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Short communication

A new temperature-insensitive allele of the Arabidopsis *AXR6/CUL1* locus derived from a missense mutation in the C-terminal RBX1 binding region

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Keywords: *Arabidopsis thaliana*, auxin signaling, auxin resistance, *axr6*, cullin, mutant

Abbreviations: AXR, auxin resistant; CUL, cullin; 2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4-DB, 2,4-dichlorophenoxybutyric acid; L*er*, Landsberg *erecta*; RBX1, RING-box 1

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Abstract

We isolated a new recessive allele at the *AUXIN RESISTANT6/CULLIN1* (*AXR6/CUL1*) locus, *axr6-101*, from an EMS-mutagenized population of *Arabidopsis thaliana*, the Landsberg *erecta* ecotype. *axr6-101* is auxin resistant and semi-dwarf similar to the other recessive *axr6* mutants. The *axr6-101* phenotype is caused by the E716K substitution of the CUL1 protein, which is likely to affect its ability to bind to the C-terminal RING domain of RING-box 1 (RBX1). The previously reported allele of AXR6, *cul1-7*, is caused by a substitution at T510 that binds to the N-terminal β-strand of RBX1. Although *cul1-7* shows temperature-sensitive phenotype, the *axr6-101* phenotype is largely unaffected by temperature. *axr6-101* may provide an important genetic resource for study of the structure–function relationship of the CUL1 protein.

CULLIN1 (CUL1) is a scaffolding protein of the SCF-type ubiquitin ligase E3,[1,2] and consists of 738 amino acid residues in Arabidopsis. At its N-terminus, it binds to a substrate-recognizing subunit through a SKP1-like protein, ASK1, in Arabidopsis. In addition, it binds to RING-finger protein RING-box 1 (RBX1) at its C-terminus, which recruits the ubiquitin-charged ubiquitin conjugating enzyme E2. There are a large number of substrate-recognizing F-box proteins,[3] including the auxin F-box receptors, TIR1/AFB1-4.[4] Thus, while null alleles of *cul1* are not viable,[5] missense alleles of *cul1* show multiple phenotypic alterations, including auxin-insensitivity due to disruption of auxin signaling through the altered $SCF^{TIR1/AFB1-4}$. To date, 5 alleles that contain missense mutations at the *CUL1* locus have been reported. Interestingly, their phenotypes vary widely. The first reported alleles, *auxin resistant* (*axr*) *6-1* and *axr6-2*, were caused by missense mutations at F111 and are dominant.[6,7] They are seedling lethal because embryogenesis is affected. In contrast, *cul1-6*, harboring a L115F substitution, is recessive.[8] Furthermore, *axr6-3*, featuring a substitution of E159K, is also recessive, but is temperature-sensitive.[9] Defects observed in *axr6-3* mutants are dramatically enhanced at elevated temperatures. For example, the primary stem of *axr6-3* is about half as long as the wild type at 20°C; however, it is shorter than 1 cm at 28°C; auxin insensitivity is highly elevated at 28°C at the seedling stage. F111, L115, and E159 are located in the region that binds to ASK1.[1,8,9] To date, *cul1-7* is the only reported missense mutation in the C-terminal region that results in a T510I

substitution.[10] In the C-terminal region, CUL1 interacts with RBX1.[1] *cul1-7* is recessive and temperature-sensitive, like *axr6-3*. Besides these missense mutations, two recessive and viable alleles of *CUL1*, *icu13*[11] and *cul1-494*,[12] have been reported, which are caused by missplicing. It is not known whether they are temperature-sensitive or not.

Previously, we isolated a few Arabidopsis mutants that can grow in the presence of 2,4-dichlorophenoxybutyric acid (2,4-DB), but not in the presence of 2,4-dichlorophenoxyacetic acid (2,4-D), to study glyoxysomal fatty acid β-oxidation.[13] In this screen, we carried out two successive screenings with 2,4-DB and 2,4-D. As a result, we found a recessive mutant that was resistant to 2,4-D as well as 2,4-DB. Here we report that this auxin-resistant mutant is a new, recessive allele of *AXR6/CUL1*, which we named *axr6-101*. We found that *axr6-101* has a E716K substitution in the CUL1 protein, and that its defects are not temperature-sensitive, in contrast to *cul1-7*. *axr6-101* may be an important genetic resource to study the structure–function relationship of the CUL1 protein.

The *axr6-101* mutant line was isolated from an EMS-mutagenized Arabidopsis population (ecotype Landsberg *erecta* (L*er*)) as a mutant that germinated and grew on growth medium containing 0.05 μg ml^{-1} 2,4-D (0.23 μM).[13] This mutant was recessive: when root growth was examined on media with 0.08 μM 2,4-D, 11 of the 41 F_2 seedlings obtained by a cross between our mutant and L*er* exhibited 2,4-D-resistant root growth. For positional cloning, a mapping population of 332 phenotypically mutant plants derived from a cross between our mutant and the Columbia (Col) ecotype was used for

linkage analysis using simple sequence length polymorphic (SSLP) and cleaved-amplified polymorphic sequence (CAPS) markers. Our mutation mapped to a 116-kb interval between two SSLP markers on top of chromosome 4, which were generated using two insertions/deletions of the Cereon Arabidopsis polymorphism collection,[14] CER458376 and CER458010 (Table S1). This interval encompassed 36 annotated genes, one of which was *AXR6*. Furthermore, no recombination was found at the CAPS marker that was generated using a single nucleotide polymorphism, CER441938, which is located in the 3' non-coding region of *AXR6* (Table S1). We sequenced the *AXR6* transcription unit in our mutant, finding a G -> A transition in the last exon that produced the E716K amino acid substitution. This mutation was detected by CAPS using a pair of oligonucleotide primers (5'-GTGGTGATGAAACTCATTGG-3' and 5'-CCTCTCCAAATAATCTCTGGT-3') and the restriction enzyme MnlI.

To verify that our mutation was an allele of *AXR6*, we carried out a complementation test between our mutant and *axr6-1*.[6] *axr6-1* is semi-dominant and seedling-lethal when homozygous. Therefore, we examined F_1 plants obtained from a cross between our mutant and a heterozygote for *axr6-1*. Of 17 F_1 plants examined, 8 plants grew normally, 8 were seedling-lethal, and one did not germinate, indicating that our mutant was recessive and did not complement *axr6-1*. Thus, we named it *axr6-101*. Taken together, these results show that our mutant is a new recessive allele of *AXR6*.

Next, we examined the phenotype of *axr6-101* using the

twice-backcrossed line. Root growth showed 2,4-D resistance with the half maximal inhibitory concentration (IC50) of about 0.3 μM (Fig. 1A). When the IC50 was compared, the auxin resistance of *axr6-101* was similar to that of *axr6-3*,[9] and was slightly stronger than that of *cul1-7* [10] at 23 – 24°C. *axr6-101* had wrinkled rosette leaves like *axr6-1* heterozygotes.[6] Mature *axr6-101* plants were ~20% as tall as L*er* at 23°C (Fig. 1B). Flower morphology was altered in *axr6-101*, as is reported for *cul1-6*.[8] The number of floral organs was often reduced: the most common flowers of *axr6-101* consisted of 4 sepals, no petals, 4 stamens, and 1 gynoecium, in contrast to L*er* flowers showing 4 sepals, 4 petals, 6 stamens, and 1 gynoecium (Table S2). Although fused flower organs such as sepal–petal fusion and petal–anther fusion are observed in *cul1-6*,[8] no fusion of floral organs was observed in axr*6-101* at any of the temperatures tested. At the seedling stage, the formation of lateral roots was inhibited (Fig. S1). Furthermore, the gravitropic response of the roots was compromised (Fig. S2). However, the defects of the hypocotyls were rather small in terms of gravitropism (Fig. S3) and phototropism (Fig. S4).

Then, we raised *axr6-101* plants at 20 and 28°C and investigated whether the phenotype was temperature-sensitive. The *axr6-101* plants were small at 20°C; however, the size was not reduced further at 28°C (Fig. 1B). In *cul1-7*, the morphology of the etiolated hypocotyls is more affected at higher temperatures.[10] At 28°C, *cul1-7* seedlings exhibit a de-etiolated phenotype in the dark, with short hypocotyls (~20% as long as wild-type hypocotyls) and open hooks and cotyledons. For etiolated *axr6-101* seedlings, growth of

hypocotyls and roots was reduced to ~70 − 80% of the mean size achieved by L*er*. However, the relative inhibition of growth was not affected in either organ by an elevated temperature (Fig. 2A). L*er* was affected more readily by temperature in the hook opening characteristic. As a result, at 20°C more *axr6-101* hypocotyls had open hooks than those of L*er*, but at 28°C more open hooks were observed in L*er* (Fig. 2B). Further, most etiolated *axr6-101* seedlings had closed cotyledons, similar to L*er* seedlings, a feature that did not change between 20 and 28°C (Fig. S5). Finally, we investigated the gravitropic growth orientation of roots and hypocotyls in etiolated seedlings. SD of the growth angle can be a measure of growth orientation: a larger SD indicates a more random growth orientation, which reflects weaker gravitropic control of growth direction.[15] SD of the growth angle of *axr6-101* roots was clearly larger than that of L*er* at both temperatures, indicating gravitropic malfunction in *axr6-101* roots. However, the extent of the defects was similar at both 20 and 28°C (Fig. 2C). In the case of the hypocotyl, the SD was similar between L*er* and *axr6-101* at 20°C; however, a larger SD was observed in *axr6-101* at 28°C, suggesting that gravitropic defects are promoted at higher temperatures. In conclusion, the defects of *axr6-101* were largely unaffected by elevated temperatures, in contrast to *cul1-7* and *axr6-3*.

axr6-101 has an amino-acid substitution, E716K, in the C-terminal region of CUL1 where it binds to RBX1. RBX1 consists of an N-terminal β-strand and a C-terminal RING domain. Amino acid residues of human CUL1 (HsCUL1) that interact with RBX1 have been identified by the

crystallographic study of the RBX1−HsCUL1 complex.[1] Most residues bind to the N-terminal β-strand of RBX1, and the remaining bind to the C-terminal RING domain. E716 of Arabidopsis CUL1 (AtCUL1) corresponds to D754 in HsCUL1, and the adjacent residue of HsCUL1, I755, is involved in binding to the RING domain. Furthermore, E716 is located near K682 of AtCUL1 (corresponding to K720 in HsCUL1), which is the neddylation site of CUL1. Neddylation of CUL1 is necessary for full activity of the SCF ligase E3 and auxin signaling.[2,16] Though E716 may not interact directly with RBX1, it is highly conserved and mostly occupied by E or D in the CUL1 sequences of other organisms. In contrast, *cul1-7* is defined by a T510I substitution. T510 corresponds to S541 of HsCUL1 that interacts directly with the N-terminal β-strand of RBX1.[1,10] Thus, *axr6-101* is an allele of *AtCUL1* that harbors an amino-acid substitution that could affect binding to the RING domain of RBX1. This structural difference may be the reason for the phenotypic difference between *axr6-101* and *cul1-7*. However, *axr6-101* was obtained on the L*er* genetic background,[17] whereas *cul1-7* and most of other *axr6/cul1* alleles have a Col background. Therefore, the different genetic background should be considered when comparing the phenotype of *axr6-101* with that of other alleles. Further study of *axr6-101* may result in a deeper understanding of CUL1 function in auxin signaling.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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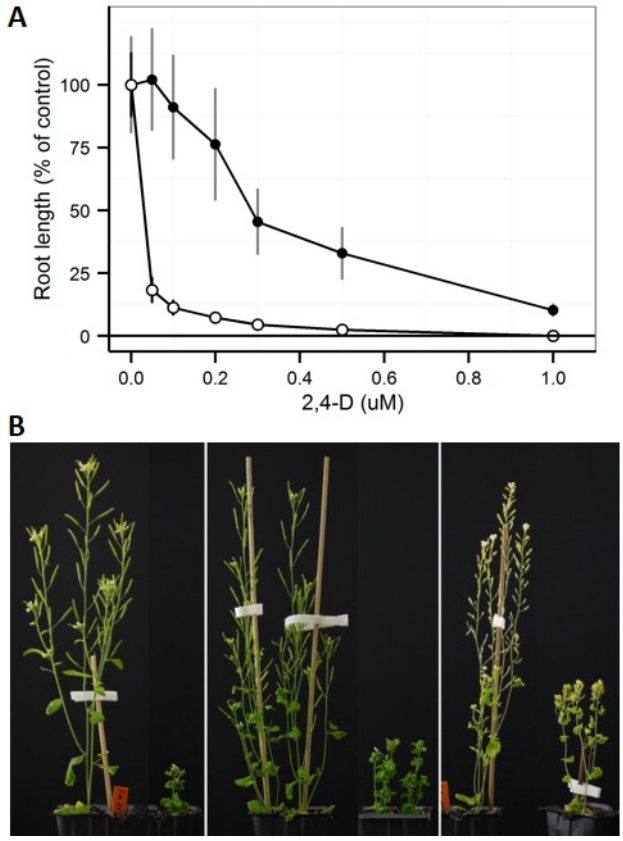

Figure 1. *axr6-101* is auxin-resistant and semi-dwarf at the mature stage. (A) Root length was measured in L*er* (open circle) or *axr6-101* (closed circle) seedlings grown on agar medium containing the indicated concentrations of 2,4-D for 6 days under continuous white-light condition at 23°C. The data represent the mean and SD of 12 seedlings. (B) L*er* (left) and *axr6-101* (right) plants grown for 5.5 weeks at 20 (left panel), 23 (middle panel), and 28°C (right panel) under continuous white light. Plants were grown in 5.5-cm-square pots.

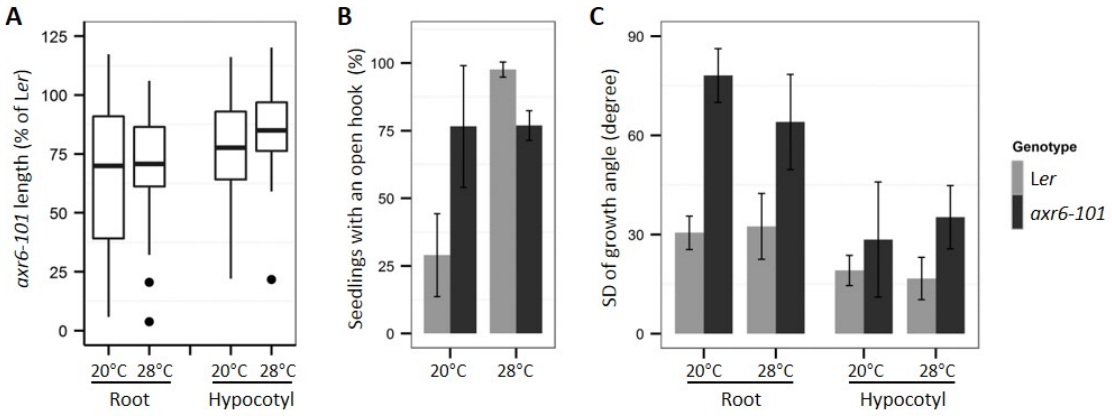

Figure 2. Phenotype of *axr6-101* is largely independent of growth temperature. (A) Growth of roots and hypocotyls. The length of each organ was examined in 4-day-old etiolated L*er* and *axr6-101* seedlings at 20 and 28°C, and the relative length (%) of *axr6-101* to L*er* is shown in each organ for 30 − 36 seedlings. The relative length of each organ was not significantly different between 20 and 28°C ($P > 0.195$ in *t*-test). (B) Hook opening. Hook structure was examined in etiolated hypocotyls grown as above. The data represent the mean and SD of 4 measurements, in which 9 − 22 seedlings were used. (C) Gravitropic growth orientation of the root and hypocotyl. The growth angle was measured in 4-day-old etiolated seedlings. The data represent the mean and SD of 5 − 6 experiments, in which 9 − 41 seedlings were measured. The measurements of each organ from *axr6-101* were not significantly different between 20 and 28°C ($P > 0.076$). The SD of the hypocotyl was larger for *axr6-101* than that of L*er* at 28°C ($P = 0.0036$).

Supplemental Material

Table S1. SSLP and CAPS markers used for linkage analysis.

Name	Forward (Fwd) and reverse (Rev) primers	Amplified fragment (bp)		Restriction enzyme
		L*er*	Col	
CER458376	Fwd: 5'-GTGTTACTACTCTGTTTCC-3'	157	171	
	Rev: 5'-GTCATGACTATCCTTGTG-3'			
CER458010	Fwd: 5'-TCTTTCTTGGGATTGTTTGG-3'	113	146	
	Rev: 5'-TGATAACACAGGGCGTAA-3'			
CER441938	Fwd: 5'-AGAAGTCCATATGGAGACTG-3'	697	172+525	HinfI
	Rev: 5'-TGAGAAACATTTGCTCGATG-3'			

Table S2. Number of flower organs. Data represent mean and SD of 12 flowers.

Genotype	Sepal	Petal	Stamen	Gynoecium
L*er*	4.0 ± 0.0	4.0 ± 0.0	6.0 ± 0.0	1.0 ± 0.0
axr6-101	3.67 ± 0.49	0.83 ± 1.11	4.33 ± 0.65	1.0 ± 0.0

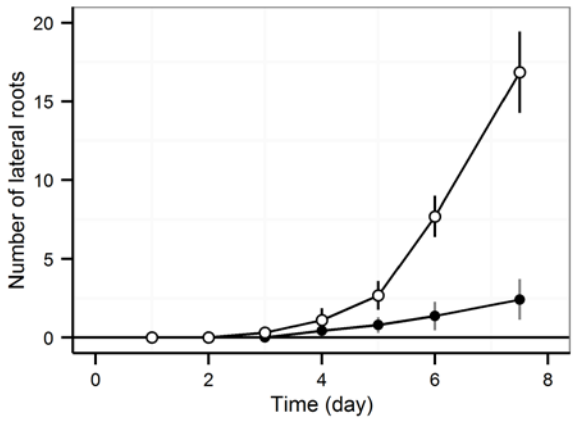

Figure S1. Lateral root formation in *axr6-101* (closed circle) and L*er* (open circle). Number of lateral roots was examined at 23°C under continuous white-light condition after induction of germination by white-light irradiation for 24 hr. Mean and SD of 12 seedlings are presented.

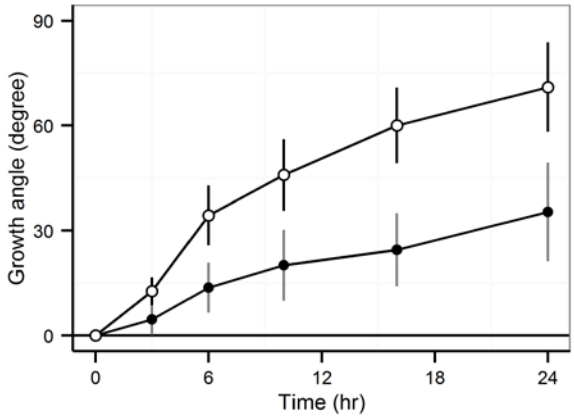

Figure S2. Gravitropic response of roots of etiolated *axr6-101* (closed circle) and L*er* (open circle) seedlings at 23°C in the dark. Seedlings were grown on vertically-held agar plates for 3 days in the dark, and then reoriented by 90°. Only roots that had grown vertically downward before reorientation were examined thereafter. Mean and SD of 12 seedlings are presented.

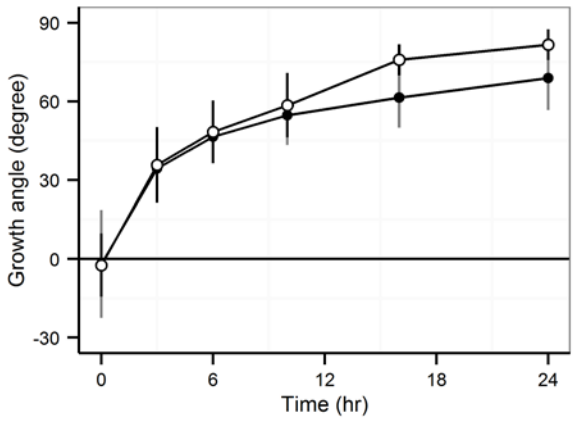

Figure S3. Gravitropic response of hypocotyls of *axr6-101* (closed circle) and L*er* (open circle) seedlings at 23°C in the dark. Three-day-old etiolated seedlings grown on vertically-held agar plates were reoriented by 90°. Mean and SD of 12 seedlings are presented. Only values at 16 and 24 hr are significantly different between L*er* and *axr6-101* ($P < 0.0078$ in *t*-test).

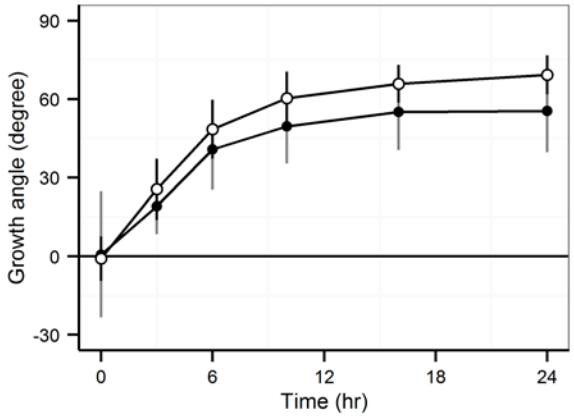

Figure S4. Phototropic response of etiolated hypocotyls of *axr6-101* (closed circle) and L*er* (open circle) seedlings at 23°C in the dark. Three-day-old etiolated seedlings grown on vertically-held agar plates were unilaterally irradiated with 0.1 μmole m^{-2} s^{-1} blue light, and then growth angle of their hypocotyls was determined thereafter. Mean and SD of 12 seedlings are

presented. Only values at 16 and 24 hr are significantly different between L*er* and *axr6-101* ($P < 0.016$ in *t*-test).

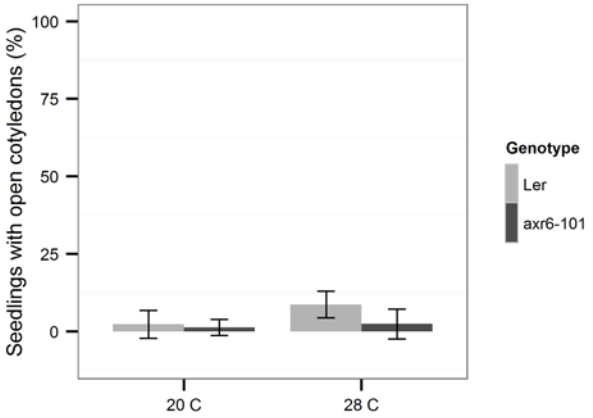

Figure S5. Cotyledon opening. Morphology of cotyledons was examined in 4-day-old etiolated seedlings grown at 20 and 28°C. Data represent mean and SD of 4 measurements, in which 9 to 22 seedlings were used. There are no significant differences between values at 20 and 28°C of each genotype or between different genotypes at each temperature ($P > 0.087$ in *t*-test).