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Ethylene and shoot regeneration: *hookless1* modulates de novo shoot organogenesis in *Arabidopsis thaliana*

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Abstract We have investigated the role of ethylene in shoot regeneration from cotyledon explants of *Arabidopsis thaliana*. We examined the ethylene sensitivity of five ecotypes representing both poor and prolific shoot regenerators and identified Dijon-G, a poor regenerator, as an ecotype with dramatically enhanced ethylene sensitivity. However, inhibiting ethylene action with silver nitrate generally reduced shoot organogenesis in ecotypes capable of regeneration. In ecotype Col-0, we found that ethylene-insensitive mutants (*etr1-1, ein2-1, ein4, ein7*) exhibited reduced shoot regeneration rates, whereas constitutive ethylene response mutants (*ctr1-1, ctr1-12*) increased the proportion of explants producing shoots. Our experiments with ethylene over-production mutants (*eto1*, *eto2* and *eto3*) indicate that the ethylene biosynthesis inhibitor gene, *ETO1*, can act as an inhibitor of shoot regeneration. Pharmacological elevation of ethylene levels was also found to significantly increase the proportion of explants regenerating shoots. We determined that the *hookless1* (*hls1-1*) mutant, a suppressor of the ethylene response phenotypes of *ctr1* and *eto1* mutants, is capable of dramatically enhancing shoot organogenesis. The effects of ACC and loss of *HLS1* function on shoot organogenesis were found to be largely additive.

Keywords *Arabidopsis* · Organogenesis · Regeneration · Ethylene · Hookless

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Introduction

Many plant species exhibit the remarkable ability to generate new functional shoot systems from differentiated somatic cells. Shoot organogenesis is also routinely stimulated in vitro by incubating explants or tissues on a nutrient media augmented with a specific ratio of the plant hormones auxin and cytokinin. In addition to enormous genetic variation in regenerative abilities in vitro, shoot organogenesis is generally very sensitive to any manipulation of culture conditions. Consequently, a significant proportion of the literature pertaining to in vitro shoot regeneration that has accumulated since the techniques were first described (Skoog and Miller 1957; Vasil and Hildebrandt 1965) is concerned with optimizing culture conditions for a specific genotype. One approach often employed to enhance or investigate regeneration in culture is to manipulate the levels of other phytohormones, in addition to the auxin or cytokinin normally supplied. For a number of reasons the gaseous plant hormone ethylene has been the subject of many of these studies (Chraibi et al. 1991; Huxter et al. 1981; Kumar et al. 1987; Pua and Chi 1993; Pua and Lee 1995). Exogenous auxin and cytokinin used to induce shoots have been shown to stimulate ethylene production by inducing (reviewed Tsuchisaka and Theologis 2004), or modifying (Vogel et al. 1998), specific biosynthetic enzymes. Ethylene is known to accumulate readily in tissue culture and can have a significant impact on regeneration (reviewed Biddington 1992). However, manipulating ethylene levels in different in vitro systems has revealed that the hormone has the potential to both inhibit and enhance shoot organogenesis, depending on the culture system and genotype used (reviewed Biddington 1992). New evidence from a study employing transgenic manipulation of polyamine levels in *Arabidopsis* suggests

that down-regulation of ethylene may contribute to stimulating shoot organogenesis in this species (Hu et al. 2006).

In recent years, molecular and genetic approaches have begun to elucidate some of the underlying mechanisms controlling or affecting shoot regeneration by a number of species in tissue culture (reviewed Zhang and Lemaux 2004). Analysis and mapping of QTLs regulating in vitro growth and regeneration led to the first successful cloning of a gene modulating tissue culture responses (Nishimura et al. 2005) and has revealed many additional, potentially important targets (DeCook et al. 2006; Holme et al. 2004; Lall et al. 2004; Mano and Komatsuda 2002; Schiantarelli et al. 2001; Taguchi-Shiobara et al. 2006). Considerable progress has also been made using gene expression profiling and promoter-reporter based studies of regenerative processes in *Arabidopsis* (Cary et al. 2002; Che et al. 2002, 2006; Zhao et al. 2002). In one of these recent studies, expression analysis revealed that some genes with putative roles in ethylene biosynthesis or signaling are up-regulated during a presumptive dedifferentiation step that precedes organogenesis (Che et al. 2006).

Ethylene signaling regulates many aspects of plant growth and development including defense and stress responses, fruit and flower development, and the triple-response and signaling changes during germination (reviewed Chen et al. 2005; Joo and Kim 2007; Wang et al. 2002). In *Arabidopsis*, the ethylene biosynthesis and signaling pathways have been studied in detail, and consequently many well-characterized mutants are available affecting specific components of these pathways (reviewed Chen et al. 2005; Joo and Kim 2007; Wang et al. 2002). However, to date a systematic analysis of the effects of these mutations on shoot regeneration has not been undertaken. In this study, we utilize a number of key mutants affecting ethylene signaling and biosynthesis, combined with pharmacological approaches, to investigate the role of ethylene in shoot organogenesis in vitro.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana ecotypes Columbia (Col-0, stock# CS1092 ABRC), Landsberg *erecta* (L*er*, stock# CS20, ABRC), Estland (Est-1, WT-06A-03, Lehle Seeds), Dijon-G (Di-G, WT-10-02, Lehle Seeds) and Nossen (No-0, WT-09-05-01, Lehle seeds) were used. All mutant lines used were obtained from the *Arabidopsis* biological resource center (ABRC, OH, USA). Seeds were germinated and grown under sterile conditions in 100 × 25 mm Petri plates (VWR International Ltd.) on 0.5× MS + B5 salts (Phytotechnology Laboratories), buffered with 4.5 mM MES to pH 5.7, amended with 1% sucrose and solidified with 3 g/l phytogel (Sigma-Aldrich Co.). Seeds were surface-sterilized with 15% bleach (Queen Bleach Co.) and 0.01% Silwet L-77 (Lehle Seeds) for 15 min, followed by 30–45 s in 70% ethanol and three to five changes of sterile distilled water. The seeds were cold-treated for 3–7 days at 4°C and resuspended in sterile 0.1% agar. Using a template, 27 seeds were sown individually onto specific, reproducible points. The plates were sealed with gas-permeable Micropore^{TM} surgical tape (3 M, 1530–1). The plants were grown under constant cool white fluorescent light at 60–80 μMol m^{-2} s^{-1} and ambient temperatures of 21–25°C.

Shoot regeneration assays

Cotyledons were excised from 7-day-old seedlings (grown as described above) and placed on 100 × 25 mm Petri plates (VWR International Ltd.) of the appropriate medium at a density of 26 cotyledons per plate. The cotyledons were arranged in a reproducible, evenly spaced pattern using a template. The plates were sealed with Micropore^{TM} surgical tape (3 M, 1530–1). All shoot induction media contained 1× Gamborg's B5 with vitamins (*Phyto*technology Laboratories), 20 g/l glucose, 0.5 g/l MES and 3 g/l Phytagel (Sigma, P8169) and were adjusted to pH 5.8 with KOH. Standard shoot induction medium SIM (Zhao et al. 2002) contained 4.4 μM 6-(γ,γ-dimethylallyamino) purine (2-iP) and 0.5 μM 1-naphthalene acetic acid (NAA), whereas the low-hormone version (10% SIM) contained 0.44 μM 2-iP and 0.05 μM NAA. To make stock solutions, the auxins and cytokinins were initially dissolved in 1 ml of 1 M KOH, then made up to 1,000× strength with distilled water and filter-sterilized. Comparisons of shoot organogenesis between different ecotypes were carried out on a standard SIM. Where SIM was supplemented with $AgNO_3$, this was added to the molten media at 55°C from a filter-sterilized, 1,000× stock. Assaying shoot organogenesis from ethylene mutants in a Col-0 background was carried out on 10% SIM. Shoot regeneration growth conditions were: 24 h constant low light (cool, white fluorescent light ~20 μmol m^{-2} s^{-1}) at 23°C in a Conviron growth chamber. Explants not used in pulse experiments were transferred to freshly made regeneration media 2 weeks after excision. In pulse experiments, the explants were moved to fresh media at the times stated and all treatments were moved simultaneously to control the effects of manipulation and new media. Shoot and root regeneration were usually scored at 3,4 and 5 weeks post-wounding, unless otherwise stated. Shoot regeneration was scored by the number of explants with at least one shoot (defined as a minimum of two leaves) and statistical

analysis was performed using InStat 3.0 for Mac (GraphPad Software).

Callus measurements

To measure green callus area, plates of cotyledon explants were scanned after 5 weeks on the relevant shoot-inducing medium against a red or pink background using a Snapscan flatbed scanner (Agfa Software). Using hue and saturation control in Photoshop Elements (Adobe), green and yellow were darkened to black, whereas red, magenta, cyan and blue were lightened to white. We applied an "RGB split" in ImageJ (National Institute of Health (NIH), U.S.) to the 300 dpi manipulated images, and the blue slide and threshold functions were used to convert the callus into "particles" before applying the "measure area" function.

ACC root elongation assay

Seeds of ecotypes Col-0, L.*er*, Est-1, Di-G and No-0 were surface sterilized and imbibed at 4°C for 3 days in sterile distilled water. The nutrient medium used for the assay was 0.5× MS + B5 (Phytotech Laboratories), buffered with 4.5 mM MES to pH 5.7, amended with 1% sucrose and solidified with 3 g/l phytogel (Sigma-Aldrich Co.). The ACC stock solution was made up fresh by initially dissolving ACC (Sigma-Aldrich Co.) in 1 ml of ethanol and making up to a 1000× stock in 70% ethanol. Hormone or control stocks were added to the molten media at 45°C and mixed by swirling. To measure inhibition of root elongation, the seeds were moved from 4°C to room temperature for 24 h to permit germination prior to resuspension in sterile 0.1% agar. Using a template, the seeds were placed in a line comprising 22 seeds across each 15 cm plate (VWR International Ltd.). The plates were opened at 7 days and scanned using a Snapscan flatbed scanner (Agfa software). The root lengths were measured from 300 dpi TIF files using ImageJ (NIH).

Results

Natural variation in ethylene responses and shoot regeneration in *Arabidopsis*

In an earlier study, we performed a natural variation screen for de novo shoot organogenesis from cotyledon explants that revealed a spectrum of in vitro shoot regeneration abilities in *Arabidopsis* (Chatfield and Raizada, unpublished). From this screen, we selected five ecotypes representing either the highest or lowest shoot organogenic rates. The low regenerators were Columbia (Col-0), Landsberg *erecta* (Ler) and Dijon-G (Di-G), with 0, 1.27 and 1.43% of explants producing shoots, respectively. The high regenerators were Estland (Est-1) and Nossen (No-0), with 83.9 and 97.4% of explants producing shoots, respectively. To determine if differential responses to ethylene might underlie some part of the observed variation in shoot regeneration, we began by assaying the inhibition of seedling root elongation by 1-aminocyclopropane-1-carboxylate (ACC; Eliasson et al. 1989) in the five ecotypes. When supplied exogenously, ACC increases ethylene biosynthesis (Abeles et al. 1992; Kende 1993). The inhibition of seedling primary root elongation was tested at concentrations of 1, 5, 10 and 25 μM ACC (data not shown). At 1 μM ACC, the differential responses of the ecotypes were most obvious. Of the five ecotypes tested, only Di-G showed a significantly different response ($P < 0.001$, Tukey–Kramer), with enhanced sensitivity to the 1 μM ACC treatment (Fig. 1a), resulting in a 30–35% greater reduction in root growth compared with the other ecotypes (Fig. 1a).

If increased ethylene sensitivity or enhanced ethylene biosynthesis in culture negatively affects shoot organogenesis, then inhibiting ethylene action might be expected to enhance regeneration. To test this hypothesis, we assayed shoot organogenesis in the five ecotypes cultured on SIM amended with 20 μM silver nitrate (Fig. 1b), an inhibitor of ethylene action (Beyer 1979). We had established that 20 μM silver nitrate was the highest concentration that could be used without a detrimental effect on explant survival (data not shown). Using this concentration of silver nitrate, two of the low regenerators, Col-0 and Di-G, failed to produce any shoots on SIM with, or without, silver nitrate (Fig. 1b), and in the remaining three ecotypes (L.er, Est-1 and No-0) the silver nitrate treatment caused a decrease in shoot regeneration rates compared with SIM alone (Fig. 1b). For Est-1 and No-0, the reduction in shoot regeneration associated with the silver nitrate treatment was found to be significant ($P < 0.01$, Tukey–Kramer), but this was not established for Ler ($P > 0.05$).

Shoot regeneration in vitro is anticipated to comprise multiple developmental steps (Che et al. 2006; Christianson and Warnick 1983; Hicks 1994; Sugiyama 1999) and ethylene may act to promote or inhibit the process at specific stages. We therefore hypothesized that inhibiting ethylene action for limited periods with pulsed treatments of silver nitrate might prove less inhibitory to shoot regeneration than continuous exposure, and might reveal whether the enhanced ethylene sensitivity we had observed in Di-G affected a particular stage in organogenesis. For this experiment, times selected for the 20 μM silver nitrate (in SIM media) pulses were: the first 5 days after explant

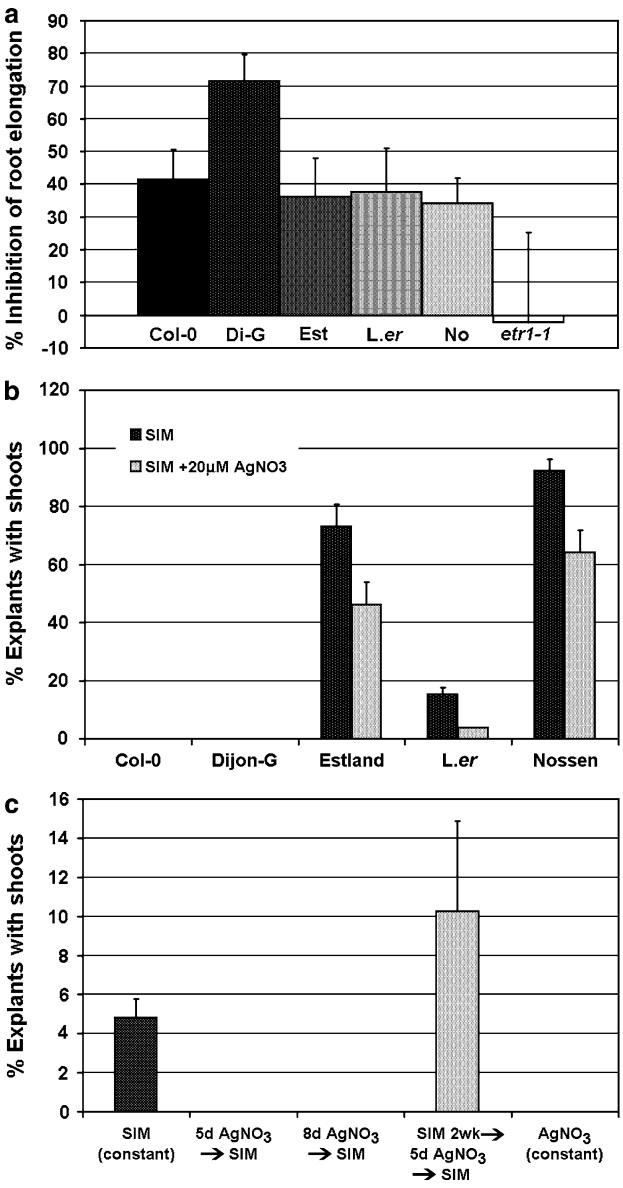

Fig. 1 Natural variation in shoot organogenesis and ethylene responses. **a** Inhibition of primary root elongation from seedlings of five ecotypes grown on half MS media containing 1 μM ACC measured at 7DAG. The ethylene-resistant mutant *etr1-1* is included as a negative control. *Error bars* represent the standard error of the mean (N = 17 to 70). **b** Shoot regeneration from cotyledon explants exposed to $AgNO_3$, an inhibitor of ethylene action. Cotyledon explants were excised at 7DAG and placed on SIM or SIM containing 20 mM $AgNO_3$. **c** Shoot regeneration response of ecotype Dijon-G to differing pulse treatments of 20 μM $AgNO_3$ in SIM. For two of the pulse treatments, cotyledon explants were initially placed on SIM + 20 μM $AgNO_3$ for the first 5 or 8 days after excision, then moved to plain SIM. For the third pulse treatment, excised cotyledons were placed on SIM for 2 weeks, and then moved to SIM + 20 μM $AgNO_3$ for 5 days and then back to SIM. The explants were moved to identical SIM or SIM + $AgNO_3$ media for constant treatments. *Error bars* represent the standard error of the mean for the percent of regenerating explants per plate (two to six plates per treatment, 26 explants per plate)

excision, the first 8 days and a 5 day pulse after 2 weeks of incubation on SIM (Fig. 1c). The early pulse treatments (5 and 8 days after excision) and continuous treatment of silver nitrate produced no shoots compared with a rate of 4.8% of explants regenerating in the control treatment. Only the latter pulse of silver nitrate, at 2 weeks post-excision, produced a modest increase in shoot regeneration to 10.25% of explants (Fig. 1c), but this increase was not found to be statistically significant ($P > 0.05$, Tukey–Kramer).

Overall, inhibition of ethylene action by silver nitrate was associated with a significant reduction in the rate of shoot production in the normally prolific regenerators (No-0 and Est-1) and did not produce a convincing improvement in the shoot organogenesis rates of the poor regenerators, including the putatively ethylene-sensitive Di-G.

Mutations in components of the ethylene-signaling pathway affect shoot organogenesis

To further explore the role of ethylene in shoot organogenesis, we hoped to exploit the numerous well-characterized mutants affecting ethylene signaling and biosynthesis in *Arabidopsis*. However, the majority of available ethylene-related mutants have been generated in a Columbia background, which did not regenerate shoots from cotyledon explants on SIM. In order to exploit these resources, we had previously developed a shoot induction medium containing a tenth of the concentration of NAA and 2-iP (10% SIM) used in standard SIM, which stimulated shoot organogenesis from 5 to 20% of Col-0 explants (Chatfield and Raizada, unpublished results). Experiments with this low-hormone SIM often yielded different rates of shoot organogenesis between experimental runs, but the relative differences between genotypes or treatments were usually consistent between each replicate.

Using 10% SIM, we compared shoot organogenesis rates in ethylene-insensitive mutants and wild type. Because inhibiting ethylene action had reduced shoot organogenesis in those ecotypes capable of regeneration, we hypothesized that mutants suppressing the ethylene response would also produce fewer shoots. In the first of these experiments, we assayed shoot organogenesis in the ethylene-insensitive mutants *etr1-1* and *ein2-1* (Fig. 2a). ETR1 is a member of a small family of ethylene receptors that are believed to act as redundant negative regulators of ethylene signaling, unless inactivated by ethylene binding, and EIN2 is a downstream transducer of the ethylene response (reviewed Chen et al. 2005; Joo and Kim 2007; Wang et al. 2002). Both the *etr1-1* and *ein2-1* mutations reduced, but did do not abolish, shoot organogenesis in Columbia (Fig. 2a). We then tested two additional ethylene-insensitive mutants *ein4* and *ein7*

against wild type and *etr1-1*. The *EIN4* gene also encodes an ethylene receptor, but of a different sub-family to *ETR1*, whereas the *ein7* mutation affects the *EXORIBONUCLEASE 4* gene and acts indirectly to interrupt ethylene signal transduction. (Guo and Ecker 2003; Potuschak et al. 2003). Rates of shoot organogenesis from *ein4* and *ein7* explants were also reduced compared with the wild type (Fig. 2b), but the effect was not as marked as observed for *etr1-1* (Fig. 2b) and not significant compared with the wild type ($P > 0.05$). Overall, mutations reducing the ethylene response were generally associated with reduced rates of shoot organogenesis.

We then tested two loss-of-function mutations in *CTR1* that bring about a constitutive ethylene response. The *ctr1-1* and *ctr1-12* mutations were both found to increase the number of explants producing shoots compared with the wild type (Fig. 2c). Shoot organogenesis was also compared between Col-0 and *ctr1-1* as the numbers of shoots produced by each regenerating explant (Fig. 2d). The *ctr1-1* mutant generally produced one to two shoots per explant, with only a small increase in the number of explants with multiple shoots compared with the wild type (Fig. 2d). Overall, the constitutive triple-response mutants enhanced shoot regeneration, increasing the proportion of explants successfully generating at least one shoot.

Although the ethylene signal transduction mutants differed in the frequency of shoot regeneration when incubated on 10% SIM, none of them overcame the inability of the Col-0 background to produce shoots on standard SIM. The ethylene signal-transduction mutants also differed in the quantity and character of the undifferentiated callus produced by each explant incubated on both full-strength and 10% SIM. The quantity of callus produced by a given genotype (data not shown) did not predict the amount of shoot regeneration, but qualitative differences in the type of callus produced were associated with differing rates of shoot organogenesis. Ethylene-insensitive mutants with reduced rates of shoot organogenesis, such as *etr1-1* and *ein2-1*, produced a more filamentous, friable callus than the wild type, whereas *ctr1-1* explants usually generated a darker green callus than the Columbia background (data not shown).

A mutant conferring endogenous ethylene over-production enhances shoot regeneration

The constitutive activation of the ethylene signal transduction pathway downstream of *CTR1* was found to enhance shoot organogenesis on low hormone SIM. Some

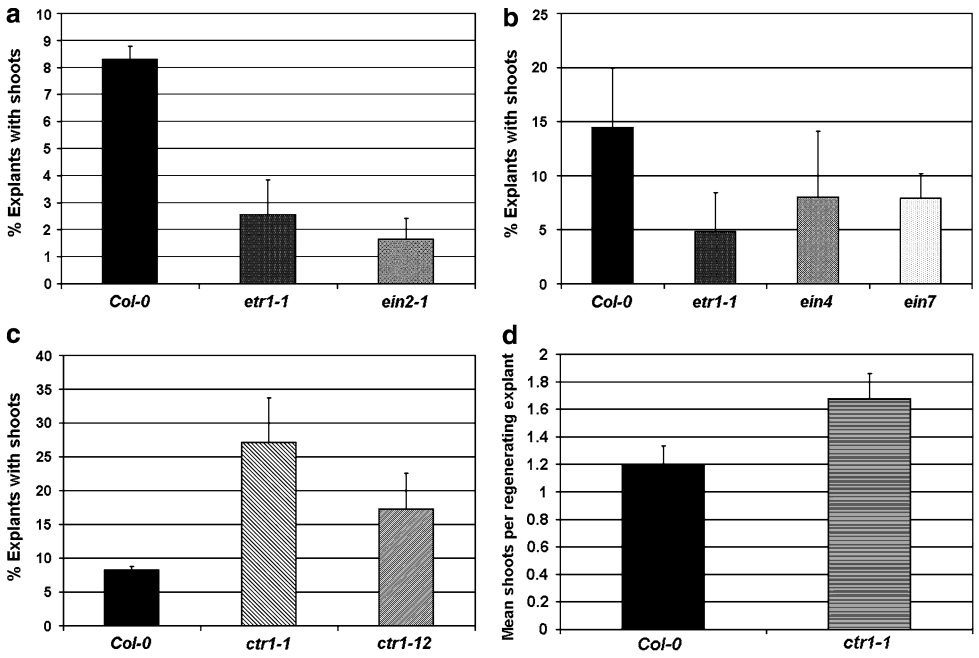

Fig. 2 Shoot regeneration from cotyledon explants of ethylene-response mutants. **a–c** Shoot regeneration from cotyledon explants excised at 7DAG and incubated on 10% SIM for 5 weeks, measured as the percentage of explants producing shoots. **a** Comparison of shoot organogenesis from the ethylene-insensitive mutants *etr1-1* and *ein2-1* with Col-0 wild type. **b** The percentage of explants producing shoots were compared between the ethylene-insensitive mutants *ein4*, *ein7* and *etr1-1* and wild type. **c** The rates of shoot organogenesis from explants of wild type (Col-0) is compared with two alleles of the *constitutive triple-response1* mutant as the percentage of explants producing shoots. **d** Shoot regeneration rates in Col-0 and *ctr1-1* are also compared as the mean number of shoots per regenerating explant. *Error bars* represent the standard error of the mean for the percent regenerating explants per plate (**a–c**; three to six plates per treatment, 26 explants per plate) or for the number of shoots per explant (**d**; $N = 130–156$)

constitutive ethylene responses have also been observed in ethylene over-production (*eto*) mutants, which generate elevated levels of endogenous ethylene (Chae et al. 2003). The dominant mutants *eto2* and *eto3* yield produce a 20- and 100-fold increase in ethylene biosynthesis in etiolated seedlings, respectively (Kieber et al. 1993), whereas the recessive *eto1-1* mutation results in a 10-fold increase in ethylene synthesis in intact seedlings (Chae et al. 2003; Guzman and Ecker 1990; Wang et al. 2004; Yoshida et al. 2005). In two separate experiments, we compared Col-0 and *eto1-1* with either *eto2* or *eto3* (Fig. 3). The *eto1-1* mutation resulted in a modest 3.5–7.5% increase in shoot regeneration compared with the wild type. Conversely, the *eto2* and *eto3* mutants produced a small, but not significant ($P > 0.05$) reduction in regeneration.

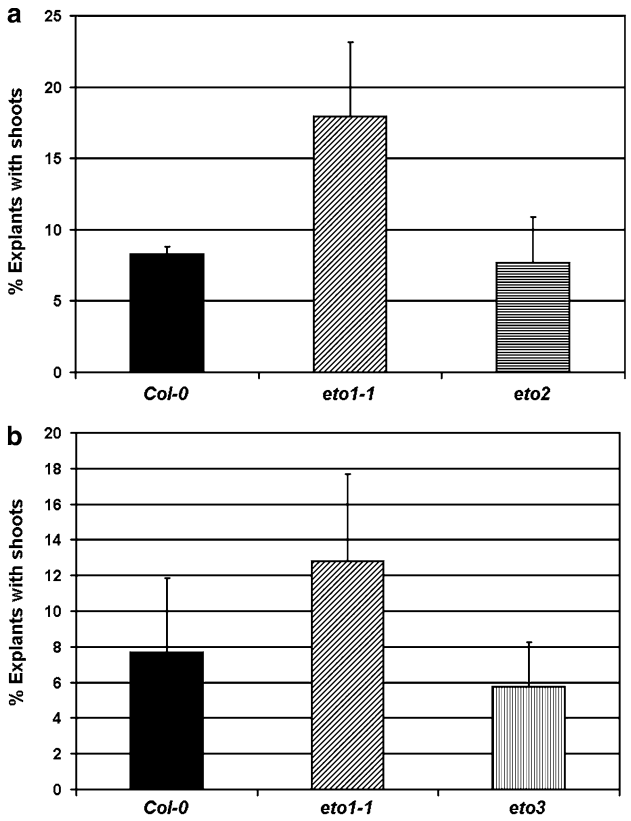

Fig. 3 Shoot regeneration responses of *ethylene over-production* (*eto*) mutants. **a**, **b** Shoot organogenesis from cotyledon explants of ethylene over-production mutants excised at 7DAG and incubated on 10% SIM. **a** Comparison of shoot organogenesis rates from the mutants *eto1-1* and *eto2*, with wild type at 5 weeks. **b** The percentage of explants producing shoots by the mutants *eto1-1* and *eto3* at 5 weeks. *Error bars* represent the standard error of the mean for the percentage of regenerating explants per plate (three to six plates per treatment, 26 explants per plate)

The *hls1-1* mutant dramatically enhances shoot organogenesis

Evidence has accumulated to support substantial cross-talk between ethylene, auxin signaling and response pathways (Chilley et al. 2006; De Grauwe et al. 2005; Harper et al. 2000; Lehman et al. 1996; Li et al. 2004; Ohto et al. 2006; Park et al. 2007; Souter et al. 2004). Several mutants have been identified that suppress aspects of the constitutive ethylene response phenotype of the *ctr1* mutant. These include two mutants known to mediate auxin responses: the *hookless1* (*hls1*) and *auxin-resistant1* (*axr1*) mutants. Strong loss-of-function alleles of both *axr1* and *hls1* mutants do not perform differential cell expansion required for proper apical hook formation and hypocotyl elongation in the triple-response, and are resistant to exogenous ethylene (Lehman et al. 1996). The ethylene-inducible *HLS1* gene encodes a putative *N*-acetyltransferase and is believed to regulate the function, or transcription, of elements of the auxin-signaling pathways (De Grauwe et al. 2005; Lehman et al. 1996; Li et al. 2004; Ohto et al. 2006). *AXR1* encodes a subunit of the RUB-activating enzyme and is believed to be required for the auxin-induced degradation of the AUX/IAA transcriptional regulators (del Pozo et al. 2002; Gray et al. 2001). Loss of *AXR1* function results in pleiotropic phenotypes consistent with an overall reduction in auxin responses (Leyser et al. 1993; Lincoln et al. 1990).

Because *ctr1* loss-of-function alleles significantly increased the rate of shoot organogenesis, we hypothesized that these suppressors of the *ctr1* mutant phenotype might reduce shoot regeneration. We tested the strong *hls1-1* allele and a weak and strong allele of *axr1* (*axr1-3* and *axr1-12*, respectively) for shoot regeneration (Fig. 4a). Loss of *AXR1* function was found to have a dramatic inhibitory effect on shoot organogenesis, which was abolished in the *axr1-12* allele and reduced to 2–3% of wild-type levels in the weaker *axr1-3* allele. Conversely, shoot regeneration rates were enhanced more than 2.5-fold in the *hls1-1* mutant compared with the wild type. Thus, *hls1-1* produced the highest regeneration rates we had observed in a Columbia background, with 54.7% of explants generating shoots.

Interestingly, both the *hls1-1* and *axr1* mutants were able to produce a number of roots on 10%SIM. The Col-0 background does not produce roots when incubated on 10%SIM, whereas *hls1-1*, *axr1-12* and *axr1-3*, developed visible roots from 46, 24.8 and 1.8% of explants, respectively (Fig. 4b). Apart from *hls1-1*, all other ethylene signaling and over-production mutants tested in the previous experiments produced roots from only 0 to 2% of explants (data not shown).

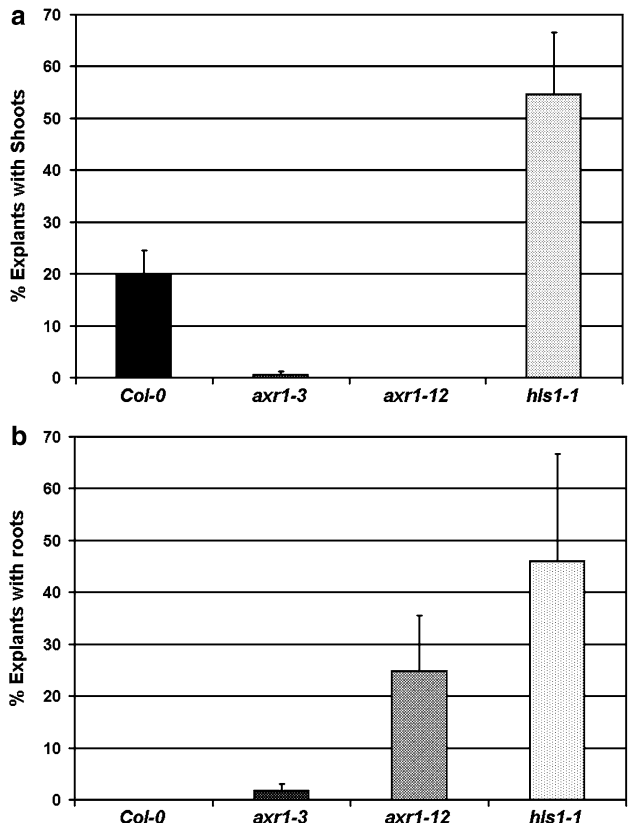

Fig. 4 Shoot regeneration responses of *hookless1* (*hls1-1*) and *auxin-resistant1* (*axr1-3* & *axr1-12*) mutants. **a** Comparison of shoot organogenesis from cotyledon explants excised at 7DAG from the *axr1-3*, *axr1-12* and *hls1-1* mutants and incubated on 10% SIM for 5 weeks. **b** Comparison of root organogenesis from cotyledon explants excised at 7DAG from the *axr1-3*, *axr1-12* and *hls1-1* mutants and incubated on 10% SIM for 5 weeks. *Error bars* represent the standard error of the mean for the percentage of explants per plate producing (**a**) shoots or (**b**) roots (five to seven plates per treatment, 26 explants per plate)

The *hls1-1* mutation and increased ethylene enhance shoot regeneration additively

The *eto1-1* mutation was associated with some enhancement of shoot regeneration rates in our earlier experiments, and we decided to test the effect of a modest pharmacological increase in ethylene on shoot regeneration by supplementing 10% SIM media with 1 μM ACC. Previous root elongation experiments had shown that this concentration of ACC produced a significant effect on ethylene-mediated developmental responses, without a deleterious effect on survival in tissue culture. To determine if increased ethylene might prove more or less beneficial to shoot organogenesis at specific times after excision, we included shift treatments in our experiment, moving the explants between 10% SIM with or without 1 μM ACC. In addition, we decided to compare the Col-0 background with the ethylene-response mutant, which had the most significant impact on shoot organogenesis (*hls1-1*), to investigate the interactions between elevated ethylene and the mutant lesion in modulating regeneration.

Four different treatments were used in the experiment. Two constant treatments were employed, namely 10% SIM (treatment label S-S-S) or 10% SIM plus 1 μM ACC (A-A-A) and two pulsed treatments. In the first of the pulse treatments, the explants spent the first week after excision on 10% SIM plus 1 μM ACC and were then moved to normal 10% SIM for the two succeeding weeks (treatment label A-S-S). Explants subjected to the second pulse treatment spent the first and third weeks after excision on 10% SIM, but were exposed to 10% SIM plus 1 μM ACC for the second week (treatment S-A-S). All treatments, continuous and pulsed, were shifted to fresh media at the end of each of the first 3 weekly periods. The results of this experiment are shown in Fig. 5.

All treatments in which the wild type was exposed to ACC, continuously or as a week-long pulse, induced visible shoot production (Fig. 5a) earlier (at 4 weeks) and significantly ($P < 0.05$, Tukey–Kramer) enhanced the rate of shoot regeneration (Fig. 5a, b). The percentage of wild-type explants producing shoots at 6 weeks after excision was similarly enhanced by approximately threefold in each of the ACC treatments (Fig. 5a). The *hls1-1* mutant also produced visible shoots on 10% SIM earlier than the wild-type control (Fig. 5a) In addition, the *hls1-1* mutant also responded to some ACC treatments with enhanced shoot regeneration. However, *hls1-1* only showed a significant increase in the numbers of explants producing shoots on two of the ACC treatments; a pulse of ACC for the second week after excision ($P < 0.01$, Tukey–Kramer), or continuous 1 μM ACC ($P < 0.001$, Tukey–Kramer), enhanced shoot regeneration at 6 weeks from approximately 20% of explants to 45 and 52%, respectively (Fig. 5a). In contrast, a pulse of ACC in the first week after excision did not enhance shoot regeneration substantially in the *hls1-1* mutant, with only 24% of explants producing shoots (Fig. 5a). The first week pulse of ACC was also the only treatment in which Col-0 produced shoots from more explants (35%) than the *hls1-1* mutant (Fig. 5a).

Shoot regeneration was also measured as the mean number of shoots per regenerating explant (Fig. 5b). In the wild type, the majority of regenerating explants exposed to both constant 10% SIM and the ACC treatments produced only one shoot (Fig. 5b). Conversely, the *hls1-1* mutation increased the mean number of shoots produced by each regenerating explant approximately twofold (Fig. 5b). In addition, none of the ACC treatments increased the number of shoots produced by regenerating *hls1-1* explants (Fig. 5b).

The quantity of green callus produced by the explants subjected to each condition was also measured (Fig. 5c). It

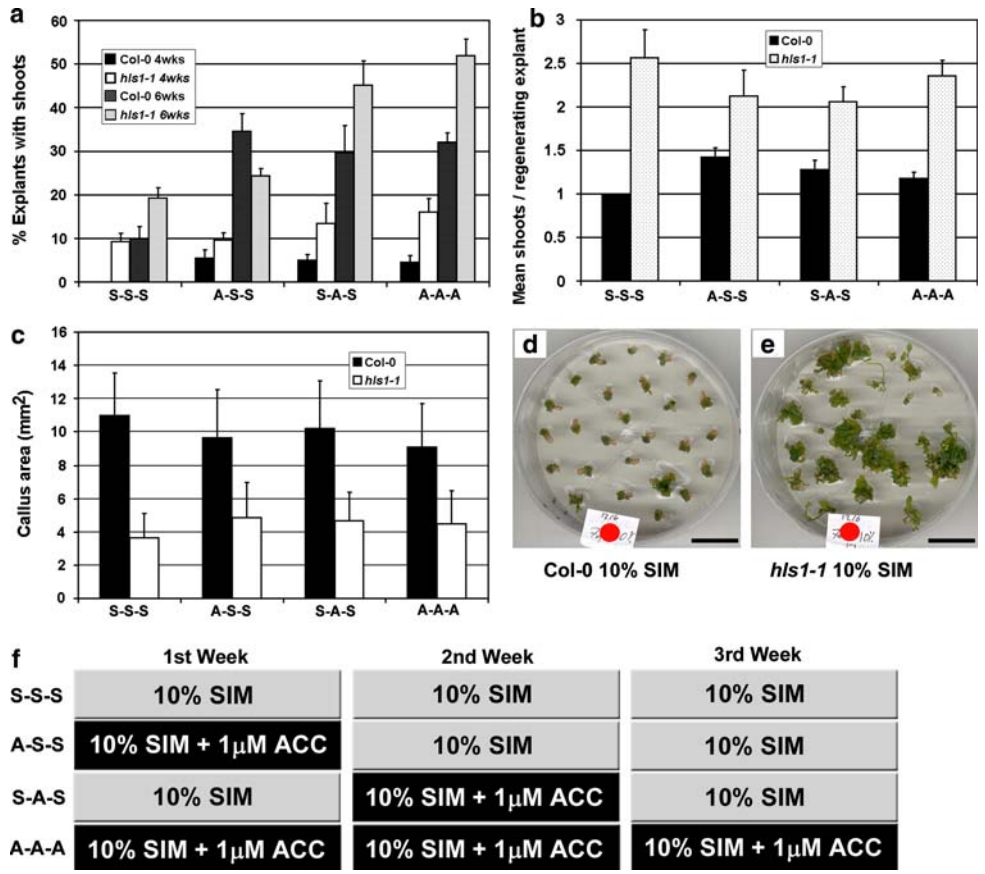

Fig. 5 Shoot regeneration and callus growth responses of wild-type (Col-0) and the *hookless1 (hls1-1)* mutant to shift/pulse treatments of ACC. **a** Rates of shoot organogenesis from Col-0 and *hls1-1* cotyledon explants (measured as percentage of explants with shoots) subjected to differing combinations of 10% SIM or 10% SIM + 1 μM ACC and scored at 4 and 6 weeks after excision. **b** Shoot organogenesis rates were also measured at 5 weeks as the mean number of shoots per regenerating explant. Shoots were defined as: two or more leaves growing from a single origin. **c** The area covered by green callus produced by the explants on the different treatments was measured at 6 weeks after excision. **d**, **e** Sample plates comparing shoot regeneration from (**d**) Col-0 and (**e**) *hls1-1* explants cultured on 10% SIM for 6 weeks (scale bars = 2 cm). **f** A key detailing the shift treatments corresponding to the three letter codes (e.g., S-S-S) used in the graphs. *Error bars* represent the standard error of the mean for the percentage of regenerating explants per plate (**a**) and mean shoots per explant (**b**; N = four to six plates, 26 explants per plate) and standard deviation between explants for callus area (**c**; N = 104−156 explants)

was found that the *hls1-1* explants generated approximately half as much callus as the wild type in each of the treatments (Fig. 5c), whereas the ACC treatment did not significantly ($P > 0.05$, Tukey–Kramer) affect the amount of callus produced by either genotype (Fig. 5c). Qualitatively, it was also obvious that the shoots on *hls1-1* explants were usually more bushy or developed than those of Columbia, with larger numbers of leaves (Fig. 5d, e).

Overall, 1 μM ACC substantially increased the proportion of regenerating explants in the wild type, but not the number of shoots produced by each of these organogenic explants. The *hls1-1* mutation increased both the number of regenerating explants and the number of shoots produced by each of these. For the most part, the effects on shoot regeneration of increasing ethylene and of the loss of *HLS1* function are additive, except where the two conditions are combined in a pulse treatment of ACC in the first week after excision.

Discussion

Ethylene accumulation has frequently been found to have a negative effect on shoot organogenesis in culture (reviewed Biddington 1992) and this negative correlation has been specifically shown in regeneration of the model plant *Arabidopsis thaliana* (Hu et al. 2006) and the related species *Brassica juncea* (Pua and Lee 1995). However, our data demonstrate a functional requirement for some components of the ethylene-signaling pathway in shoot regeneration, namely *ETR1* and *EIN2*. Additionally, we have shown that up-regulation of the ethylene-response pathway downstream

of *CTR1*, or increased ethylene biosynthesis, can enhance rates of shoot regeneration. The contrasting effect of ethylene in our studies may reflect differences in the culture system and explants used. For example, our study employed a single reduced-hormone shoot induction step, without pre-incubation on callus induction medium. Higher concentrations of auxin and cytokinin may induce supra-optimal levels of endogenous ethylene that reduce shoot regeneration. Further, our culture system employed deep-dish culture plates, ventilated by sealing with gas-permeable tape, in which head-space ethylene was unlikely to accumulate. In this way we have been able to identify a positive role for ethylene in shoot organogenesis and in our work with the *hls1-1* mutant, implicate a subset of interactions between the auxin and ethylene response pathways in promoting shoot regeneration.

Natural variation in ethylene sensitivity and shoot regeneration

Of the five ecotypes tested, only Di-G showed a marked difference in the sensitivity of root elongation to the ACC treatment (Fig. 1a). This suggests that global, *in planta*, ethylene sensitivity does not underlie the substantial differences in shoot regeneration between the other four ecotypes, Col-0, Ler, Est-1 and No-0. However, ethylene biosynthesis and responses in isolated organs incubated on exogenous auxin and cytokinin may differ markedly from intact seedlings. Therefore, a role for ethylene in contributing to at least some of the variation in shoot regeneration in these ecotypes cannot be eliminated.

Inhibition of ethylene action with silver nitrate was not found to improve regeneration in the ecotypes we tested (Fig. 1b). For the most part, 20 μM $AgNO_3$ was found to reduce the numbers of explants producing shoots in those ecotypes capable of regeneration on SIM. The modest enhancement of shoot regeneration seen in one of the pulsed treatments of the putatively ethylene-sensitive Di-G ecotype appears to be too late to influence developmental programs associated with the key early stages in organogenesis (Che et al. 2006; Christianson and Warnick 1985), and this treatment could act to improve organogenesis by reducing ethylene-induced senescence in culture. Regardless of the mode of action, this result is not sufficient to support the hypothesis that ethylene sensitivity underlies the poor shoot regeneration of Di-G.

Ethylene signaling and shoot regeneration

Our studies revealed a consistent correlation between ethylene signaling and shoot regeneration. Mutations down-regulating the ethylene response were associated with decreased rates of shoot organogenesis, whereas up-regulation of the ethylene-response pathway in the *ctr1* mutants was associated with enhanced regeneration. Differences in the impact of the two redundant ethylene receptor mutants, *etr1-1* and *ein4*, on shoot regeneration may reflect different relative contributions of receptor groups 1 and 2 to ethylene signaling, or differing expression levels in the explants (Qu et al. 2007).

In addition to producing fewer shoots, we found that mutants with reduced ethylene sensitivity produced a faster growing callus than the wild type, with larger numbers of filamentous projections (data not shown). This morphology resembles type II callus required for efficient regeneration via somatic embryogenesis in many cereals (Armstrong and Green 1985). Thus, down-regulating the ethylene pathway may promote a more open-ended multipotent fate in *Arabidopsis* callus cells. A study by Hamant et al. (2002) has revealed a negative feedback relationship between ethylene signaling and the expression of the meristem-associated gene *KNAT2*. Exogenous ACC or constitutive ethylene responses in a *ctr1* mutant background were found to down-regulate *KNAT2* expression, whereas in an ethylene-insensitive background the *KNAT2* domain expands within the meristem and ectopic expression is also seen. These results seem inconsistent with our observations indicating that up-regulation of ethylene signaling enhances shoot regeneration, a process presumed to include the expression of shoot meristem-specific genes (Zhang and Lemaux 2004). However, it is likely that the formation of a new shoot from the callus will also depend upon a subset of cells undergoing differentiation to initiate and form lateral organs. There is strong evidence that feedback systems operate between the meristematic and the adaxial cell fate of developing lateral organs to maintain and co-ordinate development (McConnell and Barton 1998; Siegfried et al. 1999; Sussex 1954). Therefore, suppression of the stem-cell fate to allow differentiation of cells within a mass of callus may be an important step in promoting the establishment of an organized de novo meristem. Thus, ethylene signaling may enhance shoot organogenesis by promoting differentiation or delimiting the stem cell fate.

Effects of ethylene over-production mutations and pharmacological manipulation of ethylene levels on shoot regeneration

The *ethylene over-production* mutants *eto2* and *eto3* were found to produce marginally fewer shoots from cotyledon explants than the wild type (Fig. 3a,b). The dramatic increase in ethylene production seen in these mutants results in phenotypically similar effects on dark-grown seedlings as

the constitutive ethylene responses associated with the *ctr1* mutants, which were associated with enhanced shoot regeneration (Fig. 2c). However, normal development in light-grown plants yields a dark green, compact and later-flowering phenotype from *ctr1* in contrast to the more precocious senescence seen in the *ethylene over-production* mutants. Therefore, ethylene over-production appears to be impinging on some different response pathways than those up-regulated by loss of *CTR1* function. Promotion of senescence by *eto2* and *eto3* may negatively impact on the ability of these mutants to generate shoots, since age-related decreases in shoot organogenic competence have been seen in cotyledon culture (Zhao et al. 2002). The *eto1-1* mutant produces a more modest over-production of ethylene than *eto2* and *eto3* and also yielded a small increase in shoot regeneration (Fig. 3a, b). This is consistent with the hypothesis that there might be a threshold concentration of endogenous ethylene above which shoot organogenesis is negatively affected.

Independent effects of ethylene and HLS1 loss of function in enhancing shoot regeneration

Expression of the *HLS1* gene is up-regulated by ethylene and is known to be down-regulated in some ethylene-insensitive backgrounds (Lehman et al. 1996). Over-expression of *HLS1* is associated with some constitutive ethylene-response phenotypes, and loss of function in this gene is known to suppress aspects of the constitutive ethylene-response phenotype of the *ctr1* mutants (Lehman et al. 1996). Therefore, enhancement of shoot regeneration by *hls1-1* appears to conflict with our earlier observations regarding ethylene signaling and shoot regeneration. However, we also determined that the increases in shoot regeneration associated with the *hls1-1* mutation and exogenous ACC are largely additive, suggesting that the mutation might be acting via independent mechanisms to enhance organogenesis. In support of this view, the effect of the *hls1-1* mutation on shoot regeneration also appears to differ in character. Loss of *HLS1* function markedly increased both the proportion of explants producing shoots and the number of shoots produced by each regenerating explant, whereas ACC addition, or the *ctr1* mutations, only had a substantial impact on the number of explants that generated shoots. However, we cannot eliminate the possibility that both the *hls1-1* mutant and addition of 1 μM ACC affect the same ethylene-dependent process, but at sub-optimal levels. The absence of an additive effect in the two treatments in the first week pulse of ACC is also interesting and may indicate that there is some interaction between the two conditions during this initial culture period.

The *HLS1* gene is believed to mediate interactions between ethylene and auxin-signaling pathways and has been shown to regulate specific genes involved in mediating auxin responses, although the mechanism underlying this control remains unknown (De Grauwe et al. 2005; Lehman et al. 1996; Li et al. 2004; Ohto et al. 2006). Additionally, aspects of the *HLS1* loss-of-function phenotype can be phenocopied by exogenous application of 2,4-D (a synthetic auxin) or auxin transport inhibitors (Lehman et al. 1996). It could therefore be hypothesized that, in the *hls1-1* mutant, a subset of auxin responses may be altered in a way that favors shoot organogenesis given the specific concentrations of auxin and cytokinin supplied exogenously. Consistent with this view, the *hls1-1* mutant does appear to respond differently to the exogenous hormones in culture, producing less callus (Fig. 5c) and initiating a number of roots (Fig. 4b). The *axr1-12* mutant also shared some of these responses to the culture conditions, but failed to initiate any shoots, probably reflecting a more global and severe impact on auxin responses. If *hls1-1* were responding differently to the ratio of supplied hormones, then one might hypothesize that an alternative ratio of auxin:cytokinin could be found to enhance regeneration in wild-type *Arabidopsis*. In fact, a high auxin:cytokinin ratio is used in a callus-induction step that enhances regeneration in some *Arabidopsis* ecotypes (Cary et al. 2002; Che et al. 2002; Valvekens et al. 1988). However, in previous studies, we established that standard callus induction media did not enhance shoot regeneration in Col-0 cotyledon explants, but a reduction in the amount of endogenous hormones supplied in the shoot induction medium (10% SIM) did. Both the low-hormone SIM and the *hls1-1* mutation are associated with reduced callus and improvements in shoot regeneration rates in a Col-0 background, suggesting that achieving a critical mass of dedifferentiated cells is not an issue preventing Col-0 from regenerating shoots.

Decades of research have established that ethylene produced by plant tissues in culture can significantly affect growth and regeneration in vitro (reviewed Biddington 1992). Using ethylene biosynthetic and signaling mutants, we have identified specific genes capable of modulating the shoot regenerative abilities of *Arabidopsis* in culture. The assignment of rate-limiting targets, such as *CTR1*, *ETO1* and *HLS1*, offer an avenue for enhancing our understanding of the phenomenon and potential groundwork for the design of small molecules to improve in vitro regeneration of recalcitrant species.

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References

Abeles F, Morgan P, Saltveit M (1992) Ethylene in plant biology, 2nd edn. Academic Press, San Diego

Armstrong CL, Green CE (1985) Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-proline. Planta 164:207–214

Beyer EM (1979) Effect of silver ion, carbon dioxide, and oxygen on ethylene action and metabolism. Plant Physiol 63:169–173

Biddington NL (1992) The Influence of ethylene in plant-tissue culture. Plant Growth Regul 11:173–187

Cary AJ, Che P, Howell SH (2002) Developmental events and shoot apical meristem gene expression patterns during shoot development in *Arabidopsis thaliana*. Plant J 32:867–877

Chae HS, Faure F, Kieber JJ (2003) The eto1, eto2, and eto3 mutations and cytokinin treatment increase ethylene biosynthesis in *Arabidopsis* by increasing the stability of ACS protein. Plant Cell 15:545–559

Che P, Gingerich DJ, Lall S, Howell SH (2002) Global and hormone-induced gene expression changes during shoot development in *Arabidopsis*. Plant Cell 14:2771–2785

Che P, Lall S, Nettleton D, Howell SH (2006) Gene expression programs during shoot, root, and callus development in *Arabidopsis* tissue culture. Plant Physiol 141:620–637

Chen YF, Etheridge N, Schaller GE (2005) Ethylene signal transduction. Ann Bot 95:901–915

Chilley PM, Casson SA, Tarkowski P, Hawkins N, Wang KLC, Hussey PJ, Beale M, Ecker JR, Sandberg GK, Lindsey K (2006) The POLARIS peptide of *Arabidopsis* regulates auxin transport and root growth via effects on ethylene signaling. Plant Cell 18:3058–3072

Chraibi KM, Latche A, Roustan JP, Fallot J (1991) Stimulation of shoot regeneration from cotyledons of *Helianthus annuus* by the ethylene inhibitors, silver and cobalt. Plant Cell Reports 10:204–207

Christianson ML, Warnick DA (1983) Competence and determination in the process of in vitro shoot organogenesis. Dev Biol 95:288–293

Christianson ML, Warnick DA (1985) Temporal requirement for phytohormone balance in the control of organogenesis in vitro. Dev Biol 112:494–497

De Grauwe L, Vandenbussche F, Tietz O, Palme K, Van Der Straeten D (2005) Auxin, ethylene and brassinosteroids: Tripartite control of growth in the *Arabidopsis* hypocotyl. Plant Cell Physiol 46:827–836

DeCook R, Lall S, Nettleton D, Howell SH (2006) Genetic regulation of gene expression during shoot development in *Arabidopsis*. Genetics 172:1155–1164

del Pozo JC, Dharmasiri S, Hellmann H, Walker L, Gray WM, Estelle M (2002) AXR1-ECR1-dependent conjugation of RUB1 to the *Arabidopsis* cullin AtCUL1 is required for auxin response. Plant Cell 14:421–433

Eliasson L, Bertell G, Bolander E (1989) Inhibitory action of auxin on root elongation not mediated by ethylene. Plant Physiol 91:310–314

Gray WM, Kepinski S, Rouse D, Leyser O, Estelle M (2001) Auxin regulates SCFTIR1-dependent degradation of AUX/IAA proteins. Nature 414:271–276

Guo H, Ecker JR (2003) Plant responses to ethylene gas are mediated by SCFEBF1/EBF2-dependent proteolysis of EIN3 transcription factor. Cell 115:667–677

Guzman P, Ecker JR (1990) Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. Plant Cell 2:513–523

Hamant O, Nogue F, Belles-Boix E, Jublot D, Grandjean O, Traas J, Pautot V (2002) The KNAT2 homeodomain protein interacts with ethylene and cytokinin signaling. Plant Physiol 130:657–665

Harper RM, Stowe-Evans EL, Luesse DR, Muto H, Tatematsu K, Watahiki MK, Yamamoto K, Liscum E (2000) The NPH4 locus encodes the auxin response factor ARF7, a conditional regulator of differential growth in aerial *Arabidopsis* tissue. Plant Cell 12:757–770

Hicks GS (1994) Shoot induction and organogenesis in vitro—a developmental perspective. In Vitro Cell Dev Biol Plant 30P:10–15

Holme IB, Torp AM, Hansen LN, Andersen SB (2004) Quantitative trait loci affecting plant regeneration from protoplasts of *Brassica oleracea*. Theor Appl Genet 108:1513–1520

Hu WW, Gong HB, Pua EC (2006) Modulation of SAMDC expression in *Arabidopsis thaliana* alters in vitro shoot organogenesis. Physiol Plant 128:740–750

Huxter TJ, Thorpe TA, Reid DM (1981) Shoot initiation in light-grown and dark-grown tobacco callus—the role of ethylene. Physiol Plant 53:319–326

Joo S, Kim WT (2007) A gaseous plant hormone ethylene: the signaling pathway. J Plant Biol 50:109–116

Kende H (1993) Ethylene biosynthesis. Ann Rev Plant Physiol Plant Mol Biol 44:283–307

Kieber JJ, Rothenberg M, Roman G, Feldmann KA, Ecker JR (1993) CTR1, a negative regulator of the ethylene-response pathway in *Arabidopsis*, encodes a member of the RAF family of protein kinases. Cell 72:427–441

Kumar PP, Reid DM, Thorpe TA (1987) The role of ethylene and carbon dioxide in differentiation of shoot buds in excised cotyledons of *Pinus radiata* in vitro. Physiol Plant 69:244–252

Lall S, Nettleton D, DeCook R, Che P, Howell SH (2004) Quantitative trait loci associated with adventitious shoot formation in tissue culture and the program of shoot development in *Arabidopsis*. Genetics 167:1883–1892

Lehman A, Black R, Ecker JR (1996) HOOKLESS1, an ethylene response gene, is required for differential cell elongation in the *Arabidopsis* hypocotyl. Cell 85:183–194

Leyser HMO, Lincoln CA, Timpte C, Lammer D, Turner J, Estelle M (1993) *Arabidopsis* auxin-resistance gene AXR1 encodes a protein related to ubiquitin-activating enzyme E1. Nature 364:161–164

Li H, Johnson P, Stepanova A, Alonso JM, Ecker JR (2004) Convergence of signaling pathways in the control of differential cell growth in *Arabidopsis*. Dev Cell 7:193–204

Lincoln C, Britton JH, Estelle M (1990) Growth and development of the AXR1 mutants of *Arabidopsis*. Plant Cell 2:1071–1080

Mano Y, Komatsuda T (2002) Identification of QTLs controlling tissue-culture traits in barley (*Hordeum vulgare* L.). Theor Appl Genet 105:708–715

McConnell JR, Barton MK (1998) Leaf polarity and meristem formation in *Arabidopsis*. Development 125:2935–2942

Nishimura A, Ashikari M, Lin S, Takashi T, Angeles ER, Yamamoto T, Matsuoka M (2005) Isolation of a rice regeneration quantitative trait loci gene and its application to transformation systems. Proc Natl Acad Sci USA 102:11940–11944

Ohto MA, Hayashi S, Sawa S, Hashimoto-Ohta A, Nakamura K (2006) Involvement of HLS1 in sugar and auxin signaling in *Arabidopsis* leaves. Plant Cell Physiol 47:1603–1611

Park JE, Kim YS, Yoon HK, Park CM (2007) Functional characterization of a small auxin-up RNA gene in apical hook development in *Arabidopsis*. Plant Sci 172:150–157

Potuschak T, Lechner E, Parmentier Y, Yanagisawa S, Grava S, Koncz C, Genschik P (2003) EIN3-dependent regulation of plant

ethylene hormone signaling by two *Arabidopsis* F box proteins: EBF1 and EBF2. Cell 115:679–689

Pua EC, Chi GL (1993) De novo shoot morphogenesis and plant growth of mustard (*Brassica juncea*) in vitro in relation to ethylene. Physiol Plant 88:467–474

Pua EC, Lee JEE (1995) Enhanced de novo shoot morphogenesis in vitro by expression of antisense 1-aminocyclopropane-1-carboxylate oxidase gene in transgenic mustard plants. Planta 196:69–76

Qu X, Hall BP, Gao ZY, Schaller GE (2007) A strong constitutive ethylene-response phenotype conferred on *Arabidopsis* plants containing null mutations in the ethylene receptors ETR1 and ERS1. BMC Plant Biol 7:3

Schiantarelli E, De la Pena A, Candela M (2001) Use of recombinant inbred lines (RILs) to identify, locate and map major genes and quantitative trait loci involved with in vitro regeneration ability in *Arabidopsis thaliana*. Theor Appl Genet 102:335–341

Siegfried KR, Eshed Y, Baum SF, Otsuga D, Drews GN, Bowman JL (1999) Members of the YABBY gene family specify abaxial cell fate in *Arabidopsis*. Development 126:4117–4128

Skoog P, Miller C (1957) Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp Soc Exp Biol 54:118–130

Souter MA, Pullen ML, Topping JF, Zhang X, Lindsey K (2004) Rescue of defective auxin-mediated gene expression and root meristem function by inhibition of ethylene signalling in sterol biosynthesis mutants of *Arabidopsis*. Planta 219:773–783

Sugiyama M (1999) Organogenesis in vitro. Curr Opin Plant Biol 2:61–64

Sussex IM (1954) Experiments on the cause of dorsiventrality in leaves. Nature 174:351–352

Taguchi-Shiobara F, Yamamoto T, Yano M, Oka S (2006) Mapping QTLs that control the performance of rice tissue culture and evaluation of derived near-isogenic lines. Theor Appl Genet 112:968–976

Tsuchisaka A, Theologis A (2004) Unique and overlapping expression patterns among the *Arabidopsis* 1-amino-cyclopropane-1-carboxylate synthase gene family members. Plant Physiol 136:2982–3000

Valvekens D, Vanmontagu M, Vanlijsebettens M (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants using kanamycin selection. Proc Natl Acad Sci USA 85:5536–5540

Vasil V, Hildebrandt AC (1965) Growth and tissue formation from single, isolated tobacco cells in microculture. Science 145:1454–1455

Vogel JP, Woeste KE, Theologis A, Kieber JJ (1998) Recessive and dominant mutations in the ethylene biosynthetic gene ACS5 of *Arabidopsis* confer cytokinin insensitivity and ethylene overproduction, respectively. Proc Natl Acad Sci USA 95:4766–4771

Wang KLC, Li H, Ecker JR (2002) Ethylene biosynthesis and signaling networks. Plant Cell 14:S131–S151

Wang KLC, Yoshida H, Lurin C, Ecker JR (2004) Regulation of ethylene gas biosynthesis by the *Arabidopsis* ETO1 protein. Nature 428:945–950

Yoshida H, Nagata M, Saito K, Wang KLC, Ecker JR (2005) *Arabidopsis* ETO1 specifically interacts with and negatively regulates type 2 1-aminocyclopropane-1-carboxylate synthases. BMC Plant Biol 5:14

Zhang SB, Lemaux PG (2004) Molecular analysis of in vitro shoot organogenesis. Crit Rev Plant Sci 23:325–335

Zhao QH, Fisher R, Auer C (2002) Developmental phases and STM expression during *Arabidopsis* shoot organogenesis. Plant Growth Reg 37:223–231