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RESEARCH PAPER

Arabidopsis *aux1*^{rcr1} mutation alters AUXIN RESISTANT1 targeting and prevents expression of the auxin reporter *DR5:GUS* in the root apex

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Abstract

Multilevel interactions of the plant hormones ethylene and auxin coordinately and synergistically regulate many aspects of plant growth and development. This study isolated the *AUXIN RESISTANT1* (*AUX1*) allele *aux1*^{rcr1} (*RCR1* for *REVERSING CTR1-10 ROOT1*) that suppressed the root growth inhibition conferred by the constitutive ethylene-response *constitutive triple response1-10* (*ctr1-10*) allele. The *aux1*^{rcr1} mutation resulted from an L126F substitution at loop 2 of the plasma membrane-associated auxin influx carrier protein AUX1. *aux1*^{rcr1} and the T-DNA insertion mutant *aux1-T* were both defective in auxin transport and many aspects of the auxin response. Unexpectedly, expression of the auxin-response reporter *DR5:GUS* in the root apex was substantially prevented by the *aux1*^{rcr1} but not the *aux1-T* mutation, even in the presence of the wild-type *AUX1* allele. Following treatment with the synthetic auxin 1-naphthaleneacetic acid (NAA), *DR5:GUS* expression in *aux1*^{rcr1} and *aux1-T* occurred mainly in the root apex and mature zone. NAA-induced *DR5:GUS* expression in the root apex was markedly prevented by ethylene in genotypes with *aux1*^{rcr1} but not in *aux1-T* genotypes and the wild type. The effect of *aux1*^{rcr1} on *DR5:GUS* expression seemed to be associated with AUX1-expressing domains. Green fluorescence protein-fused aux1^{rcr1} was localized in the cytoplasm and probably not to the plasma membrane, indicating important roles of the Lys^{126} residue at loop 2 in AUX1 targeting. The possible effects of *aux1*^{rcr1} on *DR5:GUS* expression are discussed.

Key words: AUX1, *Arabidopsis*, *DR5:GUS*, auxin, ethylene, root gravitropism.

Introduction

Ethylene and auxin are plant hormones coordinately regulating various aspects of plant growth and development. Ethylene is perceived by a small family of ethylene-receptor members. The biochemical nature of the receptor signalling mechanism is unknown. Current studies suggest that ethylene-receptor signalling is mediated by the physical interaction of the receptor histidine kinase (HK) domain and the mitogen-activated protein kinase kinase kinase CONSTITUTIVE TRIPLE-RESPONSE1 (CTR1) to suppress ethylene responses (Clark *et al*., 1998; Huang *et al*., 2003). Recent studies suggest that members of the ethylene receptor can cooperatively mediate the ethylene signal to an alternative pathway independent of CTR1 (Gao *et al*., 2008; Chen *et al*., 2010; Liu and Wen, 2012; Qiu *et al*., 2012; Xie *et al*., 2006, 2012). Auxin is

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; ARF, auxin response factor; AUX, auxin; ER, endoplasmic reticulum; GFP, green fluorescent protein; GUS, β-glucuronidase; IAA, indole acetic acid; LRC, lateral root cap; MS, Murashige and Skoog; NAA, 1-naphthaleneacetic acid; PM, plasma membrane; qRT-PCR, quantitative RT-PCR; SD, standard deviation; SE, standard error; UTR, untranslated region; YFP, yellow fluorescent protein.

perceived by a small family of TRANSPORTER INHIBITOR INSENSITIVE1 (TIR1)-related F-box proteins and functions as a 'molecular glue' to facilitate the association of the receptor and auxin/indole acetic acid (AUX/ IAA) proteins that negatively modulates the expression of *AUXIN RESPONSE FACTOR*s (*ARF*s). The association of TIR1 and AUX/IAAs facilitates AUX/IAA polyubiquination, which subjects AUX/IAAs to 26S proteosome-mediated degradation, and the repression of *ARF* expression is alleviated (Dharmasiri *et al*., 2005; Dos Santos Maraschin *et al*., 2009; Parry *et al*., 2009; Tan *et al*., 2007). Activation of *ARF*s directs the expression of genes responsive to auxin.

Arabidopsis etiolated seedlings produce a long hypocotyl and root when grown without exogenous ethylene. With ethylene treatment, the curvature in the apical region is exaggerated, and the hypocotyl and root elongation is inhibited. Ethylene-induced seedling growth alterations are collectively called the seedling triple-response phenotype (Guzman and Ecker, 1990). Many ethylene-induced growth alterations depend on auxin, and seedlings of some mutants defective in auxin biosynthesis or transport show altered triple-response phenotype. *ETHYLENE INSENSITIVE ROOT1* (*EIR1*) encodes the auxin efflux carrier protein PIN-FORMED2 (PIN2), and the *eir1*/*pin2* loss-of-function mutation impacts on root gravitropism and prevents ethylene-induced root growth shortening (Roman *et al*., 1995; Luschnig *et al*., 1998; Muller *et al*., 1998). *HOOKLESS1* (*HLS1*) encodes an *N*-acetyltransferase, and the *hls1* loss-of-function mutation prevents the ethylene-induced apical hook formation. A suppressor screen for *hls1* led to the identification of *hookless1 suppressor1* (*hss1*), which is defective in the auxin-response transcription factor *AUXIN RESPONSE FACTOR2* (Lehman *et al*., 1996; Li *et al*., 2004). *AUXIN RESISTANT1* (*AUX1*) encodes an auxin influx carrier protein that associates with the plasma membrane (PM) depending on the endoplasmic reticulum protein AUXIN RESISTANT4 (AXR4) (Dharmasiri *et al*., 2006). Loss-of-function mutations of *aux1* prevent the ethylene-induced root shortening and apical hook formation (Stepanova *et al*., 2007; Vandenbussche *et al*., 2010). Previously, *weak ethylene insensitive* (*wei*) mutants were isolated by a genetic screen for components that involve ethylene signalling (Alonso *et al*., 2003). With ethylene treatment, the seedling root was longer for *wei1*, *wei2*, *wei7* and *wei8* mutants than for the wild type. *WEI1* encodes the auxin receptor protein TIR1, and *WEI2*, *WEI7* and *WEI8* are involved in auxin biosynthesis (Alonso *et al*., 2003; Stepanova *et al*., 2005, 2007, 2008). The ethylene-insensitive mutants *ethylene insensitive2* (*ein2*) and *ein3* are resistant to the inhibition of lateral root initiation by auxin, which indicates that both ethylene signalling and auxin responses are essential for this inhibitory effect (Ivanchenko *et al*., 2008). Measurement of auxin transport suggests that ethylene promotes long-distance polar auxin transport through AUX1 and results in the negative effect of ethylene on lateral root formation (Negi *et al*., 2008). The involvement of auxin in growth alterations induced by ethylene suggests that the interplay of the two hormones coordinately controls many aspects of plant growth and development.

The biologically active auxin IAA is transported across cells distantly. IAA may exist in two forms: the charged IAA^- and the protonated IAAH. With the acidic condition in the apoplast (pH ~5.5) outside the cell, a portion of IAA is protonated (IAAH) and can pass passively through the PM into the cell, whereas the charged IAA^- cannot. The PM-associated AUX1 and its homologs Like AUX1s (LAXs) are the auxin influx carriers that transport the charged IAA^- to the cytoplasm (Carrier *et al*., 2008; Péret *et al*., 2012; Robert and Friml, 2009; Zažímalová *et al*., 2010). About 17% of IAA freely enters the cells and 83% is de-protonated (Blakeslee *et al*., 2005; Zažímalová *et al*., 2010). The pH is nearly neutral in the cytoplasm, and IAA is de-protonated and exists as IAA^-. The de-protonated IAA^- is trapped in the cytoplasm and cannot permeate the membrane; it requires auxin transporters such as PIN proteins to exit the cell. With the polarized localization of different PIN proteins, IAA is transported in a polar fashion, the so-called polar auxin transport (Kerr and Bennett, 2007; Robert and Friml, 2009; Zažímalová *et al*., 2010) for acropetal and basipetal auxin transport and thus modulation of various growth and development events (Blakeslee *et al*., 2005).

To isolate components that involve synergistic functions by ethylene and auxin, we isolated the *reversing ctr1-10 root1* (*rcr1*) mutation that suppresses root growth inhibition of the hypomorphic *ctr1-10*. *rcr1* is allelic to *AUX1*, and is designated $aux1^{rcr1}$. The $aux1^{rcr1}$ mutation but not the T-DNA insertion allele *aux1-T*, prevented expression of the *DR5:GUS* construct in the root apex. The $aux1^{rcr1}$ isoform predominantly localized in the cytoplasm, whereas AUX1 localized to the PM. AUX1 loops 1 and 3 but not loop 2 have been predicted to be involved in AUX1 functioning (Swarup *et al*., 2004). The residue Lys^{126} in AUX1 loop 2 could involve correct AUX1 targeting to the PM. The $aux1^{rcr1}$ mutation may prevent *DR5:GUS* expression, possibly by affecting nuclear auxin signalling.

Materials and methods

Plant materials and seedling germination and growth conditions

The *ctr1-10* (SALK_122868.46.30.n) and *aux1-T* (CS859699) mutants were from the Arabidopsis Biological Resource Center. For seed germination and seedling growth, *Arabidopsis* seeds were stratified on Murashige and Skoog (MS) salt-containing agar (0.8% agar, pH 5.8) for 72 h at 4 °C and then germinated at 22 °C. For ethylene treatment, ethylene gas (20 µl l^{-1}) was applied. For auxin treatment, the auxin concentrations were as indicated. Seedling hypocotyl and root measurements were carried out with VideoTesT (Moscow) as described previously (Zhou *et al*., 2007); more than 30 individual seedlings were scored for each treatment, and the measurement was presented as mean ±standard deviation (SD). The cloning of transgenes in this study is described in Supplementary Data S1 at *JXB* online.

Quantitative RT-PCR (qRT-PCR) and PCR-based genotyping

qRT-PCR of the expression of *CTR1*, *AUX1*, and $aux1^{rcr1}$ involved use of StepOne Plus™ (ABI). The primer and sequence information for qRT-PCR is given in Supplementary Data S1.

Root gravity response assay

Seeds were surface sterilized, stratified in the dark at 4°C for 72 h, and germinated vertically on 0.5× strength MS (0.5× MS) salt-containing agar with constant illumination at 22 °C for 5 d. Seedlings

were then transferred to agar containing 0.5× MS salt with or without 10^{-7} mol l^{-1} 1-naphthaleneacetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D), and grown horizontally (at 90° rotation) under the same growth conditions. The root gravity response was scored by measuring the angles formed 24 h after the gravity change with use of ImageJ (NIH).

Auxin transport assay

Arabidopsis seedlings were grown for 6 d and a 10 mm segment to the root tip was excised. [^{3}H]-labelled IAA was applied to the cut and the root segments were incubated in the dark for 6 h. After incubation, a 5 mm segment to the tip was excised and washed with 0.5× MS salt. The washed root tips (15 tips for each measurement) were incubated in scintillation liquid and scintillation counting was carried out (PerkinElmer 1450 Microbeta scintillation counter) for [^{3}H] IAA measurement.

Laser-scanning confocal microscopy

The subcellular localizations of yellow fluorescent protein (YFP)–AUX1 and green fluorescent protein (GFP)–aux1^{rcr1} were examined by laser scanning confocal microscopy with an Olympus FV1000 microscope.

β-Glucuronidase (GUS) staining

Histochemical staining for GUS activity in transgenic plants was performed as described previously (Jefferson *et al*., 1987). Seedlings were grown under light on MS salt-containing agar for 4 d and transferred to MS salt-containing agar with or without auxin (100 nM NAA) in an air-tight chamber with or without ethylene treatment for 2 d. The seedlings were harvested, immersed in the reaction solution (1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, 100 mM sodium phosphate, 0.1 mM EDTA, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, and 0.1% Triton X-100, pH 7.0) and incubated at 37 °C for 16 h.

Results

The hypomorphic ctr1-10 mutation results in mild constitutive ethylene responses

Ethylene inhibits elongation of the hypocotyl and primary root of etiolated *Arabidopsis* seedlings. We sought to isolate the components involved in ethylene-induced root growth inhibition from a screen of suppressors in a mutation background that exhibited weak constitutive ethylene responses.

Currently known *constitutive triple response1* (*ctr1*) loss-of-function mutants, except for *ctr1-8* and *ctr1*^{btk}, show strong constitutive ethylene responses, with a short seedling hypocotyl and root (Huang *et al*., 2003; Ikeda *et al*., 2009; Xie *et al*., 2012). Here, we found that *ctr1-10* had a T-DNA insertion in the 5'-untranslated region (5'-UTR) and determined whether it was a weak allele that could be used for a suppressor screen (Fig. 1A).

Under dark growth conditions, the seedling hypocotyl was shorter for *ctr1-10* than for the wild type (Col-0) and was longer than *ctr1-1* without ethylene treatment. F1 seedlings of *ctr1-1* and *ctr1-10* phenotypically resembled *ctr1-10* seedlings. Hypocotyls were slightly shorter for F1 seedlings generated from the respective crosses of the wild type with *ctr1-1* and *ctr1-10* than for wild-type seedlings. Ethylene treatment inhibited seedling growth, and these genotypes showed a typical ethylene triple-response phenotype: shortening of the seedling hypocotyl and root, with an exaggerated apical hook (Fig. 1B). Hypocotyl measurement of seedlings gave the same results, with the hypocotyl shorter for *ctr1-10* seedlings than for the wild type and longer than for *ctr1-1* seedlings without ethylene treatment (Fig. 1C).

Under light growth conditions, the seedling growth inhibition phenotype was more severe for *ctr1-10* than for the wild type (Col-0) and weaker than that of *ctr1-1*. The cotyledons were small and the hypocotyls and roots were shorter for *ctr1-10* and *ctr1-1* than for the wild-type seedlings, and *ctr1-1* seedlings produced a shorter primary root and smaller cotyledons than *ctr1-10* seedlings. The mutant phenotype was weaker for F1 *ctr1-10*/*ctr1-1* than for *ctr1-1* plants but was similar to that for *ctr1-10* plants (Fig. 1D). At the adult stage, the *ctr1-1* mutant produced a relatively small rosette, but wild-type and *ctr1-10* plants did not differ in rosette size. The rosette was smaller for F1 *ctr1-10*/*ctr1-1* than *ctr1-10* plants but larger than for *ctr1-1* plants (Fig. 1E). Thus, the constitutive ethylene response was weaker with the *ctr1-10* than with the *ctr1-1* mutation. Complementation tests showed that ectopic expression of the genomic *CTR1* clone *gCTR1* (driven by the native *CTR1* promoter) rescued the *ctr1-10* seedling growth inhibition (Fig. 1F, G).

The T-DNA insertion occurs at the 5'-UTR and does not disrupt the *CTR1* open reading frame. qRT-PCR revealed greater mRNA expression of *CTR1* in *ctr1-10* than in the wild type (Fig. 1H). Ethylene promotes *CTR1* expression (Hall *et al*., 2012), and the increased *CTR1* level was consistent with the elevated constitutive ethylene response in *ctr1-10*. Sequence analysis did not identify an alternative start codon in the 5'-UTR. The mutation nature that attenuates *CTR1* functions in *ctr1-10* is unclear; one possibility is that the corresponding *CTR1* transcript may not be efficiently translated into protein. Our results indicated that *ctr1-10* is a loss-of-function mutation and a hypomorph.

Isolation of REVERSING CTR1-10 ROOT1 (RCR1)

To isolate the components of ethylene-induced root growth inhibition, we mutagenized *ctr1-10* with ethyl methanesulfonate and grew the resulting M2 seedlings under light on MS salt-containing agar. We identified a mutant that produced a longer primary root than *ctr1-10* and named the mutation *reversing ctr1-10 root1* (*rcr1*).

Etiolated seedlings of *ctr1-10 rcr1* produced a longer primary root than *ctr1-10*, regardless of ethylene treatment, but the hypocotyls were similar in length. Ethylene treatment resulted in the formation of an exaggerated apical hook curvature in wild-type (Col-0) and *ctr1-10* seedlings but not in *ctr1-10 rcr1* seedlings (Fig. 2A). Consistently, light-grown *crt1-10* and *ctr1-10 rcr1* seedlings were phenotypically similar, except that *ctr1-10 rcr1* produced a longer root, regardless of ethylene treatment (Fig. 2B). Of note, *ctr1-10 rcr1* seedlings showed an agravitropic root growth phenotype (Fig. 2A, B).

To clone *RCR1*, *crt1-10 rcr1* was crossed with the La-0 ecotype, and the resulting F2 seedlings exhibiting the *ctr1-10*

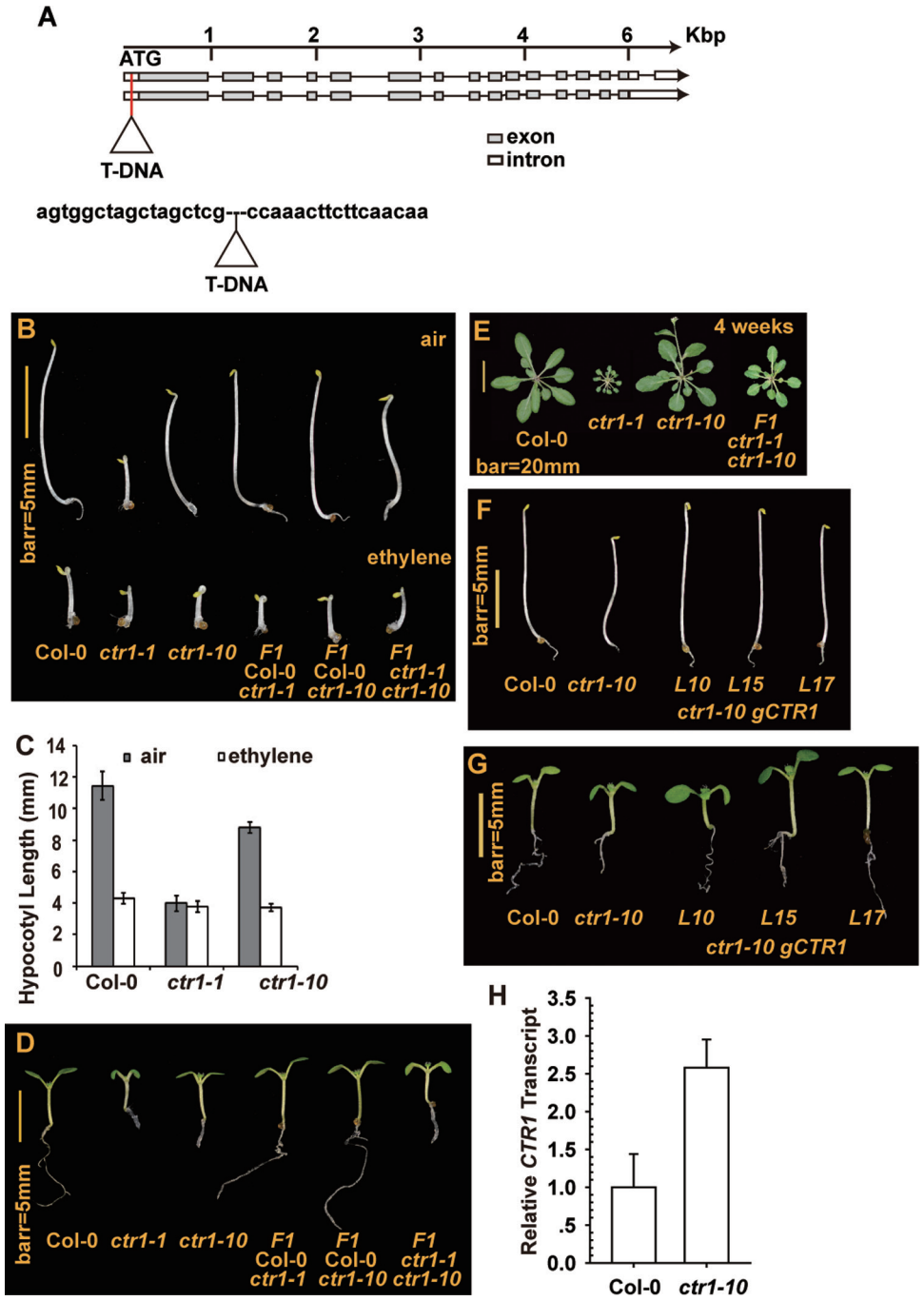

Fig. 1. The ethylene response phenotype of *ctr1-10*. (A) The structure and T-DNA insertion position, with the flanking sequence shown, for *ctr1-10*. Two *CTR1* alternative spliced isoforms are shown. (B, C) Phenotype (B) and hypocotyl measurement (C) for wild-type, *ctr1-1*, and *ctr1-10* seedlings grown in the dark with or without ethylene (20 μl l^{-1}). (D, E) Phenotype for light-grown seedlings (D) and rosettes (E) of the wild type (Col-0) and *ctr1-1* and *ctr1-10* mutants. (F, G) Expression of the genomic *gCTR1* transgene rescued the constitutive ethylene-response phenotype of etiolated (F) and light-grown (G) *ctr1-10* seedlings. (H) qRT-PCR analysis of *CTR1* expression in the wild type (Col-0) and *ctr1-10* mutant. Data are means ±SD or ±standard error (SE) for hypocotyl measurement and gene expression, respectively.

rcr1 root phenotype underwent map-based cloning. Using 472 individual F2 samples, *RCR1* was mapped to a 600 kb region on chromosome 2. Within this region, we identified a C→T transition mutation at the *AUX1* locus, which resulted in the L126F substitution (Fig. 2C). Thus, *RCR1* may be an *AUX1* allele and the mutation may prevent the ethylene-induced root growth inhibition and apical hook formation. To support this suggestion, we crossed *aux1-T* (Fig. 2C; a T-DNA insertion mutation of *aux1*) with *rcr1* for an allele test, and the root phenotype of the resulting F1 seedlings was similar to that of both parents. The ethylene-induced root growth inhibition and apical hook phenotype in wild-type (Col-0) seedlings was prevented

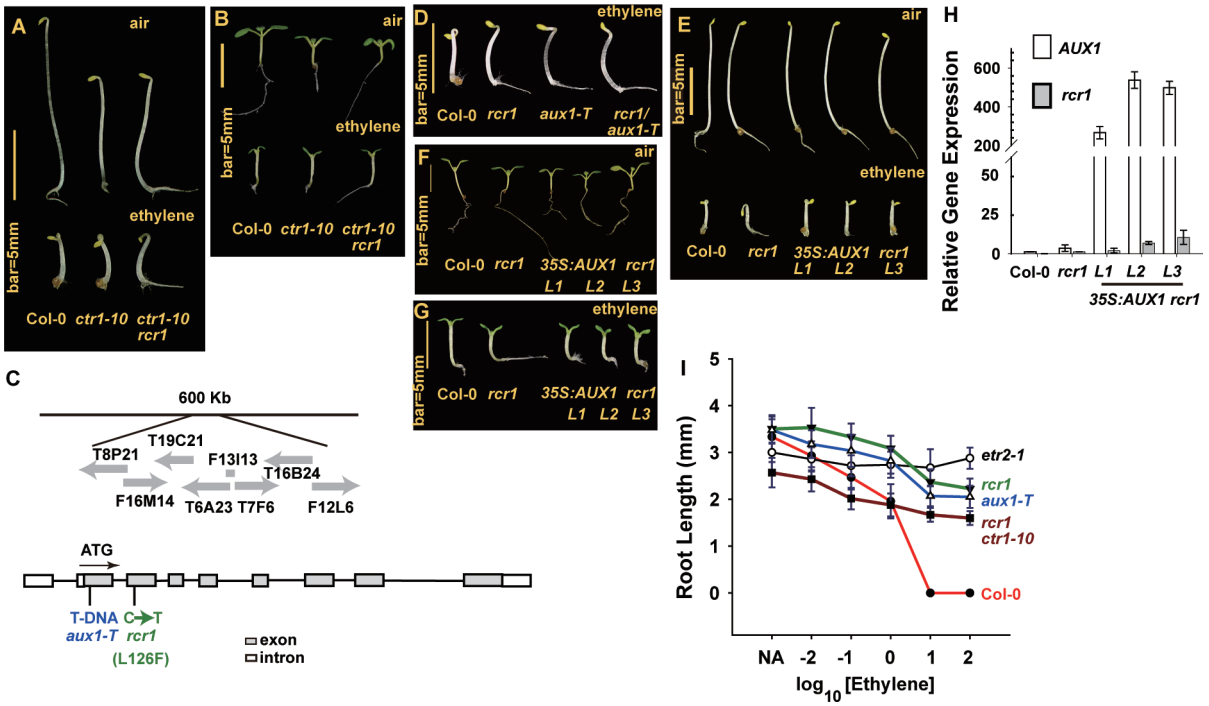

Fig. 2. *RCR1* is allelic to *AUX1*. (A, B) Phenotype for etiolated (A) and light-grown (B) seedlings of the wild type (Col-0) and *ctr1-10* and *ctr1-10 rcr1* mutants. (C) *RCR1* maps to a 600 kb region, and the *rcr1* mutation results from a C→T mutation (L126F substitution); *aux1-T* is a T-DNA insertion allele for *AUX1*. Grey arrows indicate the positions of bacterial artificial clones in this region. The *AUX1* gene structure and positions for the T-DNA insertion site and the *rcr1* lesion are indicated. (D) Phenotype of etiolated seedlings of *rcr1*, *aux1-T*, and the F1 of *rcr1* and *aux1-T* (*rcr1/aux1-T*) with ethylene (20 μl l^{-1}) treatment. (E–G) Phenotype of *rcr1* seedlings, grown in the dark (E) and light (F, G) expressing the *35S:AUX1* transgene without (F) or with (G) ethylene (20 μl l^{-1}) treatment. (H) qRT-PCR of *AUX1* expression in *rcr1* expressing *35S:AUX1*. (I) Ethylene dose-response curve for the root growth of light-grown seedlings as indicated. Data are means ±SD or ±SE for root length and gene expression, respectively. (This figure is available in colour at *JXB* online.)

in *aux1-T*, *rcr1*, and F1 seedlings (Fig. 2D). We performed a complementation test for *rcr1* with expression of the *35S:AUX1* transgene. Following ethylene treatment, the roots were longer in etiolated *rcr1* than in wild-type seedlings, with agravitropic root growth and no exaggerated apical hook curvature. As expected, seedlings of *rcr1* lines expressing the *35S:AUX1* transgene were phenotypically similar to wild-type seedlings and showed root growth inhibition and an exaggerated apical hook with ethylene treatment (Fig. 2E). With light germination, the roots were longer for *rcr1* than for wild-type seedlings and *rcr1* lines expressing *35S:AUX1*, regardless of ethylene treatment (Fig. 2F, G). qRT-PCR of *AUX1* and *rcr1* levels suggested that the *35S:AUX1* transgene was expressed (Fig. 2H). The *aux1*/*rcr1* root ethylene-insensitive phenotype was consistent with the ethylene dose–response assay for root growth inhibition, which showed a much shorter seedling root for the wild type (Col-0) than for the *aux1*/*rcr1* and the ethylene-insensitive *etr2-1* mutant at elevated ethylene concentrations (Fig. 2I).

Genetic and complementation tests suggested that *rcr1* is an *AUX1* allele, and we designated *rcr1* as *aux1*^{rcr1}. The *aux1*^{rcr1} mutation, like the *aux1-T* mutation, largely attenuated but did not completely prevent the ethylene-induced root growth inhibition. The defect in apical hook formation with *aux1*^{rcr1} is consistent with the defective apical hook phenotype in *aux1-21* (Vandenbussche *et al*., 2010).

aux1^{rcr1} *is defective in auxin transport and has reduced sensitivity to auxin*

AUX1 is an auxin influx carrier, and its loss-of-function mutation results in reduced auxin transport. To determine whether the *aux1*^{rcr1} mutation also attenuated auxin transport, we measured acropetal auxin transport (from the shoot towards the root apex) in *aux1*^{rcr1} seedling roots.

Measurement of the uptake of the tritiated auxin IAA showed an identical level of [^{3}H]IAA in root apexes of *aux1*^{rcr1} and *aux1-T* (Fisher's LSD, P=0.4) but lower than that in the wild type (Col-0) (Fisher's LSD, P<0.003). With the level of [^{3}H]IAA in the wild type set to 1, the level of [^{3}H]IAA in *aux1-T* and *aux1*^{rcr1} was about 0.51 and 0.41, respectively, which suggested reduced acropetal auxin transport in the mutants (Fig. 3A) and was consistent with *aux1-22* showing an approximate 50% reduction in auxin transport (Rahman *et al*., 2001).

Import of the synthetic auxins NAA and 2,4-D is in part independent of and dependent on, respectively, the auxin influx carrier AUX1. Conceivably, both *aux1*^{rcr1} and *aux1-T* seedlings are responsive to NAA but not to 2,4-D. We performed a dose–response assay to evaluate the effect of NAA and 2,4-D on the root growth of wild-type, *aux1*^{rcr1}, and *aux1-T* seedlings. NAA inhibited the root elongation of light-grown

aux1^{rcr1}, *aux1-T*, and wild-type seedlings (9 d after germination) to a similar extent (Fig. 3B). Wild-type (Col-0) seedlings germinated under light conditions showed marked root growth inhibition with the auxin 2,4-D at a concentration of >10^{-8} mol l^{-1}. Root growth was strongly inhibited in *aux1*^{rcr1} and *aux1-T* with 2,4-D at >10^{-7} mol l^{-1} (Fig. 3C).

Air-grown, etiolated *aux1*^{rcr1} and *aux1-T* seedlings were phenotypically identical. Following ethylene treatment, root growth was inhibited less in *aux1*^{rcr1} and *aux1-T* seedling than in the wild type, and neither mutant produced exaggerated apical hook curvature (Fig. 3D). Of note, NAA treatment (10^{-7} mol l^{-1}) had minor effects on seedling root growth (Fig. 3B), and NAA but not 2,4-D (10^{-7} mol l^{-1}) facilitated the ethylene-induced seedling triple-response phenotype in *aux1*^{rcr1} and *aux1-T* seedlings (Fig. 3D). 2,4-D treatment inhibited the root growth of wild-type but not *aux1*^{rcr1} and *aux1-T* seedlings. With 2,4-D and ethylene treatment, root growth was inhibited less in both mutants than in the wild type. Of note, the ethylene-induced apical hook formation in wild-type seedlings was prevented by 2,4-D and was not observed in *aux1*^{rcr1} and *aux1-T* seedlings (Fig. 3D).

Grown under light with ethylene treatment, root growth was inhibited less in the mutant than in the wild-type seedlings (Fig. 3E). With NAA treatment (10^{-7} mol l^{-1}), the

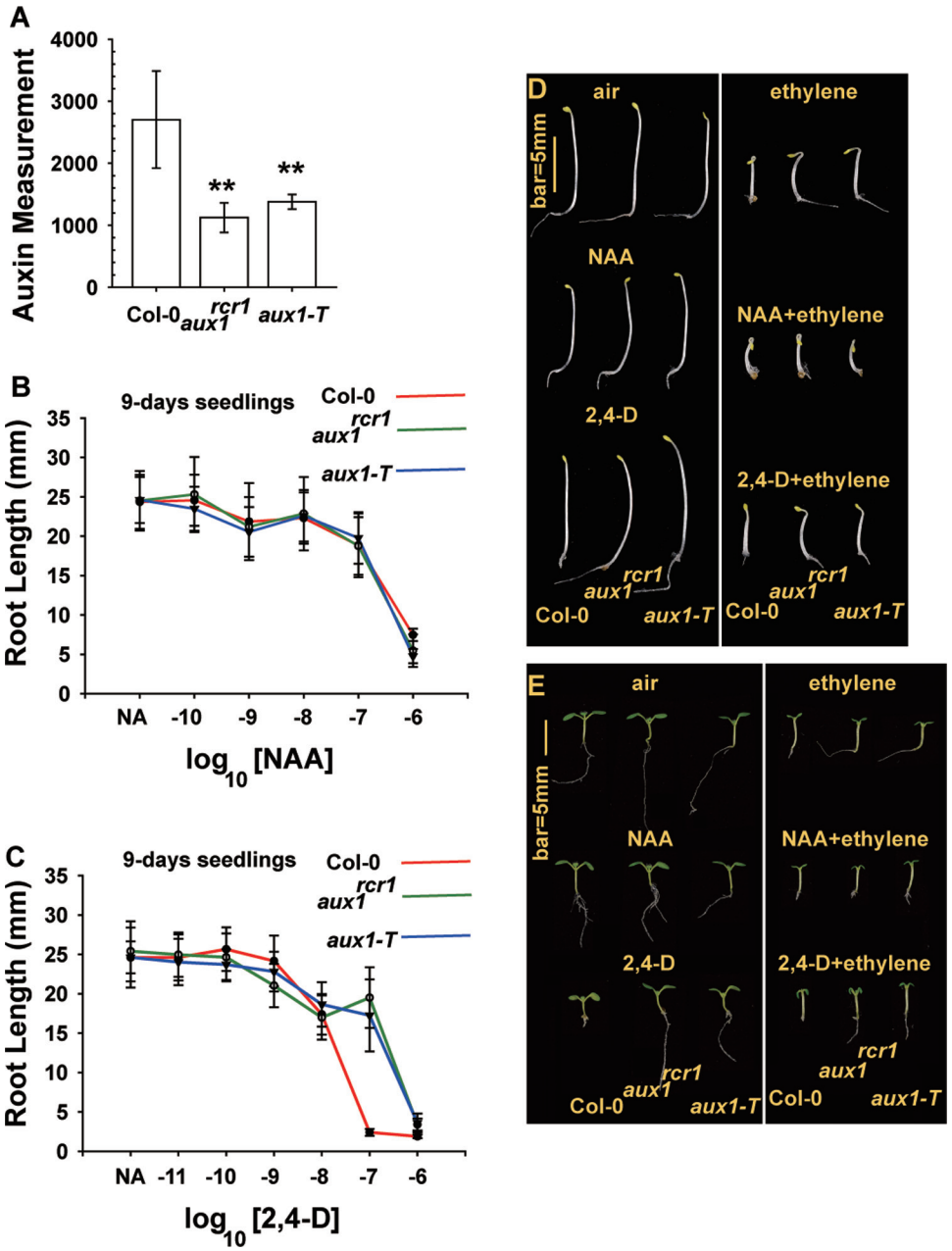

Fig. 3. The *aux1*^{rcr1} mutant is defective in IAA transport. (A) Uptake of exogenously applied [^{3}H]IAA in wild-type (Col-0), *aux1*^{rcr1}, and *aux1-T* seedling roots. (B, C) Does–response curve for root growth with NAA (B) and 2,4-D (C) treatment. (D, E) Effects of ethylene and auxin on seedling growth of *aux1*^{rcr1} compared with wild-type (Col-0) and *aux1-T* seedlings. Data are means ±SD for root length. (This figure is available in colour at *JXB* online.)

primary root of wild-type, *aux1*^{rcr1}, and *aux1-T* seedlings was similar in length. In contrast, 2,4-D treatment (10^{-7} mol l^{-1}) inhibited the root growth of the wild-type but not the *aux1*^{rcr1} and *aux1-T* seedlings (Fig. 3B, C, E). With ethylene treatment, NAA-treated wild-type, *aux1*^{rcr1}, and *aux1-T* seedlings were phenotypically identical, whereas the root was shorter for 2,4-D-treated wild-type than *aux1*^{rcr1} and *aux1-T* seedlings (Fig. 3E).

Thus, the *aux1*^{rcr1} mutation attenuated auxin transport and reduced the sensitivity to auxin. Reduced auxin transport in *aux1*^{rcr1} and *aux1-T* root apexes was consistent with the ethylene-induced root growth inhibition and apical hook phenotype of *aux1*^{rcr1} and *aux1-T* rescued by NAA but not by 2,4-D treatment.

aux1^{rcr1} but not the aux1-T allele alleviates DR5:GUS expression in the root apex

The *DR5:GUS* construct comprises a synthetic auxin-responsive promoter (DR5) fused to the GUS-encoding reporter gene. The expression of *DR5:GUS* is thus auxin inducible and has been widely used as a reporter to indicate auxin responses (Ulmasov *et al.*, 1995; Ivanchenko *et al.*, 2008; Negi *et al.*, 2008). With the *DR5:GUS* transgene, an auxin maximum in wild-type root apexes is associated with elevated GUS expression. Here, we evaluated whether the root apex auxin maximum would be altered by the *aux1*^{rcr1} allele by comparing *DR5:GUS* expression in the root apex of wild-type, *aux1-T*, and *aux1*^{rcr1} seedlings.

The *DR5:GUS* transgene was introduced into *aux1-T* and *aux1*^{rcr1} plants from a common wild-type line that carries the transgene (designated the *DR5:GUS* donor), and *GUS* expression was examined. With growth on MS medium, GUS staining was observed in wild-type (Col-0) and *aux1-T* root apexes (zone 1), as defined previously (Stepanova *et al.*, 2007). Ethylene treatment promoted acropetal auxin transport and elevated GUS staining in the transition zone (zone 2) in wild-type but not in *aux1-T* root tips. The minor effects of ethylene treatment on the *DR5:GUS* maximum in *aux1-T* root apexes were consistent with *DR5:GUS* expression in *aux1-7* root apexes unaffected by the ethylene biosynthesis precursor 1-aminocyclopropane-1-carboxylic acid (Stepanova *et al.*, 2007). Unexpectedly, GUS staining was not observed or was extremely weak in *aux1*^{rcr1} root apexes, regardless of ethylene treatment (Fig. 4A).

Therefore, the *aux1*^{rcr1} allele prevented *DR5:GUS* expression. Given that expression of the *35S:AUX1* transgene complemented the *aux1*^{rcr1} mutation (Fig. 2), we examined whether the transgene rescued *DR5:GUS* expression in *aux1*^{rcr1}. The *35S:AUX1* transgene was transformed into *aux1*^{rcr1} plants expressing *DR5:GUS* (Fig. 4A), but expression of *DR5:GUS* was not rescued by the *35S:AUX1* transgene, regardless of ethylene treatment (Fig. 4B). *AUX1* levels in the transformed lines were highly elevated (Supplementary Fig. S2 at *JXB* online); hence, *aux1*^{rcr1} could prevent *DR5:GUS* expression, even in the presence of the wild-type *AUX1*. We examined the reciprocal negative effects of *aux1*^{rcr1} on *DR5:GUS* expression in the *DR5:GUS* donor expressing *35S:aux1*^{rcr1}. The *35S:aux1*^{rcr1} transgene was introduced into the *DR5:GUS* donor (Fig. 4A) by transformation, and *DR5:GUS* was expressed at the same locus in the donor and transformed lines. In 27 independent lines that we examined, expression of *35S:aux1*^{rcr1} prevented *DR5:GUS* expression in the root apex; Fig. 4C shows *DR5:GUS* expression in three representative lines. Expression of the *35S:aux1*^{rcr1} transgene was confirmed by qRT-PCR in these three lines with reference to *AUX1* and *aux1*^{rcr1} levels in the wild-type (Col-0) and *aux1*^{rcr1}, which were each given a value of 1, respectively (Fig. 4D; i.e. *AUX1*=1 in the wild type and *aux1*^{rcr1}=1 in *aux1*^{rcr1}).

Thus, *aux1*^{rcr1} may have dominant-negative effects on *DR5:GUS* expression in the root apex. To support this scenario, we examined *DR5:GUS* expression in the heterozygous

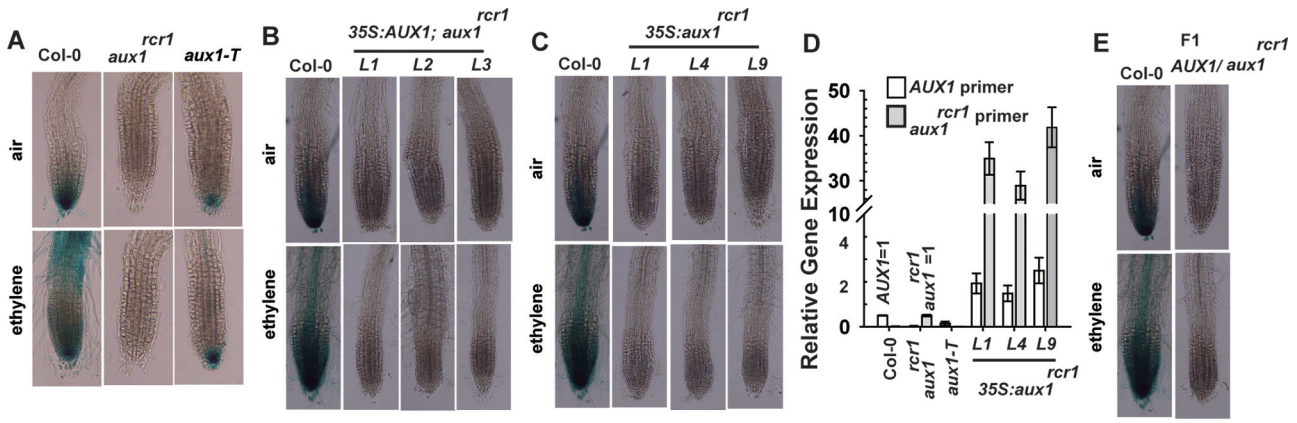

Fig. 4. *DR5:GUS* expression in the root tip is alleviated by the *aux1*^{rcr1} allele. (A) Expression of *DR5:GUS* in root tips of wild-type (Col-0), *aux1*^{rcr1}, and *aux1-T* seedlings. (B, C) Expression of *DR5:GUS* in wild-type and *aux1*^{rcr1} lines expressing *35S:AUX1* (B) and in wild-type lines expressing *35S:aux1*^{rcr1} (C). (D) qRT-PCR of *AUX1* and *aux1*^{rcr1} expression in wild-type, *aux1*^{rcr1}, and wild-type (Col-0) *DR5:GUS* lines expressing *35S:aux1*^{rcr1}. Data are means ±SE of three measurements from three independent biological samples. (E) Expression of *DR5:GUS* in root tips of the wild type (Col-0) and F1 *aux1*^{rcr1} seedlings. The ethylene concentration is 20 µl l^{-1}. *L*, transformation line. (This figure is available in colour at *JXB* online.)

AUX1/*aux1*^{rcr1} line expressing the *DR5:GUS* transgene. The wild-type *DR5:GUS* donor and *aux1*^{rcr1} that expressed the *DR5:GUS* from the donor were genetically crossed to produce the heterozygous F1 *AUX1*/*aux1*^{rcr1}; *DR5:GUS*, in which the *DR5:GUS* transgene was from a common donor. As expected, *DR5:GUS* expression was prevented (Fig. 4E).

DR5:GUS expression is NAA inducible in genotypes with aux1^{rcr1}

We showed that *DR5:GUS* expression was largely prevented by the *aux1*^{rcr1} allele, even in the presence of the wild-type *AUX1*. The mutant *aux1*^{rcr1} could have a dominant-negative effect on *DR5:GUS* expression. Alternatively, *DR5:GUS* was not expressed (or silenced) for unknown reasons. The intercellular transport of NAA is independent of AUX1. If *DR5:GUS* expression is NAA inducible in genotypes with *aux1*^{rcr1}, prevention of *DR5:GUS* expression by *aux1*^{rcr1} was probably not due to the silencing of *DR5:GUS*.

The *DR5:GUS* donor (the wild type expressing *DR5:GUS*) showed strong GUS staining in the root apex, zone 2, and mature zone with NAA treatment. Expression of *DR5:GUS* was induced by NAA in the root apex and mature zone in *aux1*^{rcr1}, the wild type (Col-0) expressing *35S:aux1*^{rcr1}, *aux1*^{rcr1} lines expressing *35S:AUX1*, and the F1 *aux1*^{rcr1}/*AUX1* expressing the *DR5:GUS* transgene; however, GUS staining in the elongation zone was barely detectable (Fig. 5A). These results did not favour the *DR5:GUS* transgene being silenced in these genotypes. The *aux1*^{rcr1} mutation had dominant-negative effects on *DR5:GUS* expression in a domain-specific manner.

Ethylene promotes auxin biosynthesis and acropetal transport, and *DR5:GUS* expression is elevated in the root tip. We examined whether ethylene treatment could synergistically elevate NAA-induced *DR5:GUS* levels in the root tip of genotypes with *aux1*^{rcr1}. Of note, ethylene inhibited root elongation, and the region below the mature zone was largely shortened compared with no-ethylene treatment (Fig. 5B, C). In wild-type root tips, GUS staining was strong in the root apex, zone 2, and the mature zone with ethylene and NAA treatment. Unexpectedly, *DR5:GUS* expression was strong in the mature zone but nearly abolished in the root apex and elongation zone in genotypes with *aux1*^{rcr1} (Fig. 5B). Thus, the *DR5:GUS* transgene was probably not silenced; rather, its expression was affected by the *aux1*^{rcr1} allele in a domain-specific manner. Measurement of GUS staining intensity supported the association of *DR5:GUS* expression inhibition with *aux1*^{rcr1} in the root apex (Supplementary Fig. S3 at *JXB* online). In contrast to the *aux1*^{rcr1} allele, which prevented *DR5:GUS* expression, *DR5:GUS* expression was not prevented in the root apex of *aux1-T*. NAA treatment induced *DR5:GUS* levels in the root apex and mature zone but in not the region in between, and the induction was not prevented by ethylene treatment (Fig. 5C).

AUX1 is expressed mainly in the root apex (Péret *et al.*, 2012). Our results suggested that *aux1*^{rcr1} affected *DR5:GUS* expression in AUX1-expressing domains.

aux1^{rcr1} *and aux1-T mutations but not aux1*^{rcr1} *overexpression impair root gravitropism*

In response to gravity changes, AUX1 and the auxin efflux carrier PIN2 protein mediate differential, basipetal auxin transport from the columella via the lateral root cap (LRC) cells to the expanding epidermis (Swarup *et al.*, 2005; Rahman *et al.*, 2010). As a result, root cells grow faster with lower than with higher auxin concentrations, and differential cell growth is facilitated. The differential root cell growth facilitates a curvature formation that re-orients the root growth towards gravity (Luschnig *et al.*, 1998; Marchant *et al.*, 1999; Rashotte *et al.*, 2000; Ottenschläger *et al.*, 2003).

Both *aux1*^{rcr1} and *aux1-T* seedling roots showed an agravitropic growth phenotype (Fig. 2) and showed distinct *DR5:GUS* expression patterns in air and ethylene. We evaluated the association of the root gravity response with root *DR5:GUS* expression in *aux1-T*, *aux1*^{rcr1}, and *aux1*^{rcr1}*ox* lines (wild-type lines expressing *35S:aux1*^{rcr1}).

We quantified the extent of altered root gravity by measuring the root angles formed after a gravity change of 90° for vertically grown seedlings. The root growth angles were grouped in 12 classes of 30°, and we have presented the gravity response for the wild type (Col-0), *aux1-T*, *aux1*^{rcr1}, and *aux1*^{rcr1}*ox* lines diagrammatically (Fig. 6). Both *aux1-T* and *aux1*^{rcr1} seedlings showed a root-growth lack of gravitropism after the gravistimulation, whereas the gravity response of wild-type seedlings and *aux1*^{rcr1}*ox* lines was similar. 2,4-D treatment did not rescue the agravitropic phenotype in *aux1-T* and *aux1*^{rcr1} seedlings, and its effects on the gravity response in wild-type seedlings and *aux1*^{rcr1}*ox* lines were minor. As expected, NAA treatment rescued the agravitropic phenotype in *aux1-T* and *aux1*^{rcr1} seedlings to a similar degree as in the wild type and *aux1-T* seedlings.

Roots of *aux1-T* and *aux1*^{rcr1} but not *aux1*^{rcr1}*ox* seedlings showed defects in response to a 90° gravity stimulus, and the *aux1*^{rcr1} allele and *aux1*^{rcr1} overexpression impaired *DR5:GUS* expression in the root apex. The aux1^{rcr1} isoform could actively affect auxin distribution or concentration in the root apex but was insufficient to affect the root gravitropism in the presence of the wild-type AUX1. The wild-type *AUX1* had a role in the root gravity response, even in the presence of the *aux1*^{rcr1} allele that has dominant-negative effects on *DR5:GUS* expression maximum.

Subcellular localization of GFP-fused aux1^{rcr1}

AUX1 is predicted to have ten transmembrane helixes. YFP fused to AUX1 at position 116 (loop 2) has revealed the fused YFP portion localized within the cytoplasm. However, YFP does not generate fluorescence when fused at position 165 (loop 3), and the YFP portion is extracytoplasmic (Swarup *et al.*, 2004) (Fig. 7A). We examined the fluorescence of GFP fused to aux1^{rcr1} at positions 116 (designated GFP-116-aux1^{rcr1}) and 165 (GFP-165-aux1^{rcr1}) to evaluate aux1^{rcr1} targeting (Fig. 7A).

YFP fused with the wild-type AUX1 at position 2 (loop 1) (Fig. 7A), designated YFP-2-AUX1, was expressed (driven by the native *AUX1* promoter) in columella, LRC, epidermis,

and protophloem cells, as described previously, and localized to the PM (Péret *et al*., 2012) (Fig. 7B, C). Driven by the constitutive *35S* promoter, GFP-116-aux1^{rcr1} was expressed over nearly all the root tip cells (Fig. 7B). GFP fluorescence with GFP-165-aux1^{rcr1} (driven by the *35S* promoter) was observed mainly in cells of the outer layers (Fig. 7B). Unexpectedly, the fluorescence of GFP-116-aux1^{rcr1} and GFP-165-aux1^{rcr1} showed a pattern characteristic of the endoplasmic reticulum (ER) structure and in part of the PM (Fig. 7C, D). Subcellular compartments that GFP-aux1^{rcr1} could be associated with need to be investigated. AUX1 recycles between the PM and cytoplasm (Kleine-Vehn *et al*., 2006; Spitzer *et al*., 2009); the L126F substitution by the *aux1*^{rcr1} mutation could impact on AUX1 trafficking. AUXIN RESISTANT4 (AXR4) is an ER protein important to AUX1 targeting (Dharmasiri *et al*., 2006; Hobbie, 2006). The L126F mutation could impair aux1^{rcr1} targeting mediated by AXR4.

Of note, the YFP-165-AUX1 fusion did not produce fluorescence, possibly because the YFP portion may face the acidic apolastic space (Swarup *et al*., 2004). The fused GFP portion of GFP-165-aux1^{rcr1} was expected to be endocytoplasmic because the fusion produced fluorescence. GFP-116-aux1^{rcr1}

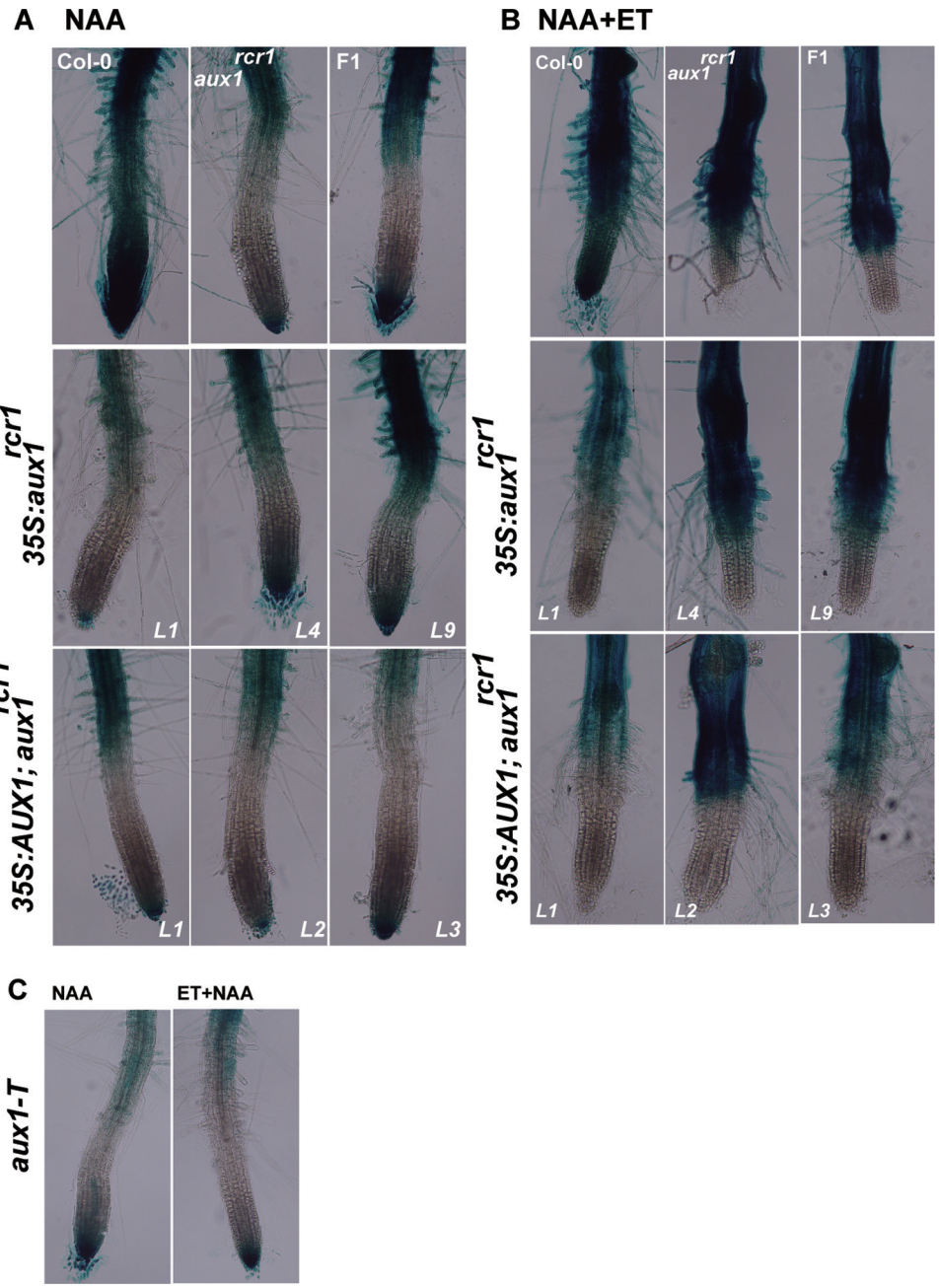

Fig. 5. Expression of *DR5:GUS* expression with NAA treatment and NAA plus ethylene treatment. *DR5:GUS* expression in the wild type, *aux1*^{rcr1}, F1 *aux1*^{rcr1} and the wild type, wild-type lines expressing *35S:aux1*^{rcr1}, and *aux1*^{rcr1} lines expressing *35S:AUX1* following NAA (A) and NAA plus ethylene treatment (B). (C) *DR5:GUS* expression in *aux1-T* in response to NAA and NAA plus ethylene treatment. *L*, transformation line. NAA was used 10^{-7} mol l^{-1}, and ethylene (ET) at 20 µl l^{-1}. (This figure is available in colour at *JXB* online.)

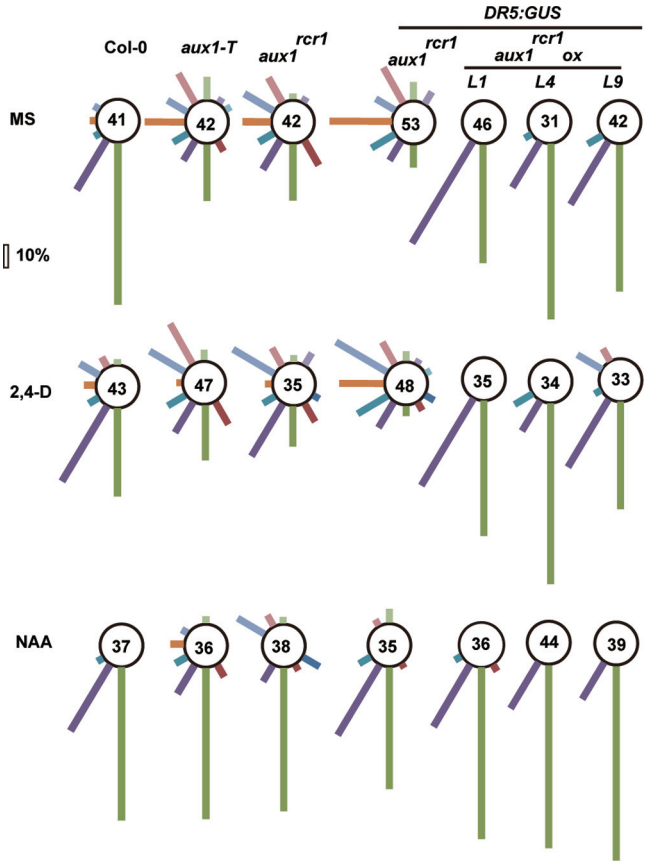

Fig. 6. Root gravity response assay. Illustration of the root response to gravistimulation with 12 classes of 30°, 24 h after a 90° rotation on agar medium containing MS salt. The bar indicates 10% of the seedlings. The 2,4-D and NAA concentrations were both 10^{-7} mol l^{-1}. Numbers indicate the population size for each scoring.

and GFP-165-aux1^{rcr1} are probably not associated with the PM; rather, both could aggregate in part near by the PM and localize to the ER.

Discussion

The plant hormone ethylene inhibits many aspects of *Arabidopsis* seedling growth and development that depend in part on or are coordinated with auxin actions. AUX1 binds and transports auxin; the association of AUX1 structure and domain functions needs to be fully addressed. The isolation of *aux1^{rcr1}*, which suppressed *ctr1-10* root growth inhibition, is consistent with the central role of AUX1 in root tip auxin transport, which involves the synergy of auxin and ethylene regulating root growth and development. aux1-22 is not serologically detectable and the mutant is probably a null mutant (Swarup *et al.*, 2004). The mutants *aux1^{rcr1}*, *aux1-T*, and *aux1-22*, but not the hypomorphic mutant *aux1-7*, have a similar effect on auxin transport in the root apex (Fig. 3) (Rahman *et al.*, 2001; Swarup *et al.*, 2004; Negi *et al.*, 2008) and a similar effect on many aspects of the auxin response, which suggests that *aux1^{rcr1}* is a strong allele.

AUX1 is a PM protein, with loops 1 and 2 being intracytoplasmic and loop 3 extracytoplasmic (Fig. 7A) (Swarup *et al.*, 2004). Both GFP-116-aux1^{rcr1} and GFP-165-aux1^{rcr1} appeared in the cytoplasm and possibly in part at the PM, which suggests that *aux1^{rcr1}* alters AUX1 localization. Given that the AUX1 loop 3 locates to the acidic apoplastic space, GFP-165-aux1^{rcr1} was probably not able to produce fluorescence if located at the PM. Therefore, GFP-116-aux1^{rcr1} and GFP-165-aux1^{rcr1} were probably not localized in part at the PM. The L126F substitution may not alter AUX1 topology for the loop 3 to face the cytoplasm. The exact subcellular localizations of these GFP–aux1^{rcr1} fusions remain for further investigation. The ER protein AXR4 is essential for AUX1 targeting to the PM (Dharmasiri *et al.*, 2006); the association of aux1^{rcr1} with the ER could be possible. Alternatively, aux1^{rcr1} may not have been recycled effectively to the PM. Our results imply an involvement of Lys^{126} of the AUX1 loop 2 in correct AUX1 targeting.

The spatial expression of both YFP-2-AUX1 (Fig. 7) and YFP-116-AUX1 (Swarup *et al.*, 2004) was consistent with AUX1 being predominant in columella, stele (protophloem), epidermis, and LRC cells in the root tip region (Swarup *et al.*, 2001; Péret *et al.*, 2012). Interestingly, a recent study showed that expression of the chimaeric protein consisting of the AUX1 N terminus and the LAX2 C terminus (DS2), but not the LAX2 N terminus and AUX1 C terminus (DS1), rescued *aux1-22* gravity responses. Driven by the native *AUX1* promoter, DS2 but not DS1 was expressed in AUX1-expressing LRC and epidermis cells, and DS1 was not targeted to the PM. Thus, the AUX1 N terminus may be involved in cell type-specific AUX1 expression and PM targeting (Péret *et al.*, 2012). Given that the AUX1 N terminus is required for correct AUX1 expression in certain cell types, cell-specific AUX1 expression could be affected by the *aux1^{rcr1}* mutation and could involve the AUX1 loop 2. Our argument for roles of Lys^{126} at the AUX loop 2 in coupling cell type-specific expression and PM targeting is consistent with DS1 not being expressed in epidermis and LRC cells, or targeted to the PM.

The aux1-7 isoform, and possibly aux1-T, fails to mediate auxin transport across the PM (Yang *et al.*, 2006). We showed that acropetal auxin transport in *aux1^{rcr1}* and *aux1-T* was prevented to a similar degree to that in *aux1-22* (Rahman *et al.*, 2001); polar auxin transport in the *aux1^{rcr1}* root tip was prevented, probably because of altered aux1^{rcr1} targeting. Whether aux1^{rcr1} can bind and transport auxin across membranes remains to be investigated. The *DR5:GUS* maximum was probably independent of acropetal auxin transport because it was affected in *aux1^{rcr1}* but not in *aux1-T* and *aux1-7*, with *aux1-T* affecting polar auxin transport and *aux1-7* not (Stepanova *et al.*, 2007; Negi *et al.*, 2008) (Fig. 3). Basipetal transport for auxin, which is *de novo* biosynthesized in the root apex, was probably affected in *aux1-T*, which facilitated auxin accumulation in the root apex, so that root gravitropism but not *DR5:GUS* expression was impaired. This argument, however, does not explain the dominant-negative effect of *aux1^{rcr1}* on maximal *DR5:GUS* expression. We do not favour a second mutation in the *aux1^{rcr1}* mutant

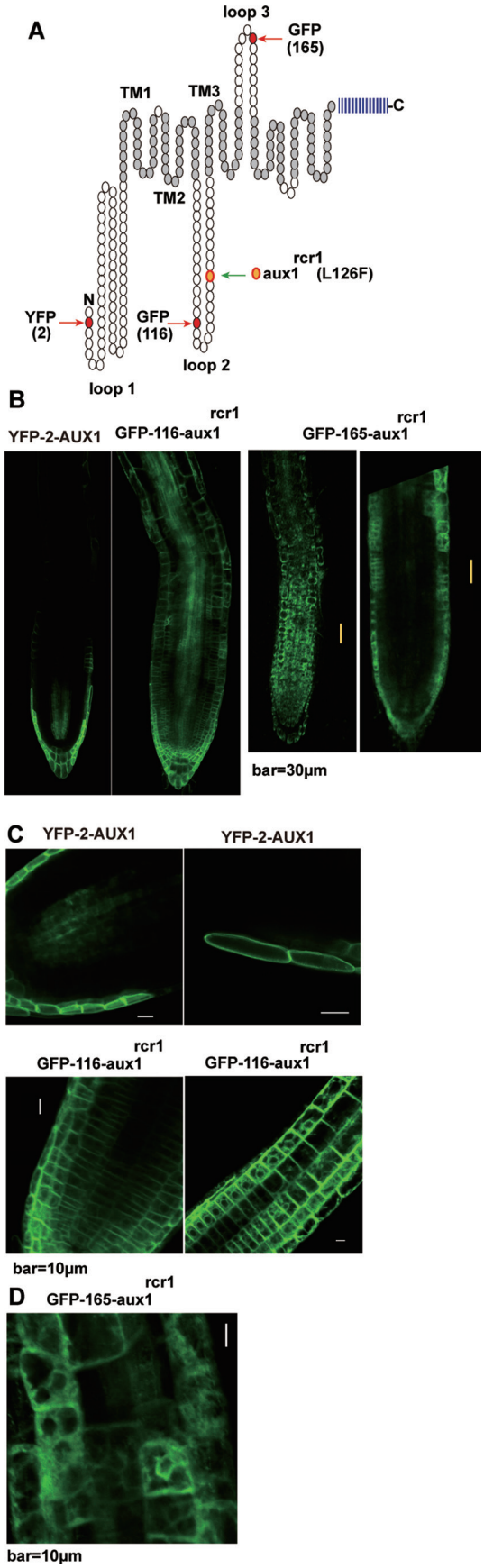

Fig. 7. Subcellular localization of GFP–aux1^{rcr1}. (A) Schematic illustration of the N-terminal structure of AUX1 and residues in which YFP or GFP were inserted. Red and green arrowheads indicate YFP/GFP insertion sites and the aux1^{rcr1} mutation, respectively. Blue vertical bars indicate the C-terminal portion of AUX1 not graphically shown. (B) Expression patterns of *AUX1p:YFP-AUX1*, *35S:GFP-116-aux1*^{rcr1}, and *35S:GFP-165-aux1*^{rcr1} in the root tip. The fluorescence of GFP-165-aux1^{rcr1} was observed at different focal planes for cells on the surface (left panel) and in the middle (right panel) of a root. (C) Subcellular localization of YFP-AUX1 in LRC cells and GFP-116-aux1^{rcr1} in cells of the root tip (left panel) and elongation zone (right panel). (D) Subcellular localization of GFP-165-aux1^{rcr1} in root tip cells. (This figure is available in colour at *JXB* online.)

preventing *DR5:GUS* expression, because *35S:aux1*^{rcr1} expression in the wild type also prevented *DR5:GUS* expression. *DR5:GUS* expression was probably not silenced after genetic crossing or transformation in the genotypes we studied, as the *aux1*^{rcr1}-containing genotypes showed a similar *DR5:GUS* induction pattern to that of *aux1-T* following NAA treatment.

The effect of *aux1*^{rcr1} on *DR5:GUS* expression was associated with sites expressing AUX1. The PM-localized PINs are auxin-efflux carrier proteins that transport intracellular auxin to the apoplast. Interestingly, ER-localized PIN5 and PIN-LIKEs (PILs) facilitate intracellular auxin transport to the ER lumen, where auxin metabolism occurs to reduce auxin availability for nuclear auxin signalling (Mravec *et al.*, 2009; Ganguly *et al.*, 2010; Barbez *et al.*, 2012; Feraru *et al.*, 2012; Swarup and Péret, 2012). AUX1 and PIN5/PILs have 10 or 11 transmembrane helixes and transport auxin across membranes, which suggests similarity in protein structure and function. These features prompted us to hypothesize that aux1^{rcr1} could localize at the ER and gain a new function to transport the intracellular auxin to the ER lumen in AUX1-expressing domains. Alternatively, *aux1*^{rcr1} could transport intracellular auxin to other subcellular compartments. Either scenario would suggest a mechanism by which the nuclear auxin is reduced to a level that is insufficient for *DR5:GUS* expression.

The polar auxin transport that facilitates auxin redistribution plays important roles in root growth and gravitropism (Marchant *et al.*, 1999; Swarup *et al.*, 2005; Swarup and Péret, 2012). With disturbed polar auxin transport, *aux1*^{rcr1} and other *aux1* alleles show the same root growth defect phenotypes. For *aux1*^{rcr1} with the *35S:AUX1* transgene and for the wild type with the *35S:aux1*^{rcr1} transgene, the wild-type AUX1 restored polar auxin transport in the presence of *aux1*^{rcr1} and thus these genotypes showed a normal root growth phenotype. In contrast, the dominant-negative effects of *aux*^{rcr1} prevented *DR5:GUS* expression, even with the wild-type AUX1, in AUX1-expressing domains. The hypothesis that aux1^{rcr1} could promote auxin transport to the ER lumen to affect auxin homeostasis needs to be demonstrated, and this scenario would suggest a higher auxin concentration required for the maximal *DR5:GUS* expression than for gravitropic root growth. Our findings could lead to further studies of AUX1 domain functions and structure.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Data S1. Primer sequences and cloning of transgenes.

Supplementary Fig. S2. qRT-PCR of *AUX1* in *aux1^{rcr1} 35S:AUX1 DR5:GUS* lines.

Supplementary Fig. S3. *DR5:GUS* expression in root apexes for genotypes with *aux1^{rcr1}*.

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