Post-transcriptional control of the *Arabidopsis* auxin efflux carrier EIR1 requires AXR1

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The auxin efflux carrier EIR1 (also known as AGR and AtPIN2) is a key mediator of the response of *Arabidopsis* roots to gravity [1,2]. This response is thought to require the establishment of a transient auxin gradient in the root meristem, resulting in differential cell elongation [3]. Recent reports suggest that EIR1 is essential for the asymmetric distribution of auxin in the root meristem [4–7], but the regulatory aspects of this process are still not fully understood. Here, we studied the regulation of *EIR1* in *Arabidopsis* using two reporters: one was a translational fusion that contained the entire EIR1 coding sequence, and the other a transcriptional fusion that had no EIR1 coding sequence. We found that EIR1 is controlled at the post-transcriptional level. The translational fusion was unstable in response to changes in auxin homeostasis, and was destabilized by cycloheximide. In contrast, the protein was stabilized in the *axr1-3* mutant, which is auxin resistant and defective in auxin responses such as root gravitropism [8,9]. AXR1 is thought to participate in ubiquitin-mediated control of protein stability [10–12]. The dependence of EIR1 reporter expression on auxin concentrations and AXR1 suggests that auxin transport is regulated through a feedback regulatory loop that affects protein stability in response to auxin.

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Results and discussion

The tissue-specific expression of EIR1 was examined *in planta* using two reporter constructs (Figure 1a). The first was a promoter fusion with the open reading frame of the β-glucuronidase (GUS) gene under the control of the *EIR1* promoter (EIP–GUS). The second was a translational fusion (EIF–GUS) containing the entire *EIR1* genomic coding region, fused to the amino terminus of the GUS reporter (order: *EIR1* promoter, EIR1 coding region, GUS). Each of these constructs was used to make transgenic *Arabidopsis* that were analyzed for GUS expression. Expression of the promoter fusion EIP–GUS was found in the entire root (Figure 1b). Expression of the full-length EIF–GUS translational fusion, in contrast, was restricted to the root meristem (Figure 1b). Thus, although both constructs shared the identical 5′ region of the *EIR1* gene, they had different expression patterns. Presumably, the *EIR1* promoter segment of EIP–GUS was incapable of conferring tissue-specific expression by itself. Additional *cis*-acting elements, located downstream of the EIR1 start codon and absent in EIP–GUS, might restrict expression of *EIR1* to the root tip. Similar mechanisms have been suggested for the expression of the flower specification gene *AGAMOUS*, as parts of the transcribed region of this gene are required to coordinate expression at the spatial and temporal level [13].

To visualize the translational fusion at the cellular level, we performed whole-mount immunostaining on EIF–GUS seedlings using an anti-GUS antibody (Figure 1c). Immunohistochemical analysis of the staining pattern revealed that EIF–GUS localized preferentially to the transverse cell walls of epidermal and cortical cells in the root meristem. The distribution of EIF–GUS mirrored exactly the localization of EIR1/AtPIN2 obtained by Müller *et al.* [6]. Additional experiments performed with X-Gluc as a substrate for the β-glucuronidase, demonstrated that the fusion protein localized preferentially to the cortical cell layer with some additional weaker staining in the epidermis and endodermis (Figure 1c). These findings implicate the root cortex as the major site for the suggested basipetal auxin transport from the root tip into the root elongation zone [2].

At the subcellular level, GUS staining was most prominent next to the transverse cell walls. We also found an additional intense signal at the periphery of the nucleus (Figure 1c). The subcellular localization of EIF–GUS both at the plasma membrane and around the nucleus was similar to that found for a full-length *EIR1* cDNA construct expressed in yeast [4]. Although the functional relevance of the perinuclear localization of EIR1 is not known, it could reflect an additional role for EIR1 in the intracellular transport of auxin.

The availability of a reporter that reflects the expression levels and the distribution of the EIR1 protein in the root

Figure 1

Reporter expression in *Arabidopsis*.
(a) Schematic illustration of the EIP–GUS and
EIF–GUS reporters. EIP–GUS contains the
5′ region of the presumptive EIR1 open
reading frame, which was used previously for
complementation of the *eir1-1* mutant [4].
uidA is the gene for GUS. Expression of
EIP–GUS gives rise to a fusion protein
between the first six amino acids of EIR1 and
GUS (grey box). EIF–GUS was made with the
identical 5′ region plus the entire genomic
coding region of the EIR1 gene product. The
GUS gene was introduced after the codon for
amino acid 647 of the presumptive EIR1
protein. (b) Expression of the promoter fusion
EIP–GUS was found in the entire root,
whereas the full-length EIF–GUS translational
fusion was only detectable in root meristems.
(c) Subcellular localization of EIF–GUS at
5 days after germination (DAG). Left, confocal
image of an EIF–GUS root immunostained
with an anti-GUS antibody. The protein was
found in the vicinity of the transverse cell walls
of epidermal and cortical cells. The scale bar
represents 50 μm. No comparable staining
was detected in plants devoid of the reporter
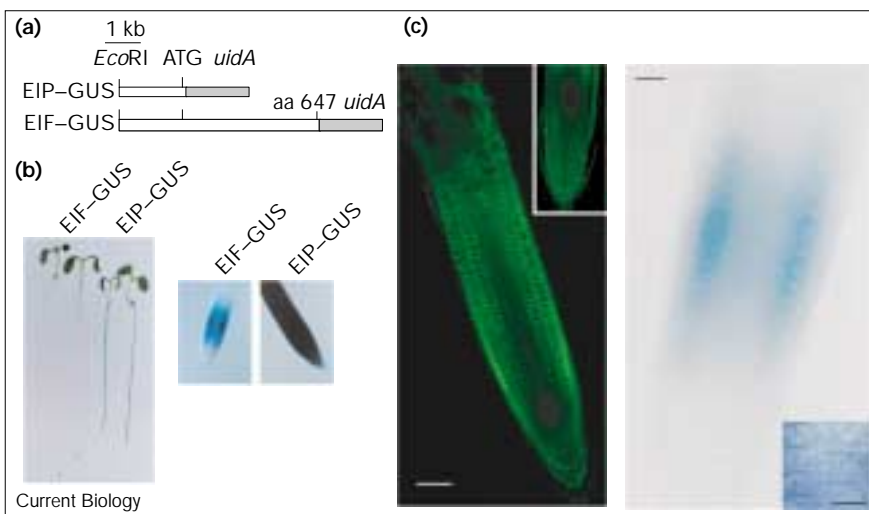
constructs (inset). Right, GUS staining of an
EIF–GUS primary root meristem. Expression
was strongest in the root cortex, with some
additional staining in the epidermis and
endodermis. The scale bar represents 30 μm.

The most prominent signal was observed in
cortical cells next to the transverse cell walls
and in the nuclear periphery (inset; the scale
bar represents 10 μm).

meristem allowed us to test responses of EIR1 to changes in auxin homeostasis. Treatment of EIF–GUS seedlings with the potent auxin analogue α-napthaleneacetic acid (1-NAA) or the inhibitor of polar auxin transport 2,3,5-tri-iodobenzoic acid (TIBA) for 24 hours caused a pronounced reduction of the EIF–GUS expression pattern and reduced β-glucuronidase activities in crude plant extracts (Figure 2a,c). These results suggest that an alteration in auxin homeostasis gives rise to a reduced expression of the auxin efflux carrier. To test whether this response requires a transcriptional regulation, we compared levels of the *EIR1* transcription as well as the expression of the EIP–GUS transgene in the presence of either 1-NAA or TIBA. Unlike the translational fusion, the EIP–GUS reporter remained highly active when incubated with 1-NAA or TIBA (Figure 2a,c). Thus, the 5′ *EIR1* promoter region is not sufficient to confer a response to the inhibitory effects of an altered auxin homeostasis on EIR1. To test whether auxin or TIBA treatment have an impact on transcript levels of EIR1, we performed northern blots with total root RNA. EIF–GUS plants grown in the presence of TIBA or 1-NAA for 24 hours — a treatment that causes a reduction in EIF–GUS protein levels — showed no significant alterations in the levels of *EIF–GUS* and *EIR1* mRNA (Figure 2b). Thus, *EIR1* mRNA levels are not affected by extensive auxin treatment.

Further evidence for a post-transcriptional regulation of EIR1 in response to auxin came from equivalent experiments performed with the auxin response mutant *axr1-3*.

This mutant, thought to be required for the regulation of the SCF[TIR1] complex [10–12], rendered EIF–GUS expression essentially insensitive to alterations in auxin homeostasis. Neither TIBA nor 1-NAA treatment led to a significant reduction of EIF–GUS activity in *axr1-3* (Figure 2a,c). Moreover, as in the wild type, *EIR1* mRNA levels in the *axr1-3* mutant remained unaffected by treatment with TIBA or auxin (Figure 2b).

To test whether the abundance of EIF–GUS is regulated through the control of protein stability, we performed quantitative GUS assays with EIF–GUS and EIP–GUS seedlings grown in the presence of the translational inhibitor cycloheximide. Cycloheximide treatment had no significant impact on EIP–GUS expression. Nevertheless, the same treatment performed with EIF–GUS seedlings reduced the enzyme activity by 70% after 4 hours incubation (Figure 2c). Thus, the coding region of *EIR1* seems to destabilize the fusion protein, giving rise to an enhanced protein turnover (Figure 2c). Presumably, the degradation of EIF–GUS becomes apparent under conditions where the levels cannot be restored by new protein synthesis. This explanation is also in agreement with previous suggestions that elements involved in auxin efflux are short-lived [14,15]. In contrast, when comparing wild-type and *axr1-3* seedlings expressing EIF–GUS in the presence of cycloheximide, we found that GUS activities in *axr1-3* plants remained at levels of 80–90% after 2 hours, whereas levels in the wild type dropped to about 50% (Figure 2c). These results suggest that EIF–GUS is

Figure 2
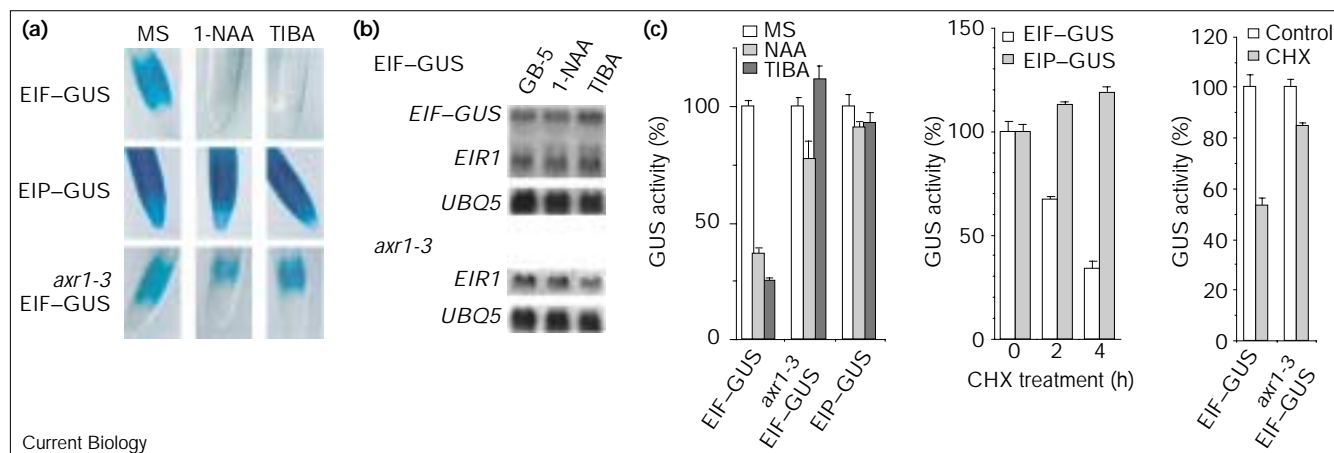
EIR1 expression depends on auxin homeostasis and elements involved in auxin signal transduction. **(a)** GUS staining in EIF–GUS and EIP–GUS seedlings at 5 DAG. Both TIBA and 1-NAA led to a significant reduction of EIF–GUS staining in the root meristem of ColO wild-type seedlings. EIP–GUS staining was not affected by 1-NAA or TIBA. EIF–GUS staining remained visible in the presence of 1-NAA or TIBA when expressed in *axr1-3* mutants. MS, Murashige and Skoog medium. **(b)** Northern blots of total root RNA from EIF–GUS and *axr1-3* seedlings. The transcript levels of *EIR1* and *EIF–GUS* were essentially unaffected by treatment with either 1-NAA or TIBA. *UBQ5* represents the loading control. GB-5, Gamborg's B-5 Medium. **(c)** Fluorometric quantitation of GUS activity. Relative activity is shown normalized to the protein content in plant extracts. Activities are plotted as percent of activity found in the untreated controls.

Standard deviations of parallel samples are indicated as bars. Left, EIF–GUS activity in the wild type responded to alterations in auxin homeostasis by a decrease of about 65–75%. In *axr1-3* mutants, EIF–GUS showed a significantly reduced response to 1-NAA treatment. No significant alterations were seen in the presence of TIBA. EIP–GUS activity remained essentially unaltered in the presence of either 1-NAA or TIBA. Middle, cycloheximide (CHX) treatment of EIF–GUS and EIP–GUS seedlings performed in liquid medium. After 4 h, EIF–GUS activities were reduced to about 30%. Right, EIF–GUS was stabilized in *axr1-3* mutants. After 2 h incubation in the presence of cycloheximide, EIF–GUS activity in *axr1-3* mutants remained at levels between 80% and 90%. EIF–GUS activity in the wild type was reduced to approximately 50% when compared with untreated controls.

stabilized to the effects of cycloheximide in the putative proteolysis mutant *axr1-3*.

Our results suggest that protein stability in response to alterations in auxin homeostasis is critical for EIF–GUS reporter expression. Thus, EIR1 activity might be regulated at a post-transcriptional level as well. To test whether the function of the EIR1 protein in auxin transport can be completely uncoupled from transcriptional control, we analyzed transgenic plants expressing the *EIR1* cDNA under the control of the heterologous cauliflower mosaic virus (CaMV) 35S promoter. Expression of this construct completely suppressed the defect caused by the *eir1-1* mutation (Figure 3). The response of transgenic plants to gravity and to 1 μM 1-aminocyclopropane-1-carboxylic acid (ACC; the precursor to ethylene) was similar to the wild type (Figure 3b,c). The reduction of longitudinal cell elongation and the induction of root hairs by ethylene in the transgenic plants was similar to that manifested by wild-type plants (Figure 3b). Those transgenic lines with no detectable expression of the EIR1 gene product still exhibited the *eir1* mutant phenotypes, suggesting that the rescue of *eir1-1* defects correlates with the ectopic expression of the *EIR1* cDNA (Figure 3). Nevertheless,

no additional, stably inherited phenotypes were observed in these transgenic lines. Thus, the expression of the *EIR1* cDNA, whose transcript was detectable in the entire transgenic seedlings, did not cause any phenotypic alterations that are unrelated to the rescue of *eir1-1* defects. Although we cannot exclude the possibility that other mechanisms regulate the stability of the ectopically expressed *EIR1* cDNA, our findings provide further support for the suggestion that EIR1 expression is controlled at the level of protein stability.

Assuming that EIR1 is required for basipetal transport of auxin from the root tip into the elongation zone, control of EIR1 by protein degradation would permit a rapid response to gravistimulation. This mechanism would also be consistent with the postulated establishment of transient auxin gradients required for root bending. Proteolytic degradation of EIR1 via activation of AXR1 could be essential for the establishment of an auxin gradient either during the initial response to gravity or, alternatively, in rapidly switching back to default levels once the root has reoriented. In either scenario, if AXR1 responds to auxin levels, then there is a mechanism for feedback inhibition of auxin transport into the root elongation zone.

Figure 3
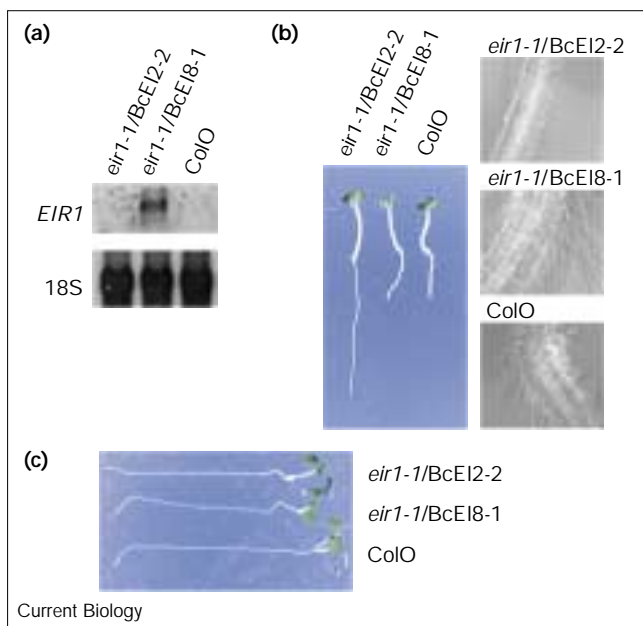
Ectopic *EIR1* expression rescues *eir1-1* defects. **(a)** Northern blot of total RNA from transgenic ColO plants transformed with pBcEI, which expresses the *EIR1* cDNA under control of a CaMV 35S promoter. Total RNA was hybridized with an *EIR1* probe, and the level of 18S rRNA confirmed equal loading. Note the lack of a hybridizing band in the ColO RNA lane, indicating the high level of constitutive *EIR1* overexpression in the transgenic line *eir1-1* BcEI8-1. No expression was seen in line *eir1-1* BcEI2-2.
(b) Response of transgenic plants towards ethylene. Left, comparison of ColO, *eir1-1* BcEI8-1 and *eir1-1* BcEI2-2 seedlings germinated on 1 μM ACC. The *eir1-1* BcEI8-1 line, which showed strong expression of the transgene, exhibited ethylene responses similar to the wild type. Root growth of *eir1-1* BcEI2-2 remained resistant to ACC. Right, parts of the primary root differentiation zone viewed under differential interference contrast (40 ×). ColO roots on 1 μM ACC formed short, expanded root cells and an increased number of root hairs. The *eir1-1* BcEI8-1 plants, which expressed *EIR1* cDNA, responded to ACC in a similar way to ColO wild-type plants whereas *eir1-1* BcEI2-2 plants did not respond to the growth regulator. **(c)** Ectopic expression of *EIR1* rescues the gravitropic defects of the *eir1-1* mutant. In the line *eir1-1* BcEI8-1 as in the wild type, root growth was reoriented when the plant was grown on vertically oriented plates. The line *eir1-1* BcE2-2, which had no detectable *EIR1* transcript, did not respond to gravity.

As a consequence, the degradation of EIR1 would provide a rapid method for equilibrating the flow of auxin within the root meristem.

Supplementary material
Supplementary material including additional methodological detail is available at http://current-biology.com/supmat/supmatin.htm.

Acknowledgements

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