Genetic characterization of mutants resistant to the antiauxin *p*-chlorophenoxyisobutyric acid (PCIB) reveals that *AAR3,* a gene encoding a DCN1-like protein, regulates responses to the synthetic auxin 2,4-dichlorophenoxyacetic acid in Arabidopsis roots.

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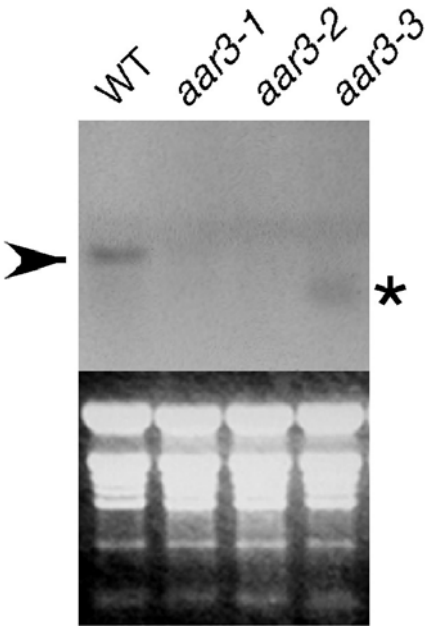
Supplemental Data

Supplemental Figure S1

RNA hybridization analysis in the *aar3* mutants.

Upper panel: RNA blot probed with the digoxigenin-labeled *AAR3* antisense RNA. Expression of *AAR3* mRNA (indicated by arrow head) is detected in wild type (WT) but is drastically reduced in *aar* mutants, consistent to the results of RT-PCR in Figure 5B and 5C. Putative truncated *aar3-3* RNA is indicated by an asterisk.

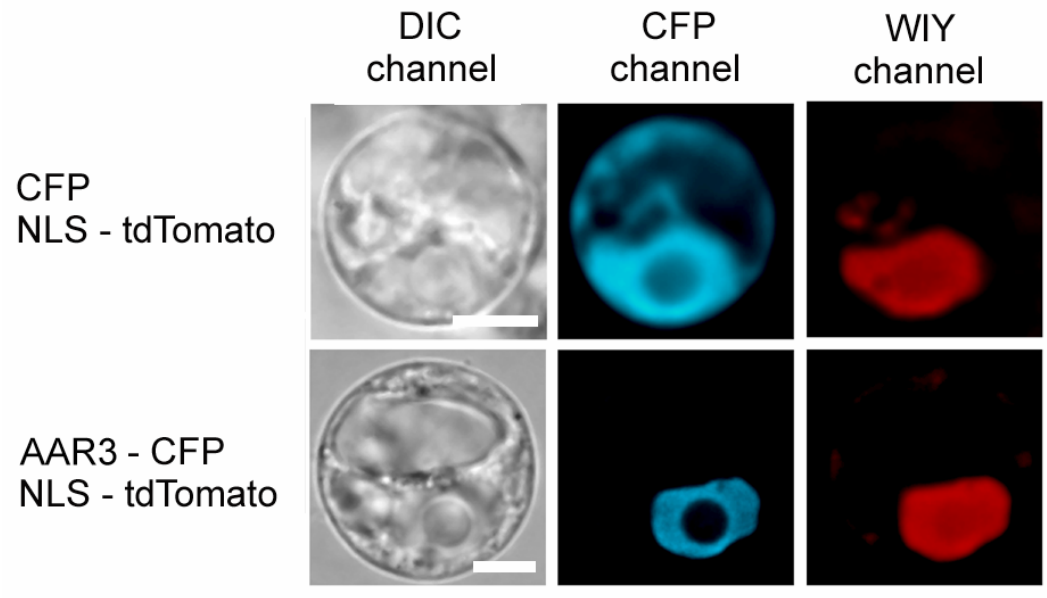
Lower panel: Ethidium bromide staining of total RNA before blotting indicates RNA samples are intact.



Supplemental Figure S2

Nuclear localization of AAR3-CFP in *Arabidopsis* protoplast.

Protoplasts of *Arabidopsis* T87 suspension-cultured cells co-expressing *35S::CFP* and *35S::NLS-tdTomato* (upper panels) and *35S::AAR3-CFP* and *35S::NLS-tdTomato* (lower panels). AAR3-CFP associated fluorescence is localized in nucleus but non-fused CFP fluorescence is distributed in both nucleus and cytosol. NLS-tdTomato was used for nuclear maker and its fluorescent signal was captured in WIY channel. Small dot signals in WIY channel corresponded to auto-fluorescence from chloroplasts. Bars, 10 nm.

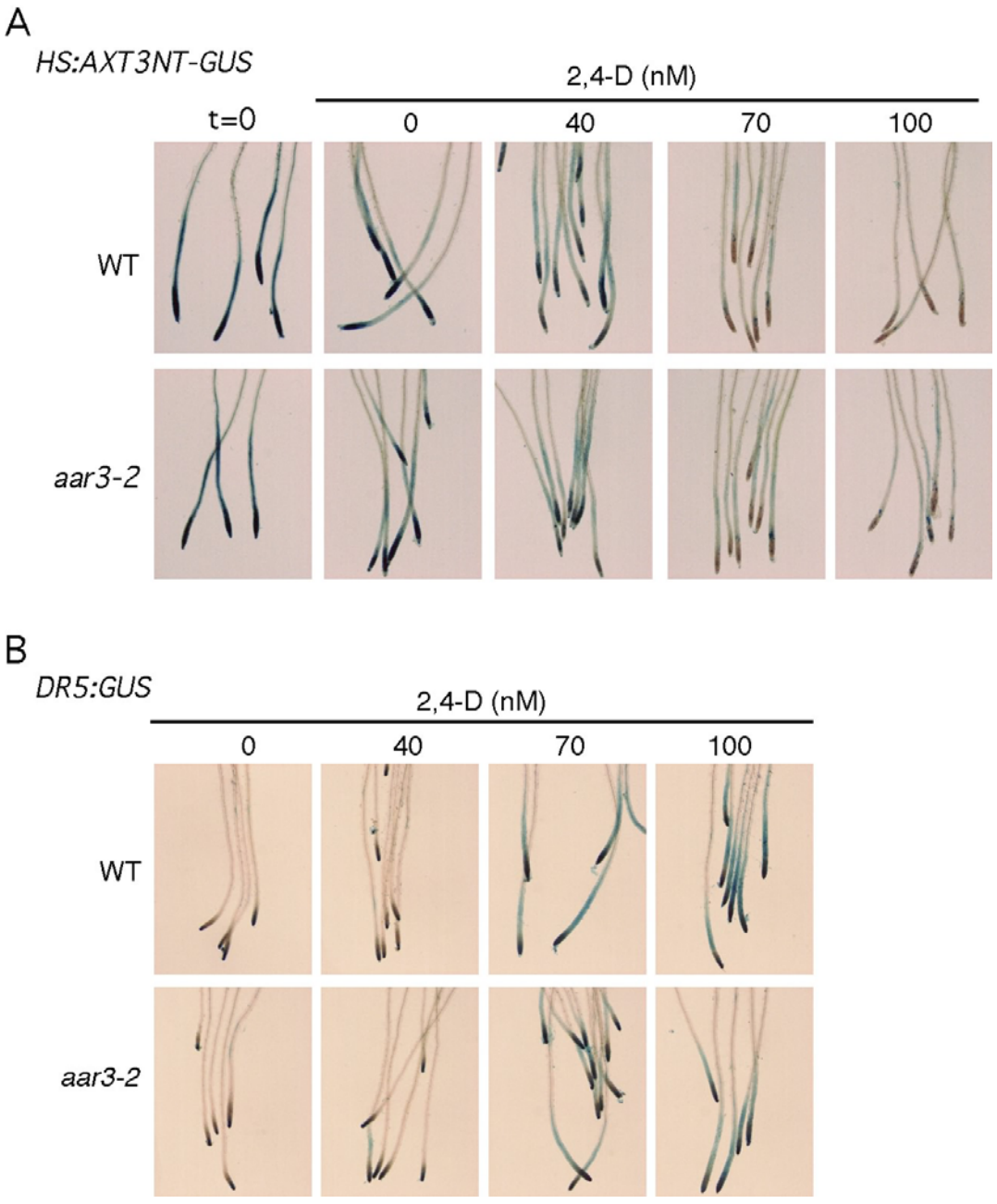


Supplemental Figure S3

Comparison of 2,4-D-induced protein degradation (A) and gene expression (B) in wild type (WT) and *aar3-2*.

A. Seedlings of *HS::AXR3NT-GUS* in the wild-type and *aar3-2* background were germinated on GM-MES media and grown for 5 days. Then, plates were incubated at 37 ˚C for 2.5 h to give heat shock. After heat shock, the seedlings were incubated in GM-MES solution with indicated concentration of 2,4-D for 2 h at 23 ˚C, followed by detection of GUS activity in X-gluc solution.

B. Five-day–old *DR5::GUS* seedlings were incubated in GM-MES solution with indicated concentration of 2,4-D for 2 h, followed by detection of GUS activity in X-gluc solution.


A
HS:AXT3NT-GUS
2,4-D (nM)
t=0
0
40
70
100
WT
aar3-2
B
DR5:GUS
2,4-D (nM)
0
40
70
100
WT
aar3-2

Supplemental Table S1. New SSLP/CAPS markers used for *aar3-1* mapping [a].

Name	Type	Position	Primer sequence	Size of PCR product[a]	Restriction enzyme	Size of digested product[a]
F16J14-1	SSLP	7.96 MB	F: ggtaagcttcaggtcgtgct R: gtcaacactttgacccgaca	224 (C) 202(L)	-	-
F5N5-1	SSLP	8.11 MB	F: gaaacatttctcggcctcat R: tcggtggagttttcccatac	204 (C) 196 (L)	-	-
MUJ8-1	SSLP	8.74 MB	F: tattgcgatgtggttttgga R: accttggtttttcggctttt	127 (C) 139 (L)	-	-
F20C19-1	SSLP	9.64 MB	F: gctctcttggccctaaacaa R: gtgatgcatttgatgcttgg	234 (C) 196 (L)	-	-
MFE16-1	SSLP	9.71 MB	F: ctacgcaacgcaatggtaaa R: ggggaacctccatggttact	222 (C) 206 (L)	-	-
MMG15-2	SSLP	10.47 MB	F: tcgtaacatgagttcttggaga R: ccacgtacgtttttgctgttt	103 (C) 95 (L)	-	-
MFJ20-1	SSLP	10.62 MB	F: tggccttctttttcgtatgg R: tggaatgcataaatcccaca	102 (C) 124 (L)	-	-
MZN14-1	SSLP	10.70 MB	F: aaacttaattgaccatgagattgaaa R: ccacctttgatatctatgacccta	299 (C) 270 (L)	-	-
MLD15-1	SSLP	10.87 MB	F: gacacatgtctcttttatattggttca R: ccctaaaatccgatatccaaaa	200 (C) 185 (L)	-	-
MLD15-2	SSLP	10.90 MB	F: cctgaaaggatcgtctgtgc R: ggttaactcgagcaccgtgt	172 (C) 165 (L)	-	-
MYI13-1	CAPS	10.94 MB	F: gtggatggtagcggttgaat R: aaacccaaaagaccgaattt	387	Cac8I	271, 117 (C) 245, 117, 26 (L)
K5K13-8	SSLP	10.96 MB	F: gggatgaaaactcttgtaaatcc R: tccaaaacaatttcgcatga	279 (C) 300 (L)	-	-
K5K13-9	SSLP	10.98 MB	F: cggtaaaatcgcaccgatac R: cgagaaatcgacacagatgg	178 (C) 175 (L)	-	-
K5K13-2	SSLP	10.99 MB	F: tggggagttcagtttgtattga R: tttttgggttgcttaacatctg	225 (C) 216 (L)	-	-
K5K13-4	SSLP	11.02 MB	F: aagatttctatttaggtgtgcaacaa R: ccaacaaaagcatcagcaaa	165 (C) 159 (L)	-	-
MRI12-5	SSLP	11.05 MB	F: actcaaaatccgtggacgag R: gcttccgtgtaagaaggaactg	150 (C) 145 (L)	-	-
MXE2-3	CAPS	11.11 MB	F: tggataaagcagccaaggac R: gggatcaaaagattccctca	152	PstI	114, 38 (C) 152 (L)
MUO22-1	SSLP	11.14 MB	F: tcatcgttcccccatacttt R: gtcatatcgaaacgggtcgt	134 (C) 148 (L)	-	-

[a] Size of products derived from genomic DNA of Arabidopsis ecotype Columbia (C) and Landsberg (L) were shown.

Supplemental Materials and Methods S1

Isolation of *aar* mutants

The ethyl methanesulfonate (EMS)-mutagenized M_2 seeds were purchased from LEHLE SEEDS (Round rock TX, USA) and Versailles T-DNA seeds (Bechtold et al., 1993) were obtained from the Arabidopsis Biological Resource Center (Columbus, Ohio, USA). Ion beam irradiation was performed as described in Hase et al., (2000). The seeds were surface sterilized and plated on GM-MES containing 20 μM PCIB. Putative mutants with elongated roots were selected and transferred to soil. Seeds from the next generation were harvested and examined for resistance to 20 μM PCIB. PCIB-resistant plants were self-fertilized for 5 to 7 generations. Mutant lines used in phenotypic assays were homozygous plants from at least the first (*aar3-1* and *aar5*) or second (*aar4*) back-cross to the parental (Columbia-0) line. To characterize auxin and PCIB responses in root, germinated seedlings in GM-MES medium were transferred to hormone-containing medium as it is indicated in the figure legends.

Procedure for mapping and identification of *aar* mutations

PCIB-resistant *aar* mutants (homozygote or heterozygote) were out-crossed to Landsberg *erecta* plants. The resulting F_2 seeds were plated on 20 μM PCIB-supplemented GM-MES medium. DNA from PCIB-resistant (for recessive lines) or PCIB-sensitive (for dominant or semi-dominant lines) individual seedlings was isolated by an Automatic DNA isolation system (Kurabo, PI-50α, Tokyo, Japan).

Mutants were mapped using published SSLP (Bell and Ecker, 1994) and CAPS (Konieczny and Ausubell, 1993) and additionally generated markers shown below.

The primer sets NGA112-1a (5’-acacaacgaacgatctgatagc-3’) and NGA112-1b (5’-tcacgaatccaccatttttc-3’), T20O10-1a (5’-tgccaggggaatagatgaaa-3’) and T20O10-1b (5’-cgctgtagcaaacgtggtaa-3’), CIW5-1a (5’-ggttaaaaattagggttacga-3’) and CIW5-1b (5’-agatttacgtggaagcaat-3’), T10P11-1a (5’-gcataagcggtgacaaggat-3’) and T10P11-1b (5’-ccctctatattaaatgcaagaatga-3’), RCI1B F (5'-atcgatttggtggcagaaac-3') and RCI1B R (5'- cagctcgttacaggcgctac-3'), and T24H18 F (5'-taccagtgcatggtttgcat-3') and T24H18 R (5'-acgcaggacatgtttcctct-3’) were generated to detect polymorphism in nga112, T20O10, ciw5, T10P11, RCI1 and T24H18 region, respectively.

For sequence analysis of the *TIR1* gene, DNA was harvested from homozygous *m31* and *m34* seedlings and long PCR amplified *TIR1* region by using two primers *tir1*a (5′-gatccagtgagtgaaacagagtgat-3’) and *tir1*b (5′-tagattccctcttctcctacacctt-3’).

For *aar2-1*, thermal asymmetric interlaced PCR was performed for mapping the integration site of the T-DNA following the procedure described by Liu et al. (1995) and McElver et al. (2001). The primers TLBAR1 (5’-caagagcgtggtcgctgtcatc-3’), TLBAR2 (5’-atgcacgaggcgctcggatatg-3’), and TLBAR3 (5’-caagcacgggaactggcatgac-3’) were used as specific primers. Amplified DNA fragments were subjected direct sequencing using primer TLBAR4 (5’-gtttctggcagctggacttc-3’).

For fine mapping of the *aar3-1* mutation, additional makers shown in Supplemental Table II were generated by referencing MONSANTO ARABIDOPSIS POLYMORPHISM AND LER SEQUENCE COLLECTION database

(http://www.arabidopsis.org/Cereon/index.jsp). Genomic DNA from *aar3-1* plants were amplified by using 6 pairs of primer set [*aar3-1*a (5’-gtagcgagtattaggcgagtaacag-3’ and 5’-taaccaaactgaacccgaaatataa-3’), *aar3-1*b (5’-gttctaagaaacgccacattatagg-3’ and 5’-ggttataactggagtgtctgatgct-3’), *aar3-1*c (5’-gctccctatagagaatgaagaaacc-3’ and 5’-acaccaaccgtgaacactataaaag-3’), *aar3-1*d (5’-atgatgattgttgctcgtacaactc-3’ and 5’-tccgatttgacttacccaatattta-3’), *aar3-1*e (5’-tgaaatcaaatagacaaaactgaactg-3’ and 5’-agagatttgacattagagtgacgattt-3’), and *aar3-1*f (5’-ttcaaacgaaaactaaacgaaagac-3’ and 5’-tgcaagttgtgactattactcattg-3’)]. Amplified products were purified with QIAquick PCR Purification Kit (QIAGEN K.K. Tokyo Japan) and sequenced directly using standard procedures.

Presence of T-DNA in the Salk lines was confirmed by PCR with left border primer, LB1 (5′-caaaccagcgtggaccgcttgctgcaactc-3′), and specific primers, T10P11 R78367 (5′-ggactctgatgccacgttct-3′), *AAR3-2*.1 (5′-tattgcttctttagacaagttaaatctttg-3′), and K5K13 R38396 (5’-gcgagaatgggcaaaagaat-3’) for *aar2-2*, *aar3-2*, and *aar3-3*, respectively. The amplified fragments were sequenced directly to determine the precise location of the T-DNA.

RNA hybridization

Total RNA (10 μg) was isolated from 5-d-old seedlings by the procedure of Theologis et al. (1985), further purified with QIAGEN RNeasy kit, size-fractionated with a 1.2% agarose gel containing formaldehyde and transferred onto a nylon membrane. Hybridization and immunological detection was performed according to DIG Northern

Starter Kit (Roche Applied Science, Mannheim, Germany) by using a digoxigenin-labeled RNA probe, polyclonal sheep anti-digoxigenin Fab-fragments conjugated to alkaline phosphatase and CDP-*Star* for chemiluminescent substrate for alkaline phosphatase. Chemiluminescent signals were visualized with a LumiImager F1 workstation (Roche).

AAR3 cDNA fragments were amplified by PCR with the template plasmid U51023, which contains the full length AAR3 cDNA and was obtained from Arabidopsis Biological Resources Center, and primers Xho-AAR3ATG (5'-tagacctcgagatggattcttcgccagtttct-3') and AAR3R+TGA-Spe (5'-gactagttcatggtttatctccagtcgataa-3'), and *PfuTurbo* DNA polymerase (Stratagene Japan, Tokyo Japan), followed by A-tailing reaction and ligation into the pGEM-T easy vector according to manufactures instruction (Promega, Maidson WI). The resulting plasmid, *AAR3*/pGEMT-easy, was digested by *Xho* I and subjected to *in vitro* transcription with SP6 RNA polymerase in DIG Northern Starter Kit to generate the digoxigenin-labeled *AAR3* antisense RNA probe.

Construction of *35S::YFP-AAR3, 35S::AAR3-CFP*, and *35S::NLS-tdTomato*

Full length *AAR3* cDNA was amplified by PCR to add *Bam* HI sites or *Nco* I sites at both ends. Each PCR fragment was inserted into pAVA554 or pAVA574 to form *YFP-AAR3* or *ARR3-CFP* fusions, respectively. The primers used for PCR were 5'-tggatccatggattcttcgccagtttctgc-3' and 5'- tggatcctcatggtttatctccagtcgata-3' for *Bam* HI sites and 5'- accatggattcttcgccagtttctgctcga-3'and 5'- tccatggatggtttatctccagtcgataaa-3'

for *Nco* I sites.

For the nuclear marker, a NLS from SV40 large T antigen was fused to *tdTomato* (Shaner et al., 2004) by PCR with the primers of 5'-aggatccatggctccaaagaagaagagaaaggtcgtgagcaagggcgaggaggtc-3' and 5'-agagctcttacttgtacagctcgtcc-3'. The *NLS-tdTomato* PCR fragment was inserted into pBI221 (Clontech) using *Bam* HI and *Sac* I sites, replacing the *GUS* gene in the vector.

CFP, YFP and tdTomato signals were detected with an IX7 microscope equipped with IX2-DSU (OLYMPUS, Tokyo, Japan) with cooled CCD camera (UIC-QE1; Molecular Devices Co., Sunnyvale, CA), and standard CFP, YFP, or WIY filters (OLYMPUS). The raw images were obtained with MetaMorph Imaging Software Version 6.1 (Universal Imaging Corporation, Downingtown, PA), and deconvoluted images were constructed using Volocity Restoration (version 4.1) software (Improvision, Coventry, UK).

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