue samples were immediately fixed in a 0.5 % (v/v) glutaraldehyde and 3 % (w/v) paraformaldehyde mixture in PBS (135 mM NaCl, 2.7 mM KCl, 1.5 mM  $\text{KH}_2\text{PO}_4$ , pH 7.2) for 3 h at 4 °C. EDAC pre-treatment was substituted with a pre-embedding procedure (Chiappetta *et al.*, 2006) and fixed samples were cut using the vibratome (Leica VT1000E, Bensheim, Germany). The floating sections (30–35  $\mu\text{m}$ ) were processed for the immuno-localization as described in Moctezuma (1999) and Thomas *et al.* (2002), and incubated overnight at 4 °C with anti-IAA monoclonal primary antibody (Sigma-Aldrich, Milan, Italy) diluted 1 : 200 in PBS/BSA solution (10 mM phosphate solution, 0.8 % BSA). Subsequently, sections were incubated with the secondary antibody (anti-mouse IgG alkaline phosphatase conjugate (Promega Italia, Milan, Italy) diluted 1 : 100 in PBS/BSA solution for 4 h. After washing, the sections were developed with NBT (nitro blue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolylphosphate) mixture for 5 min, then rinsed with stop buffer (100 mM Tris-HCl, pH 8.0; 1 mM EDTA), mounted on slides and immediately observed and photographed. To verify the effectiveness of the immuno-localization technique, sections were processed with the omission of the primary IAA antibody and with a competition assay control as described by Kerk and Feldman (1995).

#### *In vitro* hormonal treatments of NEP explants of EMB-2 plants

Explants from NEP leaves of EMB-2 were used as starting material for *in vitro* experiments. A-2 leaves were used as controls. EMB-2 leaves were selected using a reflecting stereoscope, before the *in vitro* culture, to exclude the presence of epiphyllous structures (NEP leaves). Explants were placed in Petri dishes (100 mm  $\times$  15 mm) on basal medium without growth regulators (MS) and on MS medium supplemented with different growth regulators: *trans*-zeatin riboside, gibberellic acid, IAA, 2,4-dichlorophenoxyacetic acid (2,4-D), abscisic acid and an inhibitor of auxin transport, 2,3,5-triiodobenzoic acid (TIBA). In a first experiment, all substances were added at 10  $\mu\text{M}$ . In a second experiment different concentrations of 2,4-D were used. The media, supplemented with 30 g  $\text{L}^{-1}$  sucrose, were solidified with 8 g  $\text{L}^{-1}$  Bactoagar (Oxoid). Cultures were incubated in a growth chamber under the same conditions as described above. The regeneration frequencies (number of explants developing

embryogenic structures on the total of cultured explants) were estimated from 3-week-old cultures of three independent experiments each with five to six replications (Petri dishes). Each Petri dish contained 15–18 explants. NEP leaves, at 1 or 3 d from the start of the *in vitro* culture on MS medium, were collected for both quantitative ( $n = 6$ ) and immuno-cytolabelling ( $n = 5$ ) IAA analysis as described above.

#### RNA isolation from *in vitro*-cultured explants and semi-quantitative RT-PCR analyses of Ha-LIL expression

Total RNA extractions were carried out from A-2 and NEP explants collected after 0, 1 or 3 d of culture on media described above. Total RNA was extracted from samples with the TriPure Isolation Reagent, according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). The total RNA (4  $\mu\text{g}$ ) from each sample was used, with the Superscript pre-amplification kit (Invitrogen S.R.L., Life Technologies, Milan, Italy), to produce the first-strand cDNA in conditions recommended by the manufacturer. PCRs were performed using gene-specific primers from cDNA of *Ha-LIL* (CHI-TCDNA, 5'-CTAGAGAGACAATTC-3' and CHI-REL, 5'-GAAGACAATGAGTGCATTG-3') and  $\beta$ -actin (ACT5, 5'-GATTCCGTTGCCCA/TGAGGTC/T-3' and ACT3, 5'-TC/TTCTGGA/TGGA/TGCAACCACC-3'). Primers were designed to yield 175-bp and 243-bp fragments for *Ha-LIL* and  $\beta$ -actin, respectively. PCRs were carried out for 30 cycles where the cDNAs were exponentially amplified. PCRs were performed in 20  $\mu\text{L}$  of 10  $\times$  buffer (Euroclone Spa – Life Division Sciences, Pero, Italy) containing 0.1 mM dNTPs, 0.75 or 1.50  $\mu\text{M}$  of each primer for *Ha-LIL*, 0.15  $\mu\text{M}$  of each primer for  $\beta$ -actin, 1 mM  $\text{MgCl}_2$ , 1.25 U EuroTaq DNA polymerase (Euroclone) and 1  $\mu\text{L}$  of single-stranded cDNA. Amplifications were carried out according to the following temperature profile: 94 °C for 5 min for denaturation, then 94 °C for 30 s, 53 °C for 25 s, 72 °C for 25 s and a final extension of 7 min at 72 °C. The amplified fragments were quantified using Quantity One (BioRad, Hercules, CA, USA). Each *Ha-LIL* signal was normalized to the  $\beta$ -actin signal for calculation of relative amounts. Each value was the mean of four independent experiments.

#### Construction of DIG-RNA probe

A clone HaK-4, containing partly the 3'-non-coding region (157 bp), spanning across the *Ha-LIL*-specific primers, CHI-SF: 5'-TATGCTCAGTGTAAGAC-3' (located 18 nucleotides upstream the translational stop codon), and CHI-SR: 5'-GTAGATGGAGAGTGTGAG-3', was linearized with appropriate restriction enzymes and used as template to synthesize digoxigenin-labelled RNA sense and antisense probes, according to the DIG-RNA labelling kit protocol (Roche Diagnostics GmbH, Mannheim, Germany).

#### *In situ* hybridization

Excised tissues of EMB-2 and A-2 leaves were fixed, dehydrated, embedded in paraffin, cut into 8- $\mu\text{m}$  sections and hybridized (55 °C) to a digoxigenin-labelled antisense HaK-4

RNA probe according to Cañas *et al.* (1994). For immunological detection, the slides were incubated in buffer 1 [1% (w/v) blocking solution, 100 mM Tris, pH 7.5, and 150 mM NaCl] for 1 h and then equilibrated with buffer 2 [100 mM Tris, pH 7.5, and 150 mM NaCl, 0.5% (w/v) BSA, and 0.3% (w/v) Triton X-100]. Tissue sections were then incubated with anti-digoxigenin-alkaline-phosphatase conjugate diluted 1:5000 in buffer 2 in a humidified box for 2 h and then washed three times for 20 min in buffer 100 mM Tris, pH 7.5, and 150 mM NaCl. The tissue sections were equilibrated in buffer 3 (100 mM Tris, pH 9.5, and 100 mM NaCl and 50 mM MgCl<sub>2</sub>) for 10 min and then incubated for 72 h in 3.2 µg mL<sup>-1</sup> 5-bromo-4-chloro-3-indolyl-phosphate : 6.6 µg mL<sup>-1</sup> nitro-blue tetrazolium salt in buffer 3 in a humidified box. Accumulation of *Ha-L1L* transcripts is visualized as a violet-brownish staining.

#### Statistical analysis

Data were treated using analysis of variance procedures, and means were compared by Tukey's test ( $P = 0.05$ ). Homogeneity of variances was evaluated using Bartlett's test ( $P = 0.05$ ). Statistical analyses on percentage data were performed after arc sine transformation.

## RESULTS

### *Development of ectopic embryos in EMB-2 leaves*

In EMB-2 plants, ectopic embryos develop profusely from the adaxial surface of EP leaves (Fambrini *et al.*, 2000; Chiappetta *et al.*, 2006; Fig. 1A and B). Only on the petiole, can ectopic structures occasionally originate from the abaxial leaf surface (Fig. 1C and D). The first sign of somatic embryo induction is a shift from anticlinal to periclinal divisions in epidermal cell clusters (Fig. 1E). Multicellular budding and single-cell initiation of ectopic structures apparently both occur, with multicellular budding being the more frequent pattern in the present study. This multicellular origin was resolved in some leaf cross-sections, where linear clusters of epidermal cells, with densely stained cytoplasm (Fig. 1F), initiated cell division to form small protuberances (Fig. 1G, H). These structures rapidly developed into clumps of epiphyllous embryos (Fig. 1I–L). It is hypothesized that these adaxial epidermal cells are putative founders of somatic embryos. Epiphyllous embryos formed directly opposite to the xylem pole of the veins (Fig. 1I). Phenomena of recurrent embryogenesis from primary ectopic embryos were also observed (Fig. 1L).

### *In EMB-2 plants endogenous levels of free IAA are different in epiphyllous versus non-epiphyllous leaves*

Given the clear link between auxin and plant embryogenesis (Zimmerman, 1993; Thomas *et al.*, 2002; Friml *et al.*, 2003; Jenik and Barton, 2005; Ikeda *et al.*, 2006), the endogenous level of free IAA was estimated in EP and NEP leaves of EMB-2 plants compared with that of a non-epiphyllous genotype (A-2), used as control. The amount of endogenous IAA in EP leaves was 1.8-fold higher than in NEP ones (Fig. 2), in spite of a quite comparable IAA level in EP and A-2 leaves

that do not show this epiphyllous condition. Hence, total amount of IAA *per se* is not necessarily a sensitive marker of the epiphyllous morphogenetic condition.

### *A discontinuous IAA distribution marks epidermis of EP leaves*

Several observations support the hypothesis that embryogenic cells originate by change of cell polarity and subsequent asymmetric division (Zimmerman, 1993; Dodeman *et al.*, 1997). The induction and the co-ordination of cell polarity have classically been attributed to auxin and its flow (reviewed in Dhonukshe *et al.*, 2005a; Jenik and Barton, 2005; Boutté *et al.*, 2007). In order to verify whether in EMB-2 the development of somatic embryos could be related to a localized accumulation of this hormone, IAA spatial distribution was analysed through immuno-cytolocalization.

In both A-2 and NEP leaves immuno-reaction was observed in the epidermis, strongly in vascular bundles, and scattered in mesophyll tissue (Fig. 3A, C). Moreover, in mesophyll cells the immuno-reaction was mainly localized in chloroplasts (Fig. 3B), as observed in peach (Ohmiya *et al.*, 1990) and sunflower (Thomas *et al.*, 2002). In EP leaves strongly immuno-reactive cell clusters were frequently identified along the adaxial epidermis (Fig. 3D, E). More precisely, immuno-staining was detected in single cells or in a small group of cells (linear cluster) from which ectopic embryo formation would be predicted (compare Fig. 3D, E with Fig. 1F, G). These results suggest strongly an auxin pulse in the putative founder cells of ectopic embryos.

An intense immuno-staining marked the development of whole embryogenic structure from the initial cell divisions (Fig. 3F, G) through to advanced globular embryo-like structures (Fig. 3H). No immuno-reaction was observed in EP leaves processed without primary antibody (data not shown).

### *Ha-L1L is highly expressed in early developmental stages of somatic embryo-like structures*

To dissect in more detail the relationship between *Ha-L1L* and the embryogenic competence usually exhibited by EP leaves, the spatial localization of transcripts at the very early stages of ectopic embryo development was investigated. In the part of the EP leaves proximal to the petiole junction (Fig. 4A), transcripts were detected in both the adaxial (Fig. 4B) and abaxial (Fig. 4C) epidermis as well in parenchyma cells of vascular bundles (Fig. 4A). Notably, labelled cells were grouped at the leaf margin (Fig. 4B and C) and were discontinuously distributed along the blade (Fig. 4A). In more distal sections, exhibiting ectopic structures at different developmental stages (Fig. 4D, G, H), a strong accumulation of *Ha-L1L* mRNA occurred in linear cluster of cells (Fig. 4G), incipient somatic embryos (Fig. 4E), as well as developing globular embryo-like structures (Fig. 4F, G and H). Notably, in structures clearly arrested in development and showing enlarged vacuolated cells, there was a clear decline of the hybridization signal (Fig. 4G, H). Signal was not detected in leaves processed with the sense probe (data not shown). Moreover, in both A-2 and NEP leaves, *in situ* hybridization did not reveal any *Ha-L1L* transcript accumulation (Fig. S1 in Supplementary Data, available online).

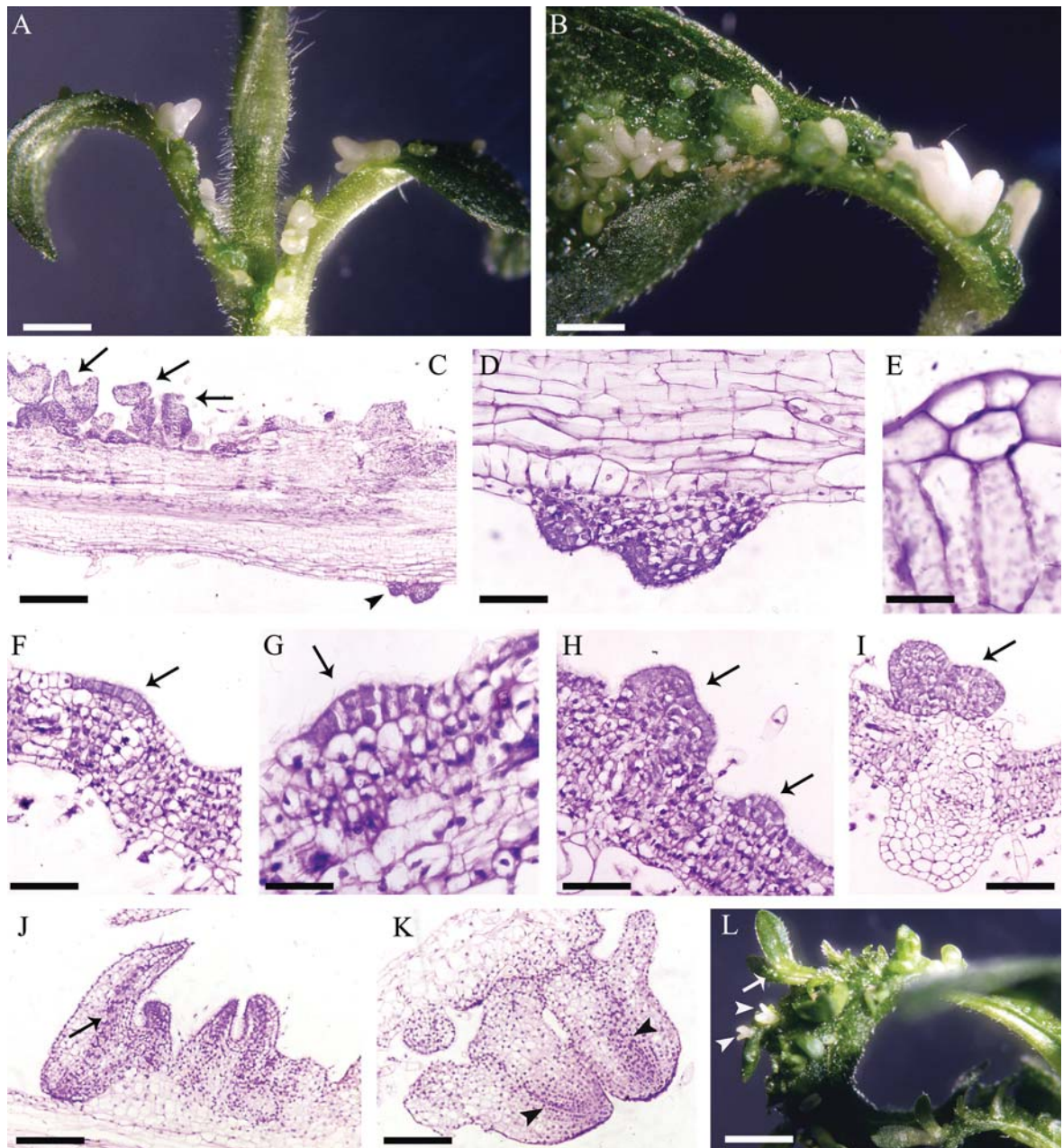


FIG. 1. Development of epiphyllous structures in EMB-2 plants of the interspecific hybrid *Helianthus annuus* × *H. tuberosus*. (A) An epiphyllous EMB-2 plant grown *in vitro*. (B) Ectopic embryos can be seen on both adaxial surface and petiole of an epiphyllous (EP) leaf. (C) Longitudinal section of an EP leaf, proximal to the petiole, along the main vein; arrows indicate ectopic structures on the adaxial surface while the arrowhead indicates an ectopic structure on the abaxial surface. (D) Magnification of (C) showing the ectopic structure originated on the abaxial surface. (E) A periclinal division in the adaxial epidermis. (F) A linear cluster of epidermal cells with densely stained cytoplasm (arrow). (G, H) Initial stages of ectopic structures (arrows). (I) An ectopic embryo formed directly opposite a xylem pole of a vein (arrow). (J, K) Epiphyllous cotyledonary embryos showing a procambial strand (arrow) and embryo axes (arrowheads). (L) Globular (arrow) and cotyledonary embryos (arrowheads) developed from primary ectopic embryos. Scale bars: (A) = 3.5 mm; (B) = 2.5 mm; (C) = 370  $\mu\text{m}$ ; (D) = 90  $\mu\text{m}$ ; (E) = 20  $\mu\text{m}$ ; (F) = 100  $\mu\text{m}$ ; (G) = 50  $\mu\text{m}$ ; (H) = 85  $\mu\text{m}$ ; (I) = 120  $\mu\text{m}$ ; (J) = 210  $\mu\text{m}$ ; (K) = 190  $\mu\text{m}$ ; (L) = 4.2 mm.

#### *Ha-LIL* activation and changes in IAA level and distribution occurred in NEP leaves cultured *in vitro*

A clear regeneration competence characterized all EMB-2 leaves since explants of NEP leaves cultured on media without growth regulators were able to differentiate somatic embryos and shoot-like structures (Chiappetta *et al.*, 2006). This prompted us to verify whether embryogenic competence

in explanted NEP leaves could be related to either an activation of *Ha-LIL* transcription or changes in IAA level or distribution. To this end, both NEP and A-2 leaves were cultured on MS basal medium without growth regulators. After 21 d, a high percentage of NEP explants developed embryo-like structures on the adaxial leaf surface (Table 1 and Fig. 5A, B), whereas A-2 explants did not.

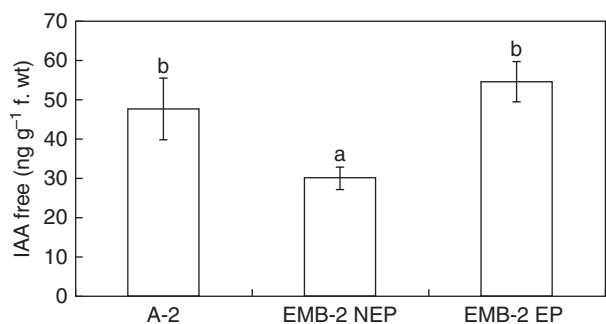


FIG. 2. Level of endogenous free IAA in the leaf from *in vitro*-grown plants of the interspecific hybrid *Helianthus annuus* × *H. tuberosus*. Free mean ( $\pm$  s.d.) IAA concentrations in three independent experiments each with three replicates. Columns marked with the same letter are not significantly different at the  $P = 0.05$  level according to Tukey's test. Abbreviations: A-2, leaves of the non-epiphyllous clone; EMB-2 EP, epiphyllous leaves of the EMB-2 clone; EMB-2 NEP, non-epiphyllous leaves of the EMB-2 clone.

After 1 d of *in vitro* culture (T1), *Ha-LIL* transcripts accumulated in NEP leaves (Fig. 6), suggesting that the up-regulation of the gene precedes somatic embryo initiation. By contrast, in cultured A-2 leaves *Ha-LIL* mRNA was not detected.

A significant decline of free IAA content also occurred in NEP leaves during the first 3 d of culture (Fig. 7), which resulted in a progressive reduction of IAA immuno-reaction in vascular bundles, mesophyll tissue and epidermis (Fig. 8A–F). A more discontinuous pattern of cell labelling was frequently detected along the adaxial epidermis in leaves cultured for 24 h (T1) compared with the uniform distribution observed in leaves detached at time 0 (Fig. 8A, C). A very faint and homogenous IAA signal was also detected in the adaxial epidermis of leaves cultured for 72 h (Fig. 8E). An immuno-reaction was not observed in EP leaves processed without primary antibody (Fig. 8D).

#### *Somatic embryogenesis and Ha-LIL transcription are slightly influenced by auxin treatments*

The transcription of the *Ha-LIL* gene was monitored in explants of NEP leaves cultured in the presence of IAA and TIBA. After 3 weeks culture, a slight increase in the frequency of somatic embryo regeneration was detected on both IAA- and TIBA-supplemented media compared with MS basal medium (Table 1).

Semi-quantitative RT-PCR analysis of *Ha-LIL* transcription was carried out in NEP leaves cultured *in vitro* for 3 d on the different media (Fig. 9). The transcript levels did not change in response to TIBA supply, whereas a slight increase of *Ha-LIL* mRNAs was detected in auxin-treated NEP leaves (IAA and 2,4-D; Fig. 9A, B). In addition, a low different dose exposure to 2,4-D revealed that *Ha-LIL* transcript levels decreased or remained unchanged in comparison to those of leaves grown on basal medium (Fig. 9C, D). Only a slight increase of *Ha-LIL* mRNA steady state levels was detected at high auxin concentrations (Fig. 9C, D). Other hormones, i.e. *t*-zeatin, abscisic acid and gibberellic acid, did not affect the *Ha-LIL* mRNA steady-state levels (Fig. S2 in Supplementary Data, available online).

## DISCUSSION

In the present work it is shown that a localized *Ha-LIL* mis-expression occurred in epidermal cell clusters before ectopic embryos became evident in EP leaves of EMB-2 plants. These ectopic structures initiate through periclinal divisions of adaxial epidermis cells. Moreover, after only 1 d of *in vitro* culture *Ha-LIL* transcripts accumulated in excised NEP leaves which later (i.e. after 14 d culture) will develop ectopic embryos, thus confirming that the up-regulation of the gene precedes somatic embryo initiation. Note that in EP leaves of the EMB-2 clone, *Ha-LIL* transcripts highly accumulated in ectopic embryos at heart and early cotyledon stages (Fambrini *et al.*, 2006b). Analogously, in maize and carrot, *LEC1* mRNA was detected in embryogenic cell clusters as well as in the first stage of somatic embryo development (Zhang *et al.*, 2002; Yazawa *et al.*, 2004). Direct somatic embryogenesis was strongly impaired in *Arabidopsis lec* mutants (Gaj *et al.*, 2005). *At-LIL* expression was detected in *Arabidopsis* zygote (Kwong *et al.*, 2003) and *Ha-LIL* was strongly expressed in early stages of *Helianthus* zygotic embryogenesis (Fambrini *et al.*, 2006b). More recently, *LIL* expression has been detected in both zygotic and somatic embryogenesis of *Theobroma cacao* (Alemanno *et al.*, 2008) and in somatic embryogenesis of *Vitis vinifera* (Schellenbaum *et al.*, 2008). In particular, a strong expression of *VvLIL* occurred during the early induction phase of *V. vinifera* somatic embryos while in developing embryos the accumulation of *VvLIL* transcripts decreased markedly (Schellenbaum *et al.*, 2008). Therefore, it is hypothesized that the *LEC1*-like homologue in *Helianthus*, *Ha-LIL*, might be a component of the molecular pathway that marks putative founder cells for ectopic embryogenesis.

Among the promoting factors of somatic embryogenesis, auxin has been long regarded as a key factor (Mordhorst *et al.*, 1997; Ikeda *et al.*, 2006). Here it is shown that ectopic embryogenesis of EMB-2 clone was related to auxin distribution pattern rather than the hormone's absolute level. Note that, auxin level was quite comparable in A-2 and EP leaves, in spite of non-epiphyllous behaviour of the A-2 clone. By contrast, a strong IAA accumulation in linear cell clusters occurred along the adaxial epidermis of EP leaves as compared with the homogenous reaction in the epidermis of both A-2 and NEP leaves. A strong and heterogeneous accumulation of auxin was also detected in the protodermis of explants from immature zygotic embryos of *Arabidopsis thaliana* transgenic lines undergoing direct somatic embryogenesis (Kurczyńska *et al.*, 2007). Interestingly, the pattern of hormone distribution correlated with the histological observations on an embryogenic-like pattern of cell divisions (Kurczyńska *et al.*, 2007). Hence, it seems that in the EMB-2 clone, the strongly IAA-reactive cells observed along adaxial epidermis of EP and NEP leaves cultured *in vitro*, also correspond to the putative founder cells of ectopic embryos.

Asymmetric distribution of auxin across plant tissues, as well as the auxin accumulation in small cell clusters, are features that accompany and mediate developmental events (reviewed in Woodward and Bartel, 2005; Tanaka *et al.*, 2006). Periclinal division of primordia founder cells is strongly indicative of the initiation of primordia (Benková *et al.*, 2003)

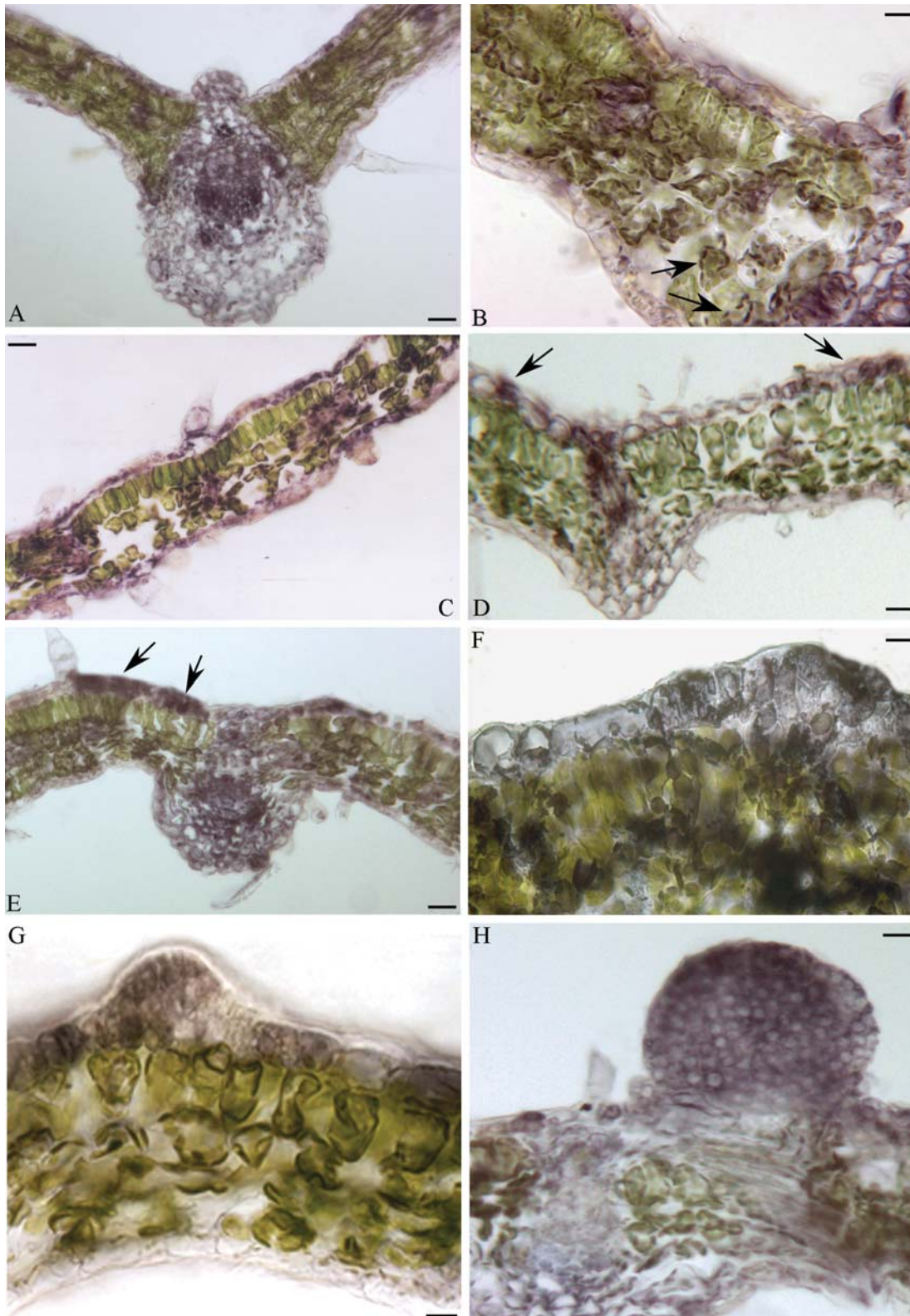


FIG. 3. Immuno-localization of free IAA in leaf cross-sections of A-2 and EMB-2 plants of the interspecific hybrid *Helianthus annuus* × *H. tuberosus*. Immuno-reaction evidenced as purple-violet staining. (A) Leaf of the non-epiphyllous A-2 clone. (B) Higher magnification of (A); arrows indicate labelled chloroplasts. (C) A non-epiphyllous (NEP) leaf of the EMB-2 clone. (D–H) Epiphyllous leaves (EP) of the EMB-2 clone. Arrows in (D) and (E) indicate strongly labelled cells. Scale bars: (A) = 35  $\mu\text{m}$ ; (B) = 15  $\mu\text{m}$ ; (C) = 40  $\mu\text{m}$ ; (D) = 20  $\mu\text{m}$ ; (E) = 30  $\mu\text{m}$ ; (F) = 8  $\mu\text{m}$ ; (G) = 12  $\mu\text{m}$ ; (H) = 15  $\mu\text{m}$ .

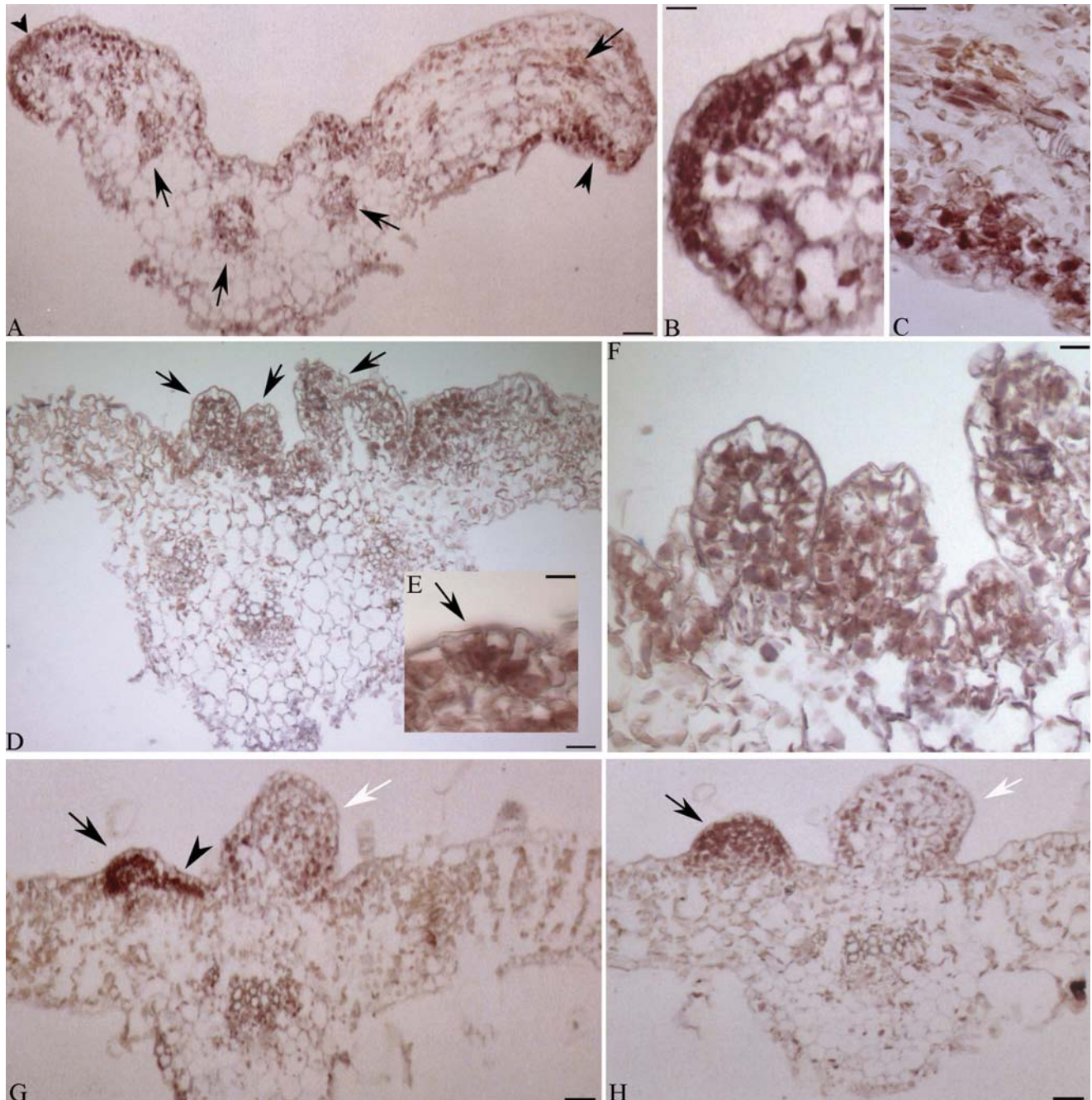


FIG. 4. Localization of *Ha-L1L* transcripts in cross-sections of epiphyllous leaves (EP) of the interspecific hybrid *Helianthus annuus* × *H. tuberosus*. The presence of *Ha-L1L* mRNAs is evidenced as a violet-brownish staining. (A) Leaf blade proximal to the petiole junction; arrowheads indicate labelled cells grouped along the leaf margin and discontinuously distributed along the blade; arrows indicate labelled vascular bundles. (B, C) Higher magnification of (A). (D) Distal leaf; arrows indicate ectopic embryos. (E, F) Higher magnification of (D); arrow in (E) indicates incipient ectopic structure. (G, H) Distal leaf blades; arrowhead indicates a linear cluster of labeled cells; arrows indicate advanced globular embryos; white arrows indicate ectopic structures arrested in development. Scale bars: (A) = 35  $\mu\text{m}$ ; (B, C) = 11  $\mu\text{m}$ ; (D) = 45  $\mu\text{m}$ ; (E) = 10  $\mu\text{m}$ ; (F) = 16  $\mu\text{m}$ ; (G, H) = 35  $\mu\text{m}$ .

and polar auxin transport is strongly linked to changes in division plane (Dhonukshe *et al.*, 2005a, b). In *Helianthus*, a transient localized auxin pulse is among the first signals leading to cell divisions that initiate somatic embryogenesis (Thomas *et al.*, 2002). Recently, live imaging of *in vitro* regeneration systems with the fusion construct *pPIN1::PIN1-GFP* have further supported the key role of asymmetric distribution of

auxin in *de novo* morphogenesis of both adventitious meristems (Gordon *et al.*, 2007) and somatic embryos (Zhao *et al.*, 2008). However, IAA accumulation in EP leaves was not restricted to founder cells of ectopic embryos (i.e. adaxial epidermal cells). The palisade and lacunose tissues also showed an IAA immuno-reaction, although embryo-like structures did not occur from these domains (Fambrini *et al.*,



TABLE 1. Regeneration from non-epiphyllous (NEP) leaves of the EMB-2 clone of *Helianthus annuus* × *H. tuberosus* cultured for 3 weeks on different media

Treatment	Regeneration (%)
MS	54.05 ± 3.98 <sup>a</sup>
MS + IAA	67.96 ± 3.10 <sup>b</sup>
MS + TIBA	70.94 ± 1.23 <sup>b</sup>

The growth regulators were added at 10 µM.

The data are means (± s.e.) from three independent experiments each with five or six replications (Petri dishes).

Values followed by a common superscript letter are not different at the 0.05 level of significance, according to Tukey's test.

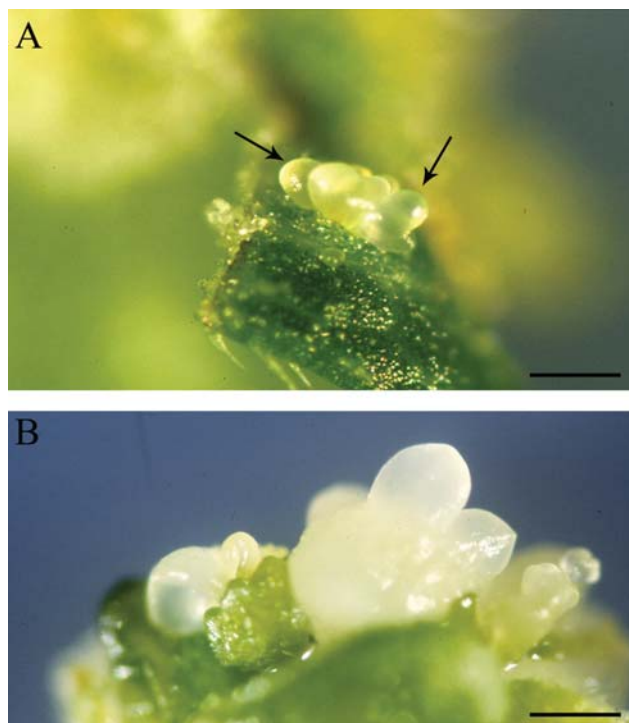


FIG. 5. Regeneration of somatic embryos from *in vitro*-cultured NEP leaves of the EMB-2 clone of the interspecific hybrid *Helianthus annuus* × *H. tuberosus*. Somatic embryos on the adaxial surface of NEP explants cultured for 9 d (A) and/or 14 d (B) on MS (Murashige and Skoog, 1962) basal medium. Arrows in (A) indicates globular somatic embryos. Scale bars: (A) = 1.7 mm; (B) = 1.2 mm.

2000). Analogously, in direct somatic embryogenesis from immature zygotic embryo of *Arabidopsis thaliana*, not all cells showing auxin signal were embryogenic (Kurcyńska *et al.*, 2007). Therefore, perhaps an auxin transient pulse is conditional for events leading to the development of somatic embryos but other unknown signals are required to trigger this event in some but not all cells.

An open question of behaviour of the EMB-2 clone is whether the localized auxin accumulation in EP leaves is due to local biosynthesis or re-distribution through polar transport. Although the present data are not sufficient to discriminate between these hypotheses, the recent evidence showing that polar auxin transport and *YUCCA*-mediated auxin

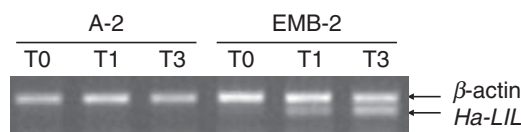


FIG. 6. RT-PCR analysis of *Ha-L1L* mRNA accumulation. The amplification of *Ha-L1L* and  $\beta$ -actin transcripts was performed from total RNA isolated from leaves of A-2 and non-epiphyllous (NEP) leaves of the EMB-2 clone cultured *in vitro* for 1 or 3 d on MS basal medium without growth regulators. The RT-PCR products were resolved on TAE 2.0% agarose gel. Abbreviations: T0, untreated leaves; T1, leaves cultured for 1 d on MS medium; T3, leaves cultured for 3 d on MS medium.

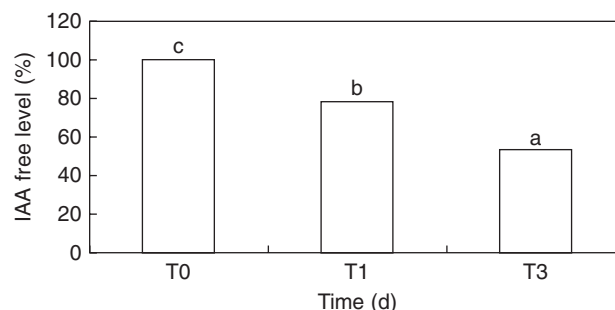


FIG. 7. Level of endogenous IAA free in non-epiphyllous (NEP) leaves of the interspecific hybrid *Helianthus annuus* × *H. tuberosus* cultured *in vitro* for 1 or 3 d on MS basal medium without growth regulators. Data were registered as percentage of IAA free with respect to control (T0, untreated leaves). Leaves cultured for 1 d (T1) or 3 d (T3) on MS medium. Data are means of six replicates. Columns marked with different letters are significantly different ( $P = 0.05$ ).

biosynthesis have strong genetic interactions can be recalled (Cheng *et al.*, 2007). It is likely that both events (i.e. auxin transport and biosynthesis) could be involved to exceed the hormonal threshold required for organ initiation (Zhao, 2008). In this context, the effect on somatic embryogenesis observed in NEP leaves cultured on medium enriched with TIBA, an auxin-transport inhibitor (Geldner *et al.*, 2001; Dhonukshe *et al.*, 2008), deserves a specific comment. It is known that TIBA treatments promote or negatively affect adventitious regeneration *in vitro* in relation to the species and/or the concentration used (Laublin *et al.*, 1991; Nissen and Minocha, 1993). Taking into account that NEP leaves showed a significant reduction of IAA content in the course of *in vitro* culture on MS basal medium, the positive effect of TIBA-enriched medium in promoting somatic embryogenesis could be related to a local auxin accumulation, as demonstrated in tobacco cells after TIBA treatment (Petrášek *et al.*, 2002). It can be predicted that both IAA and TIBA media were more suitable than MS basal medium to influence the auxin gradient in NEP leaves, enhancing the hormone accumulation in discrete regions of leaf explants through a differential uptake or transport inhibition, respectively. Moreover, TIBA could act altering the endogenous content of a different growth regulator with morphogenetic effects, as observed in the leaf of *Echinacea purpurea* (Jones *et al.*, 2007).

Finally, a basic issue is the nature of the interaction between auxin activity and the genetic control of embryogenic competence. To date, the most evident result is that the auxin biosynthetic gene *YUCCA4* is a direct target of *LEC2* (Stone *et al.*,

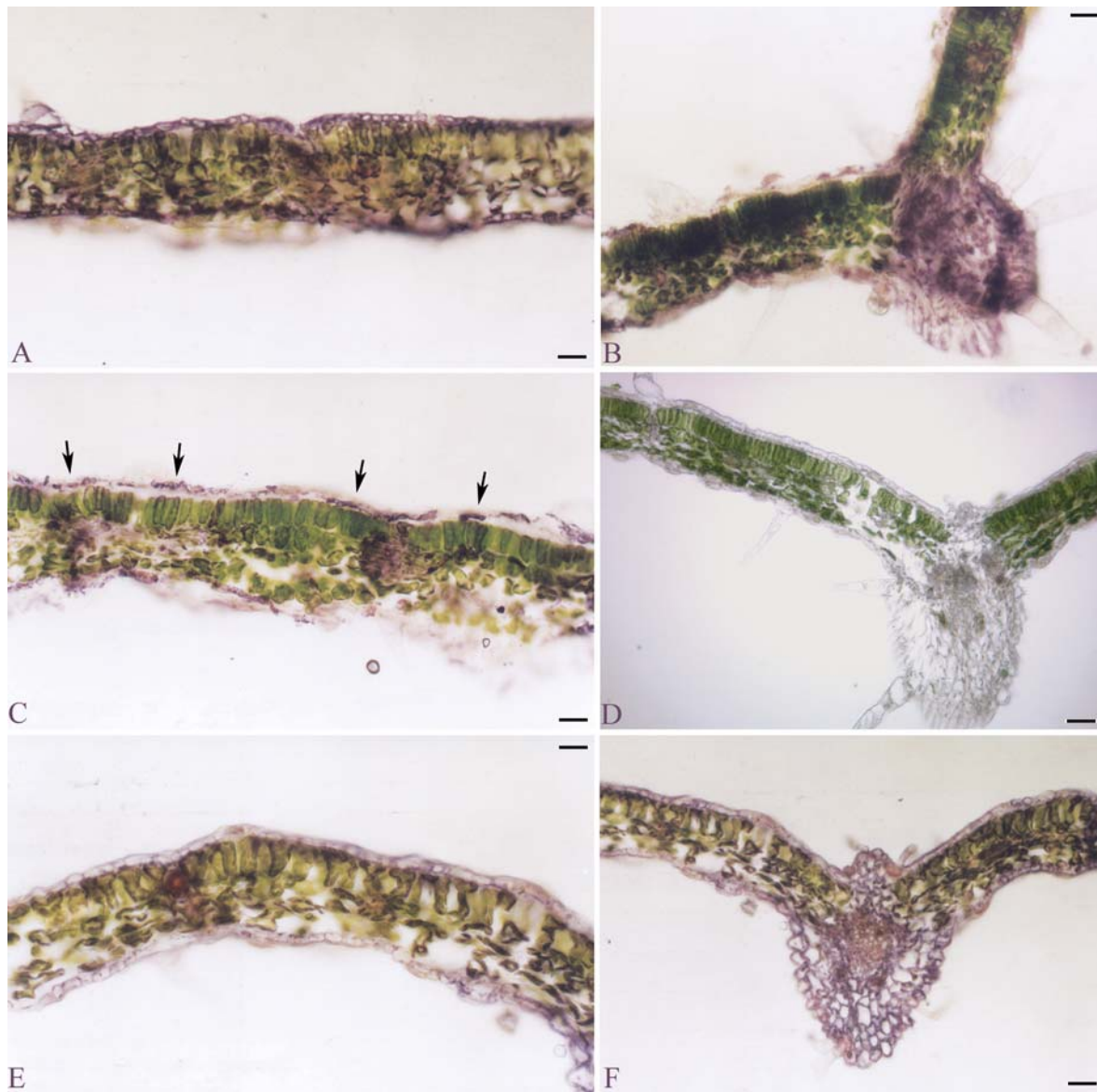


FIG. 8. Immuno-localization of free IAA in leaf cross-sections of non-epiphyllous (NEP) leaves of the interspecific hybrid *Helianthus annuus* × *H. tuberosus* cultured *in vitro* for 1 or 3 d on MS basal medium without growth regulators. Immuno-reaction is evidenced as purple-violet staining. (A) Untreated leaf (T0). (B) Untreated leaf (T0) showing the central vascular bundle. (C) Leaf cultured for 1 day on MS basal medium (T1). (D) Leaf processed without primary antibody (control). (E) Leaf cultured for 3 d on MS basal medium (T3). (F) Leaf cultured for 3 d on MS basal medium (T3) showing the central vascular bundle. Arrows in (C) indicate strongly labelled cells. Scale bars: (A, C, E) = 45  $\mu\text{m}$ ; (B, D, F) = 70  $\mu\text{m}$ .

2008). However, although the existing data are not plentiful neither univocal (Zhang *et al.*, 2002; Alemanno *et al.*, 2008; Schellenbaum *et al.*, 2008; Sharma *et al.*, 2008), the hypothesis that in the induction of embryogenesis, *LEC1/LIL* genes function downstream from auxin remains possible (Gaj *et al.*, 2005). Only a slight increase of *Ha-LIL* mRNA steady-state levels was detected in NEP leaves exposed to auxin treatments as compared with leaves grown on basal medium. However, post-transcriptional or -translational effects cannot be excluded and alternatively auxin could act by altering the expression of other key embryonic regulators to modify *in vitro* response as observed in the *turnip* mutant of *Arabidopsis* (Casson and Lindsey, 2006).

In summary, it was clearly demonstrated that localized *Ha-LIL* expression and IAA accumulation in leaf epidermis domain represent early events of the embryogenesis displayed by the epiphyllous EMB-2 clone. Further studies are required to fully understand the mechanisms underlying the relationship between these two key factors as well as the temporal correlation between ectopic *Ha-LIL* expression and IAA pulse in epidermal cells.

#### SUPPLEMENTARY DATA

Supplementary data are available online at [www.aob.oxford-journals.org/](http://www.aob.oxford-journals.org/) and consist of the following two figures.

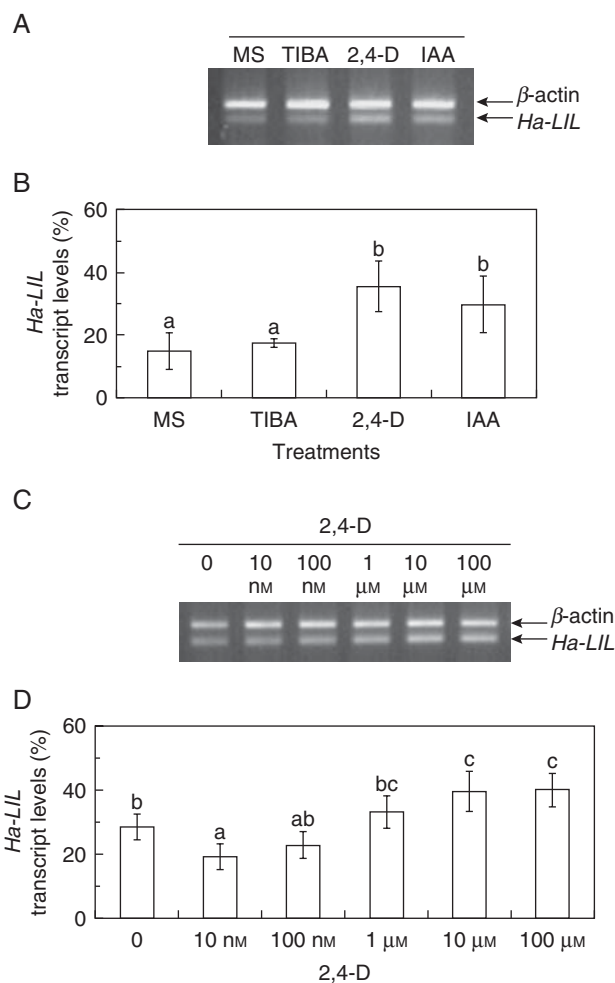


FIG. 9. RT-PCR analysis of *Ha-LIL* mRNA accumulation. (A, C) The amplification of *Ha-LIL* and  $\beta$ -actin transcripts was performed from total RNA isolated from non-epiphyllous (NEP) leaves of the EMB-2 clone cultured *in vitro* for 3 d on different media. The RT-PCR products were resolved on a TAE 2.0 % agarose gel. MS, Murashige and Skoog (1962) basal medium; TIBA, 2,3,5-triiodobenzoic acid; IAA, indol-3-acetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid. (B, D) The bar graphs show the relative levels of *Ha-LIL* transcripts, normalized with respect to the  $\beta$ -actin product, estimated by quantitative densitometry of the PCR products resolved by gel electrophoresis in (A) and (C), respectively. The columns in (B) and (D) marked by the same letter are not significantly different at the  $P < 0.05$  level according to Tukey's test. Values are shown  $\pm$  s.d. ( $n = 4$ ).

Fig S1: *Ha-LIL* *in situ* hybridization in A-2 and EMB-2 NEP of the interspecific hybrid, and cross-sections of NEP and A-2 leaves incubated with the sense and antisense probes. Fig. S2: RT-PCR analysis of *Ha-LIL* mRNA accumulation.

#### ACKNOWLEDGEMENTS

We thank Lucia Giorgetti for assistance with the *in situ* hybridization and Giuliano Cionini for technical assistance with the histological analyses. This work was supported by a grant from Ministero Italiano dell'Università e Ricerca (PRIN 2006-2008).

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