Three Auxin Response Factors Promote Hypocotyl Elongation[1,2][OPEN]

Jason W. Reed,[a,b,2,3] Miin-Feng Wu,[a,4] Paul H. Reeves,[a,5] Charles Hodgens,[a,b] Vandana Yadav,[a] Scott Hayes,[c,6] and Ronald Pierik[c]

[a]Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27599-3280
[b]Curriculum in Genetics and Molecular Biology, University of North Carolina, Chapel Hill, North Carolina 27599-3280
[c]Department of Biology, Utrecht University, 3584 CH Utrecht, The Netherlands

ORCID IDs: 0000-0001-7508-9714 (J.W.R.); 0000-0001-7272-3492 (M.-F.W.); 0000-0002-2411-8375 (P.H.R.); 0000-0002-0384-4458 (C.H.); 0000-0002-7628-894X (V.Y.); 0000-0001-8943-6238 (S.H.); 0000-0002-5320-6817 (R.P.)

The hormone auxin regulates growth largely by affecting gene expression. By studying Arabidopsis (*Arabidopsis thaliana*) mutants deficient in AUXIN RESPONSE FACTORS (ARFs), we have identified three ARF proteins that are required for auxin-responsive hypocotyl elongation. Plants deficient in these factors have reduced responses to environmental conditions that increase auxin levels, including far-red-enriched light and high temperature. Despite having decreased auxin responses, the ARF-deficient plants responded to brassinosteroid and gibberellin, indicating that different hormones can act partially independently. Aux/IAA proteins, encoded by *IAA* genes, interact with ARF proteins to repress auxin response. Silencing expression of multiple *IAA* genes increased hypocotyl elongation, suggesting that Aux/IAA proteins modulate ARF activity in hypocotyls in a potential negative feedback loop.

Plant organs grow to their final size by cell expansion. Several plant hormones, including auxin, brassinosteroids, ethylene, and GAs, regulate expansion growth, and environmental signals such as temperature and light can affect abundance of or responsiveness to these hormones (Wolters and Jürgens, 2009; Depuydt and Hardtke, 2011; Leivar and Monte, 2014). Integration of these signals with metabolic signals determines the degree of cell expansion and, hence, the final size of stems, leaves, and other organs.

Hypocotyl elongation after seed germination is sensitive to multiple hormonal and environmental signals, and occurs largely by the expansion of cells produced in the embryo. In Arabidopsis (*Arabidopsis thaliana*), mutations in genes regulating light perception, hormone response, the circadian rhythm, or transcription can each affect hypocotyl elongation. Many such mutations also affect growth in other organs, indicating that common mechanisms regulate expansion growth in multiple tissues. Thus, the hypocotyl is a useful model tissue in which to explore general control mechanisms of cell expansion.

High temperature and far-red-enriched light stimulate hypocotyl elongation, and the circadian rhythm gates hypocotyl elongation, in part by regulating auxin response. Integrated signals from these and other inputs affect the levels and activity of PHYTOCHROME INTERACTING FACTOR (PIF) transcription factors (Gray et al., 1998; Covington and Harmer, 2007; de Lucas et al., 2008; Feng et al., 2008; Leivar et al., 2008; Lorrain et al., 2008; Koini et al., 2009; Shin et al., 2009; Stavang et al., 2009; Nusinow et al., 2011; Li et al., 2012; Sairanen et al., 2012; Box et al., 2015; Nieto et al., 2015; Soy et al., 2016). PIFs can increase auxin biosynthesis and/or signaling (Franklin et al., 2011; Nozue et al., 2011; Hornitschek et al., 2012; Li et al., 2012; Sun et al., 2012; Goyal et al., 2016), and they can interact with BRASSINAZOLE RESISTANT/BRI1 EMS SUPPRESSOR (BZR/BES) and AUXIN RESPONSE FACTOR (ARF) transcription factors, which regulate responses to brassinosteroid and auxin (Oh et al., 2014). GAs also can modulate PIF activity and stability (de Lucas et al., 2008; Feng et al., 2008; Li et al., 2016). PIF, BZR/BES, and ARF transcription factors share many target genes that promote cell expansion, and may activate some of these genes cooperatively (Oh et al., 2014).

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[2]Senior author.

[3] Author for contact: jreed@email.unc.edu.

[4]Current address: Monsanto, 700 Chesterfield Parkway, West Chesterfield, MO 63017.

[5]Current address: UK Research and Innovation, Polaris House, Swindon SN2 1FL, UK.

[6]Current address: Department of Plant Molecular Genetics, Centro Nacional de Biotechnología, Calle Darwin 3, 28049 Madrid, Spain.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Jason W. Reed (jreed@email.unc.edu).

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ARFs bind to conserved DNA sequence elements in auxin-responsive promoters (Ulmasov et al., 1997, 1999a, 1999b; Boer et al., 2014). Class II ARFs have Q-rich middle domains that can activate gene expression. Conversely, the same ARF proteins can repress gene expression by interacting with Aux/IAA proteins that recruit histone deacetylase complexes (Tiwari et al., 2004; Szemenyei et al., 2008; Vernoux et al., 2011; Piya et al., 2014; Guilfoyle, 2015). Auxin-stimulated turnover of Aux/IAA proteins switches gene-repressing ARF-Aux/IAA complexes to gene-activating ARF complexes (Salehin et al., 2015). In addition, class I ARF proteins lacking the Q-rich middle domain can repress gene expression, possibly independently of recruiting Aux/IAA proteins (Ulmasov et al., 1999a; Tiwari et al., 2003; Ellis et al., 2005; Vernoux et al., 2011; Piya et al., 2014).

Loss-of-function mutations in several Arabidopsis *arf* genes decrease auxin-induced gene expression and cause growth defects associated with decreased auxin response. For example, *arf6* and *arf8* mutations decrease inflorescence stem and flower organ growth (Nagpal et al., 2005; Tabata et al., 2010; Reeves et al., 2012), and *nph4/arf7* and *arf19* mutations decrease leaf cell expansion and lateral root formation (Okushima et al., 2005; Wilmoth et al., 2005). *nph4/arf7 arf19* double mutants also have decreased sensitivity to root growth inhibition by auxin. Similarly, gain-of-function *iaa* mutations that reduce auxin-induced turnover of Aux/IAA proteins also inhibit auxin-responsive gene induction (Timpte et al., 1994; Nagpal et al., 2000; Gray et al., 2001; Tian et al., 2002; Overvoorde et al., 2005). Many of these mutations cause phenotypes similar to those of loss-of-function *arf* mutations.

Several gain-of-function *iaa* mutations affect hypocotyl elongation. *shy2/iaa3-2*, *shy2/iaa3-3*, and *axr2/iaa7-1* gain-of-function mutations cause short hypocotyls (Timpte et al., 1992; Reed et al., 1998; Tian and Reed, 1999; Nagpal et al., 2000; Chapman et al., 2012), suggesting that interacting ARF proteins normally promote hypocotyl elongation. However, whereas an *arf6 arf8* double mutant had somewhat shorter hypocotyls than did wild-type plants in darkness (Nagpal et al., 2005), *arf8* single mutant light-grown seedlings had longer hypocotyls than did wild-type seedlings (Tian et al., 2004). Similarly, the *axr3-1* mutation stabilized AXR3/IAA17 protein and caused accelerated hypocotyl elongation at early time points, although the final hypocotyl length was less than that of wild-type seedlings (Leyser et al., 1996; Nagpal et al., 2000) and mutations that stabilize IAA18 increased hypocotyl elongation (Uehara et al., 2008; Ploense et al., 2009). These contrasting phenotypes suggest that quantitative or tissue-specific aspects of ARF activity may influence the kinetics and extent of hypocotyl growth, and that additional redundancy may exist among the *ARF* genes that promote elongation.

To explore how ARF proteins regulate hypocotyl elongation, we characterized hypocotyl growth in higher order mutants deficient in multiple class II *ARF* genes known to affect cell expansion and in plants deficient in multiple *IAA* genes. We found that three ARF proteins together have a major role in controlling hypocotyl elongation downstream of auxin, high temperature, far-red enrichment, and circadian regulation; that other hormone response pathways act partially independently of these ARF proteins; and that Aux/IAA proteins repress hypocotyl elongation.

RESULTS

ARF6, NPH4/ARF7, and ARF8 Promote Hypocotyl Elongation

The class II ARF proteins ARF6 (At1g30330), NPH4/ARF7 (At5g20730), ARF8 (At5g37020), and ARF19 (At1g19220) promote cell expansion in leaf, inflorescence, and/or flower organs (Nagpal et al., 2005; Wilmoth et al., 2005). These four ARF proteins form a clade together with MP/ARF5 (At1g19850; Remington et al., 2004), can each mediate auxin-induced gene expression (Ulmasov et al., 1999a; Tiwari et al., 2003; Wilmoth et al., 2005), and interact strongly with Aux/IAA proteins in yeast two-hybrid assays (Vernoux et al., 2011; Piya et al., 2014). *ARF6*, *NPH4/ARF7*, and *ARF8* were each expressed in dissected hypocotyls (Chapman et al., 2012; Kohnen et al., 2016).

We assessed hypocotyl growth in seedlings with combinations of *arf6*, *nph4/arf7*, *arf8*, and *arf19* null mutations. We did not include *mp/arf5* mutants in these experiments because these have defective vasculature and lack a hypocotyl entirely (Berleth and Jürgens, 1993). We also silenced *ARF6* and *ARF8* by driving the expression of the microRNA precursor *MIR167a* with the broadly expressed P_{RPS5a}*:GAL4-VP16* transactivation line (Weijers et al., 2005). The mature microRNA miR167 targets *ARF6* and *ARF8* transcripts for turnover, and overexpressing *MIR167a* behind the P_{35S} viral promoter confers phenotypes very similar to those of *arf6-2 arf8-3* mutant plants (Wu et al., 2006). In most experiments, transactivated P_{RPS5a}*>MIR167a* F1 seedlings had hypocotyl lengths and adult phenotypes indistinguishable from those of *arf6-2 arf8-3* plants, and *nph4* P_{RPS5a}*>MIR167a* and *arf6-2 nph4 arf8-3* genotypes had phenotypes indistinguishable from each other (Supplemental Figs. S1, A–D, and S2, B–D and G). P_{RPS5a}*>MIR167a* and *nph4* P_{RPS5a}*>MIR167a* F1 plants were used for many experiments below because they did not require genotyping of individual seedlings in populations segregating for the sterile *arf6-2 arf8-3* mutant combination.

Dark-grown seedlings lacking combinations of ARF6, NPH4/ARF7, and ARF8 had shorter hypocotyls than did wild-type seedlings (Fig. 1, A and B, control data; Supplemental Fig. S1A). Hypocotyl lengths of single mutants were 80% to 90% of wild-type hypocotyl lengths, those of plants lacking two of these ARFs were

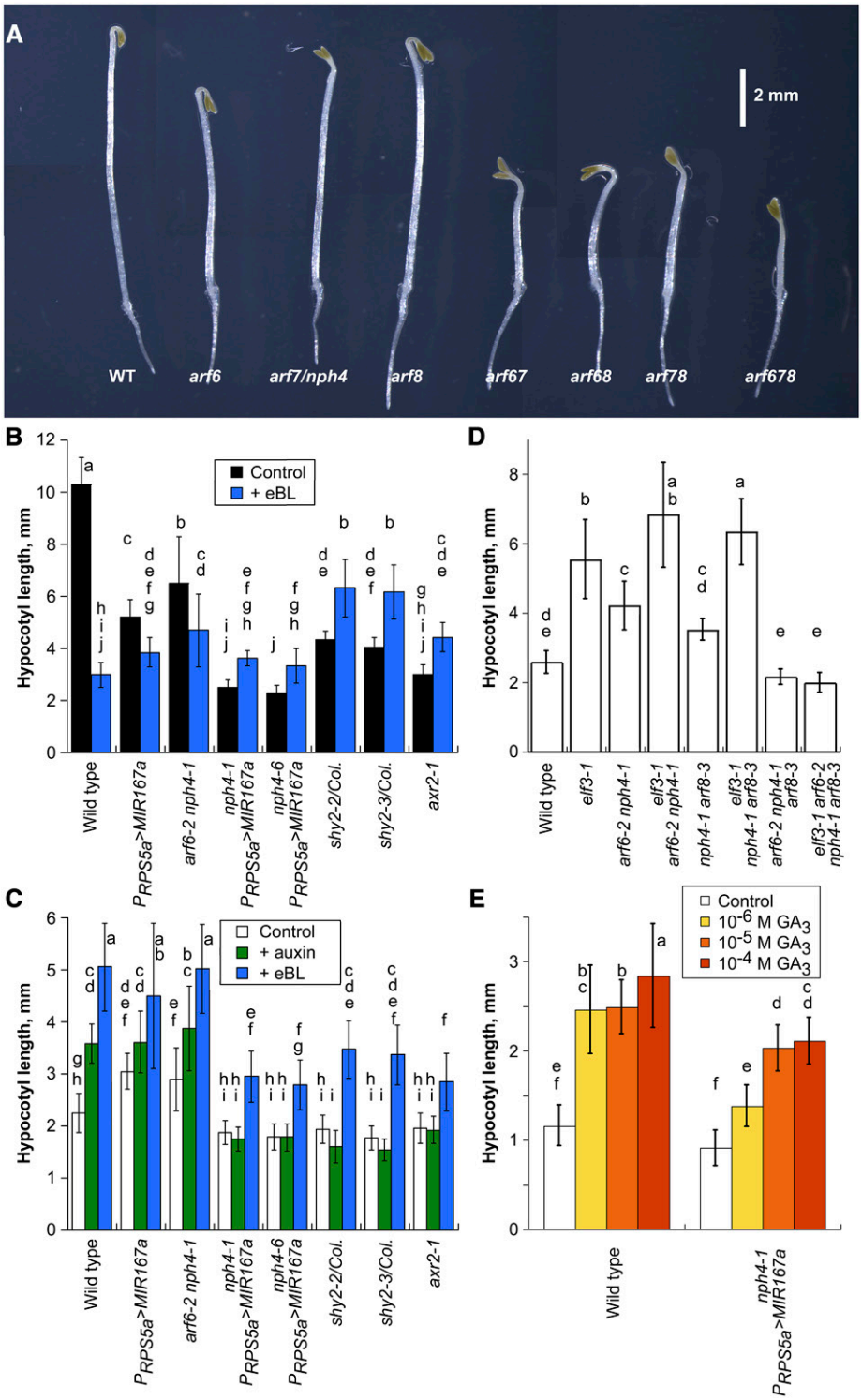

Figure 1. Hypocotyl lengths of seedlings deficient in ARFs. A, Three-day-old dark-grown seedlings of the indicated genotypes. Transactivation of *MIR167a* was used for the *arf6 arf8* and *arf6 arf7 arf8* combinations. WT, Wild type. Bar = 2 mm. B, Hypocotyl lengths of 3-d-old dark-grown seedlings of the indicated genotypes in the absence or presence of 1 μM epibrassinolide (eBL). n = 16 to 27. Supplemental Figure S1 shows data for additional genotypes without brassinolide. C, Hypocotyl lengths of 4-d-old short-day-grown seedlings of the indicated genotypes grown in the absence or presence of the synthetic auxin 30 nM 533 or 1 μM eBL. n = 17 to 26. Supplemental Figure S1 shows data for additional genotypes without added hormones. D, Effects of *elf3-1* on hypocotyl lengths of 4-d-old short-day-grown seedlings. n = 15 to 103. E, Response to GA_3. The GA biosynthesis inhibitor paclobutrazol was included on the plates at 1 μM to decrease endogenous GA production. Seeds were subjected to 6 d of cold treatment before plating to ensure germination even in the absence of new GA synthesis or exogenous GA. n = 12 to 31. In B to E, data are means ± SD, and lowercase letters indicate statistically distinguishable values as assessed by Tukey's honestly significant difference test.

50% to 80% of wild-type hypocotyl lengths, and those of plants deficient in all three ARFs were 20% to 30% of wild-type hypocotyl lengths. As found previously, dark-grown *nph4-1* seedlings had partially open apical hooks (Stowe-Evans et al., 1998). The *arf19-4* mutation did not affect elongation in the *nph4-1* or *arf6-2 nph4-1 arf8-3* background (Supplemental Fig. S1, A and C). Thus, *arf6-2 nph4-1 arf8-3* triple and *arf6-2 nph4-1 arf8-3 arf19-4* quadruple mutants had equally short hypocotyls (of 2–3 mm [i.e. 1–2 mm growth from the mature embryo]; Supplemental Fig. S1C). These results indicate that, together, ARF6, NPH4/ARF7, and ARF8

control hypocotyl elongation in darkness. Therefore, we focused further experiments on plants deficient in these three ARFs.

Dark-grown *shy2-2*, *shy2-3*, and *axr2-1* seedlings, with mutations that stabilize the Aux/IAA proteins SHY2/IAA3 or AXR2/IAA7 (Timpte et al., 1992; Reed et al., 1998; Tian and Reed, 1999; Nagpal et al., 2000), had hypocotyls either the same length as or slightly longer than those of *nph4* P_{RPS5a}*>MIR167a* seedlings (Fig. 1B). These results suggest that the stabilized Aux/IAA proteins in these mutants inhibit gene activation by ARF6, NPH4/ARF7, and ARF8 in hypocotyls, albeit incompletely in some cases.

Partial ARF Deficiency Increases Hypocotyl Elongation in Light-Grown Seedlings

In diurnal conditions, wild-type hypocotyls elongate much less than in darkness. When grown in short days at 22°C, *arf6 nph4 arf8* triple mutant, *arf6 nph4 arf8 arf19* quadruple mutant, *nph4* P_{RPS5a}*>MIR167a*, *shy2-2*, *shy2-3*, and *axr2-1* seedlings had hypocotyls only slightly shorter than, and often not statistically distinguishable from, those of wild-type seedlings (typically about 2 mm after 4 d; Fig. 1C, control data; Supplemental Fig. S1, B and D). However, even in these diurnal cycles, these mutants had shorter hypocotyls than did wild-type seedlings when subjected to some hormone treatments or environmental conditions that increase wild-type hypocotyl growth (see below).

Whereas seedlings grown in darkness had hypocotyl lengths corresponding to their *ARF6/7/8* gene dosage, short-day-grown seedlings deficient in one or two of these ARFs often had slightly longer hypocotyls than did wild-type seedlings (Fig. 1C; Supplemental Fig. S1B). These results indicate that, although *ARF6*, *NPH4/ARF7*, and *ARF8* together promote hypocotyl elongation, under day/night cycles, greater elongation occurred when ARF activity was partially compromised by knocking out just one or two of these genes. Thus, the same ARF proteins also can inhibit growth under these conditions.

We used time-lapse imaging to determine the diurnal stage at which ARF proteins may inhibit growth. In short days, hypocotyls of wild-type plants grew in the latter part of the night and early in the day, with the growth rate peaking at about dawn 2, 3, and 4 d after transfer from cold to growth conditions (Supplemental Fig. S3A; Nozue et al., 2007; Michael et al., 2008; Stewart et al., 2011). *nph4-1 arf8-3* seedlings also grew most quickly at these stages, but they had a higher maximum hypocotyl growth rate and longer growth window than wild-type seedlings during the second period of rapid growth 3 d after transfer to growth conditions (Supplemental Fig. S3A).

To explore the regulatory basis for these phenotypes, we assayed the expression levels of several auxin-responsive genes in ARF-deficient seedlings grown in short days. *nph4* P_{RPS5a}*>MIR167a* seedlings had decreased expression of the auxin-induced genes *IAA1*, *AXR2/IAA7*, *IAA19*, *SAUR19* (*SMALL AUXIN UP-RNA19*), and *SAUR63* (Fig. 2), consistent with an overall decrease in auxin response. *SAUR19* and *SAUR63* promote hypocotyl elongation (Chae et al., 2012; Spartz et al., 2012), whereas the *IAA* genes encode Aux/IAA proteins that can inhibit ARF action. These results indicate that these three ARF proteins are required for expression both of growth outputs mediated by SAUR proteins and of Aux/IAA-mediated negative feedback. The *At2g23170/GH3.3* gene, which also is auxin-inducible and encodes an auxin-amino acid-conjugating enzyme, was overexpressed in *nph4* P_{RPS5a}*>MIR167a* seedlings, and may have been activated independently of auxin response. In *arf6-2 nph4-1* and *nph4-1 arf8-3* seedlings with partial ARF deficiency, expression of *IAA1* and *AXR2/IAA7* was reduced slightly, although to a much lesser degree than in *nph4* P_{RPS5a}*>MIR167a* seedlings. In contrast, *MSG2/IAA19* was expressed at about twice the level as in wild-type seedlings (Fig. 2). *SAUR19* and *SAUR63* were expressed at similar levels in wild-type, *arf6-2 nph4-1*, and *nph4-1 arf8-3* seedlings.

ARF Proteins Are Required for Auxin-Induced Hypocotyl Elongation

We explored seedling hypocotyl elongation responses to the synthetic auxin 533, which promoted growth more strongly than did the natural auxin IAA (Savaldi-Goldstein et al., 2008). At the most effective dose (30 nM), short-day-grown wild-type seedling hypocotyl growth was stimulated by about 75% (Fig. 1C; Supplemental Fig. S4A). In contrast, auxin did not increase hypocotyl lengths of *nph4* P_{RPS5a}*>MIR167a*, *axr2-1*, or *shy2* seedlings (Fig. 1C; Supplemental Fig. S4A).

Both shade light simulated by far-red enrichment, which creates a low red:far red (R:FR) ratio, and high growth temperature increase hypocotyl elongation, in part by inducing changes in auxin levels (Gray et al., 1998; Tao et al., 2008; Keuskamp et al., 2010; Franklin et al., 2011). Indeed, *nph4* P_{RPS5a}*>MIR167a* and *arf6-2 nph4-1 arf8-3* seedlings lacked a hypocotyl elongation response to low R:FR light (Fig. 3A; Supplemental Fig. S4B). When grown at 28°C, *nph4* P_{RPS5a}*>MIR167a*, *shy2*, and *axr2-1* seedlings increased hypocotyl elongation less than half as much as did wild-type seedlings compared with growth at 22°C, although they did retain some high-temperature response (Fig. 3B).

Defects in the evening protein complex cause increased hypocotyl elongation in short days because of altered circadian rhythm outputs and are thought to arise from increased PIF expression and resultant auxin biosynthesis (Zagotta et al., 1996; Nozue et al., 2011; Nusinow et al., 2011). The *elf3-1* mutant is deficient in a component of the evening complex and also may affect PIF4 protein activity directly (Nieto et al., 2015). Consistent with the model that auxin response is required for the *elf3-1* effect on hypocotyl elongation, hypocotyls of an *elf3-1 nph4-1 arf6-2 arf8-3* quadruple mutant were as short as those of the *nph4-1 arf6-2 arf8-3* triple mutant (Fig. 1D). However,

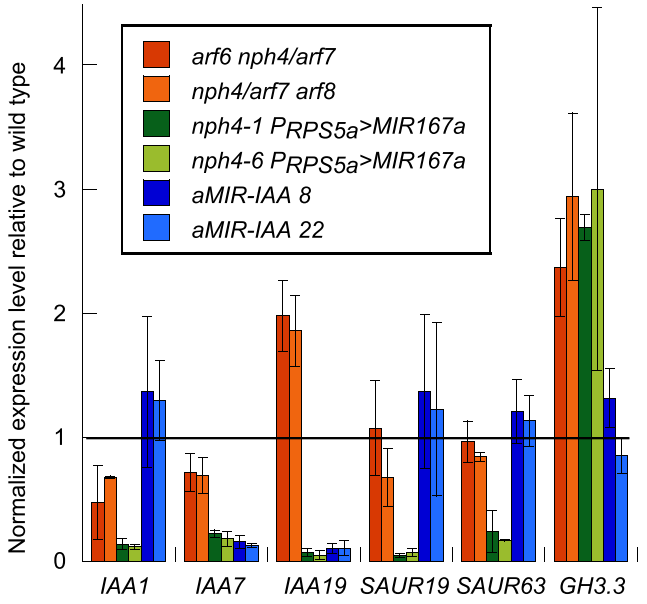

Figure 2. Gene expression in mutant seedlings. Real-time quantitative PCR (RT-qPCR) of the indicated genes is shown normalized to the *UBQ* transcript level. Tissue was harvested in the morning, 1 to 2 h after dawn. The horizontal line indicates normalized wild-type expression level of 1 for each gene. n = 2 to 3 per genotype. Error bars indicate SD.

the *elf3-1 arf6-2 nph4-1 arf8-3* quadruple mutant flowered earlier and had smaller leaves than the *arf6-2 nph4-1 arf8-3* triple mutant (Supplemental Fig. S2, E and F), indicating that ELF3 can act independently of these ARF proteins at later stages of development.

In these assays, lower order *arf* mutants retained some response. Thus, exogenous auxin and low R:FR light each stimulated hypocotyl elongation of P_{RPS5a}>*MIR167a*, *nph4 arf6*, and *nph4 arf8* seedlings, in most cases to an extent similar to that seen in the wild type (Figs. 1C and 3A; Supplemental Fig. S4, A and B). Similarly, high temperature stimulated hypocotyl elongation in *arf* double mutants to a greater extent than in the triple mutant (Fig. 3B), and *elf3-1* enhanced the long-hypocotyl phenotype of *arf6-2 nph4-1* and *nph4-1 arf8-3* double mutant combinations (Fig. 1D).

In a recent transcriptomic study, *elf3-1* seedlings overexpressed a number of auxin-regulated genes, including *SAUR63* (Ezer et al., 2017). *elf3-1 SAUR63:GUS* seedlings, with a gain-of-function form of the growth-promoting protein SAUR63 driven by its native auxin-inducible promoter (Chae et al., 2012), had hypocotyls longer than either *elf3-1* or *SAUR63:GUS* seedlings, about 3 times the length of wild-type hypocotyls (Supplemental Fig. S1E).

GA and Brassinosteroid Can Act Independently of ARF6, NPH4/ARF7, and ARF8

Although hypocotyls of seedlings deficient in ARF6, NPH4/ARF7, and ARF8 failed to elongate in response to

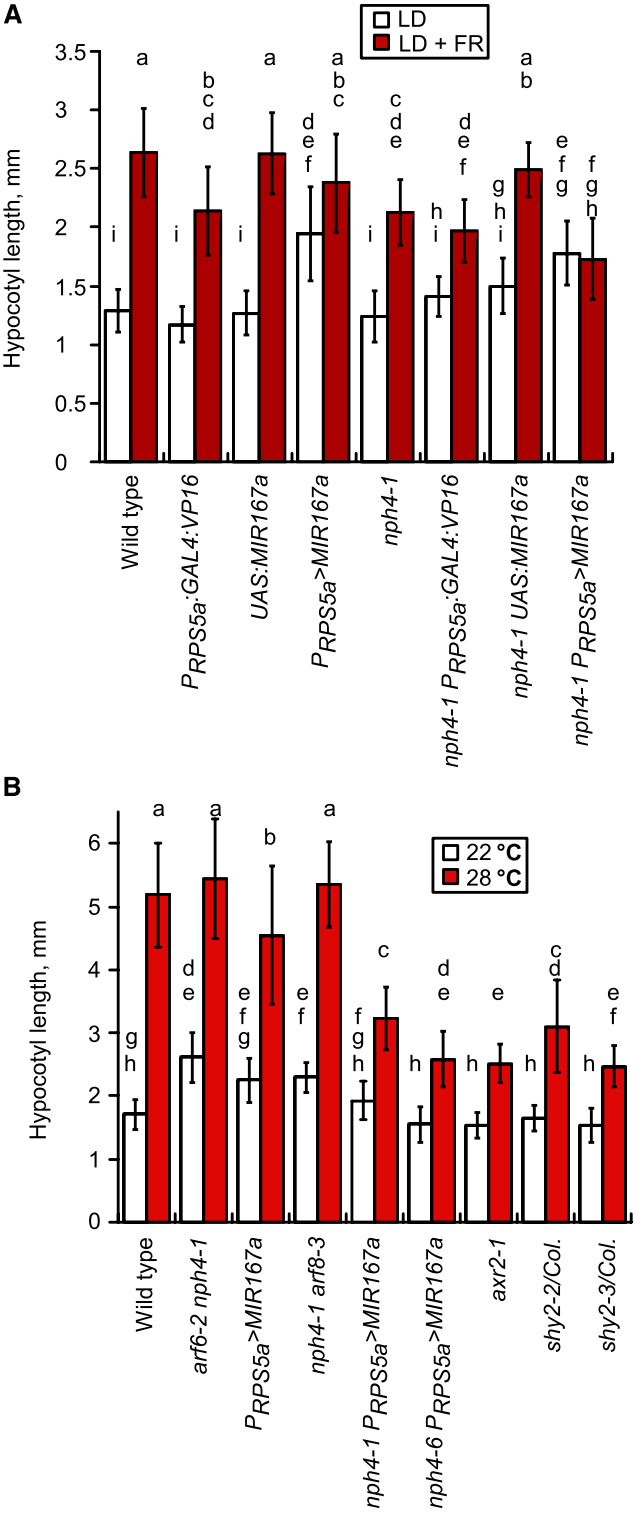

Figure 3. Hypocotyl elongation responses to treatments that affect auxin levels. A, Hypocotyl growth response to low R:FR light. LD, long days; LD + FR, long days with supplemental far-red light. n = 15 to 16. B, Hypocotyl growth after 4 short days at 22°C or 28°C. n = 17 to 29. Data are means ± SD, and lowercase letters indicate statistically distinguishable values as assessed by Tukey's honestly significant difference test.

exogenous auxin, they did elongate relative to their length in the embryo, and they elongated more in darkness than in short days, indicating that additional growth-promoting pathways were still active. The hormones brassinolide and GAs each can promote hypocotyl elongation. Exogenous eBL and GA_3 each increased elongation of short-day-grown *nph4-1* P_{RPS5a}*>MIR167a* seedling hypocotyls, although to a lesser extent than in the wild type (Fig. 1, C and E). eBL also increased hypocotyl elongation of *shy2-2*, *shy2-3*, and *axr2-1* seedlings. Conversely, the brassinosteroid biosynthesis inhibitor brassinazole (Asami et al., 2000) and the GA biosynthesis inhibitor paclobutrazol (Rademacher, 2000) each decreased hypocotyl elongation of these seedlings in short days (Fig. 1E, note the short hypocotyl lengths in the absence of GA_3; Supplemental Fig. S4D). While these inhibitors may have delayed germination, which could have decreased hypocotyl elongation at the single time points measured in these assays, taken together these results suggest that both brassinosteroid and GA response pathways are active in the ARF-deficient hypocotyls.

Whereas the concentration of exogenous eBL we used promotes hypocotyl elongation in light-grown seedlings, it inhibits elongation in wild-type dark-grown seedlings. eBL also inhibited hypocotyl elongation in dark-grown P_{RPS5a}*>MIR167a* and *nph4-1 arf6-2* seedlings deficient in two *ARF* genes. However, eBL stimulated elongation in dark-grown *nph4* P_{RPS5a}*>MIR167a*, *shy2*, and *axr2-1* seedlings (Fig. 1B). Conversely, dark-grown *nph4* P_{RPS5a}*>MIR167a* had shorter hypocotyls when grown in the presence of the inhibitor brassinazole than in its absence (Supplemental Fig. S4C).

ARF6, NPH4/ARF7, and ARF8 Affect Leaf and Inflorescence Growth

arf6 nph4 arf8 triple mutant, *nph4* P_{RPS5a}*>MIR167a*, and *arf6 nph4 arf8 arf19* quadruple mutant plants also had more severe defects in rosette leaf growth and inflorescence stem elongation than did the lower order mutants (Supplemental Figs. S2, B–D, and S5). In these respects, the triple and quadruple mutants resembled gain-of-function *axr2-1* or *shy2* plants having increased AXR2/IAA7 or SHY2/IAA3 protein level (Wilson et al., 1990; Tian and Reed, 1999). However, *arf6 nph4 arf8* and *nph4* P_{RPS5a}*>MIR167a* inflorescence stems grew upright, unlike the agravitropic inflorescences of *axr2-1* plants. *arf6 nph4 arf8* and *nph4* P_{RPS5a}*>MIR167a* plants had closed flower buds resembling those of *arf6 arf8* or P_{RPS5a}*>MIR167a* plants, and with similar final flower organ lengths (Nagpal et al., 2005; Supplemental Fig. S2, B–D and G). Thus, ARF6, NPH4/ARF7, ARF8, and ARF19 each promotes growth in leaves and inflorescences, whereas ARF6 and ARF8 are the main drivers of rapid organ growth in opening flowers.

Wild-Type Aux/IAA Proteins Inhibit Hypocotyl Elongation

ARF6, NPH4/ARF7, and ARF8 have gene activation activity but can be converted to repressors by multimerization with Aux/IAA proteins (Ulmasov et al., 1999a; Tiwari et al., 2003; Han et al., 2014; Korasick et al., 2014; Nanao et al., 2014; Dinesh et al., 2015). Auxin relieves this repression by stimulating turnover of Aux/IAA proteins. To assess how *IAA* genes regulate hypocotyl elongation, we generated plants overexpressing an artificial microRNA designed to target multiple *IAA* genes (Fig. 4A). We tested expression of eight representative *IAA* genes by RT-qPCR in 4-d-old seedlings of two stable single-locus P_{35S}*:aMIR-IAA* lines. *AXR2/IAA7*, *IAA8*, *AXR3/IAA17*, and *MSG2/IAA19* transcript levels were each lower in P_{35S}*:aMIR-IAA* than in wild-type seedlings (Figs. 2 and 4H). In contrast, *AXR5/IAA1*, *SHY2/IAA3*, *IAA9*, and *IAA26* transcript levels were similar between wild-type and P_{35S}*:aMIR-IAA* seedlings. The apparent increase in *IAA26* expression could potentially reflect a compensatory negative feedback leading to increased expression of some *IAA* genes. Consistent with these expression data, P_{35S}*:aMIR-IAA* suppressed the morphological phenotypes of the *axr2-1* gain-of function mutation in *AXR2/IAA7* but did not appreciably suppress the phenotypes of the *shy2-3* mutation in *SHY2/IAA3* (Fig. 4, C–G). The growth-promoting genes *SAUR19* and *SAUR63* had normal expression levels in *aMIR-IAA* seedlings (Fig. 2).

In the wild-type, *arf6-2*, *nph4-1*, and *arf8-3* backgrounds, P_{35S}*:aMIR-IAA* increased hypocotyl elongation in short days, indicating that Aux/IAA proteins were inhibiting elongation (Fig. 4I). Time-lapse measurements of growth indicated that the increase in growth rate occurred primarily surrounding dawn 3 d after transfer from cold to growth conditions, as was observed for *nph4/arf7-1 arf8-3* (Supplemental Fig. S3B). P_{35S}*:aMIR-IAA* seedlings did elongate in response to exogenous auxin (Supplemental Fig. S4A), suggesting that additional Aux/IAA proteins are present and could regulate ARFs in these plants.

We found previously that the *axr2/iaa7-5* loss-of-function allele in ecotype Wassilewskija had a slightly longer hypocotyl than did wild-type plants (Nagpal et al., 2000). Unlike the Wassilewskija mutant, the Columbia mutant *axr2/iaa7-7* had a similar hypocotyl length to wild-type seedlings when grown in short days. However, hypocotyls of *axr2/iaa7-7 axr3/iaa17-7* double mutant seedlings were longer than those of wild-type seedlings and as long as those of *aMIR-IAA* seedlings (Fig. 4I). Thus, decreased AXR2/IAA7 and AXR3/IAA17 activity can account for the increased hypocotyl growth of *aMIR-IAA* seedlings, although decreased levels of other Aux/IAA proteins also may contribute.

DISCUSSION

Our results show that ARF6, NPH4/ARF7, and ARF8 together account for the hypocotyl elongation response to auxin. They likely activate genes in hypocotyl epidermal cells, including *SAUR* genes, cell

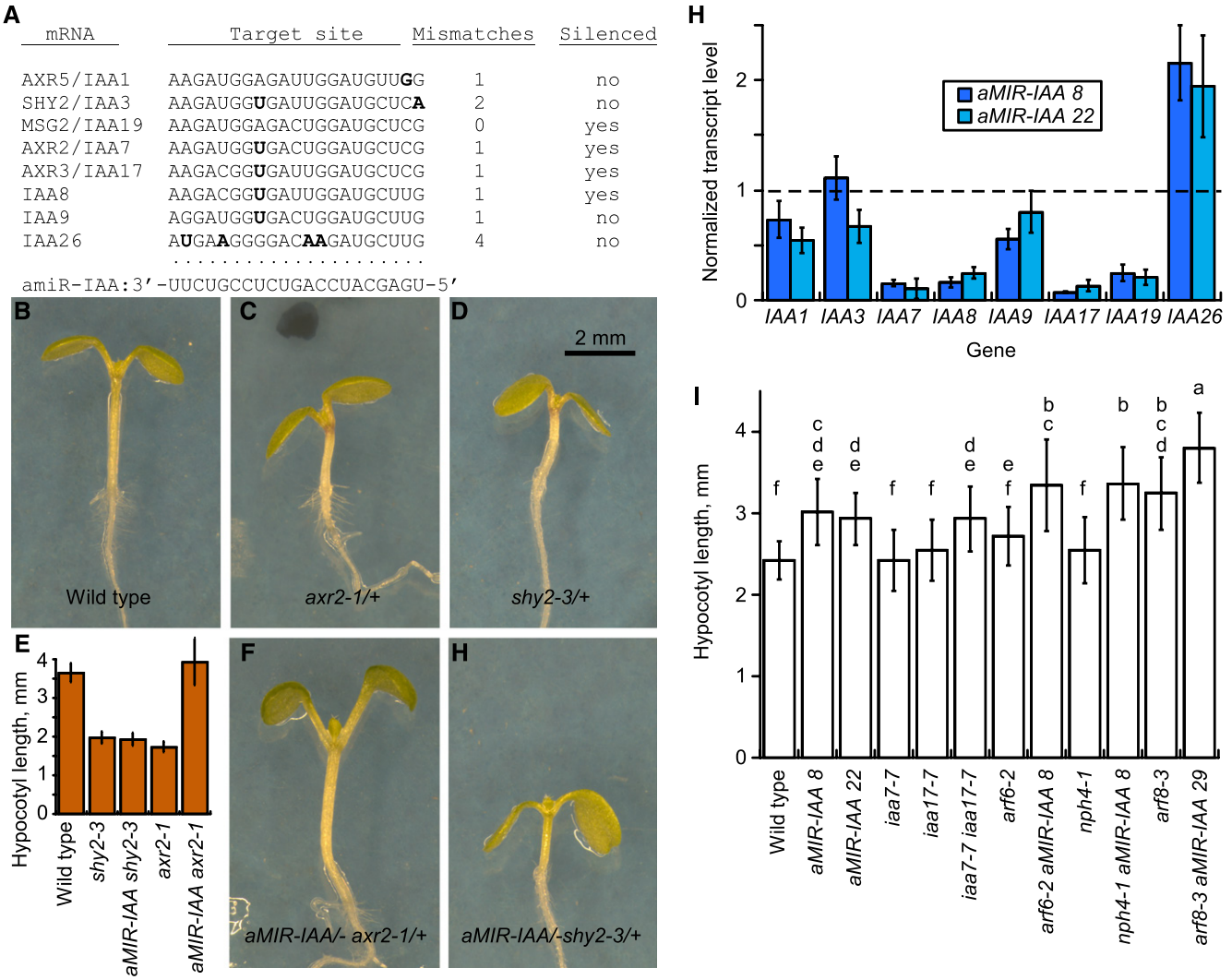

Figure 4. *aMIR-IAA* plants. A, Predicted amiR-IAA sequence base pairing to selected *IAA* genes. G:U base pairs are allowed. *IAA* gene mRNA sequences are shown 5′ to 3′. Bases not expected to pair with aMIR-IAA are indicated in boldface. B to D, F, and G Photographs of 7-d-old seedlings of the indicated genotypes. E, Mean hypocotyl lengths of the seedlings shown in B to D, F, and G, showing suppression of *axr2-1* but not *shy2-3*. $n = 9$ to 13. Error bars indicate SD. H, RT-qPCR of *IAA* genes in two different P_{35S}:*aMIR-IAA* lines. $n = 3$. Error bars indicate SD. The horizontal dashed line indicates a normalized wild-type expression level of 1 for each gene. I, Hypocotyl lengths in short days. Error bars indicate SD. $n = 13$ to 46.

wall-loosening functions, and genes acting in GA and brassinosteroid biosynthesis or signaling pathways (Chapman et al., 2012; Procko et al., 2016).

ARF-regulated hypocotyl elongation appears to be most important under specific environmental conditions. For example, ARF-deficient seedlings grew very little in darkness, lacked the shade-avoidance response to far-red enrichment, and had a decreased response to high temperature. Consistent with these results, both far-red-enriched light and high temperature can increase endogenous auxin production in cotyledons and increase hypocotyl elongation (Gray et al., 1998; Tao et al., 2008; Koini et al., 2009; Franklin et al., 2011; Li et al., 2012; Sun et al., 2012; Zheng et al., 2016). In contrast, ARF-deficient seedlings had almost normal hypocotyl growth in diurnal cycles at 22°C under fluorescent light. Thus, for hypocotyl elongation, the auxin response might act mainly under conditions that cause particularly rapid growth. Similarly, ARF6 and ARF8 promote petal and stamen filament growth just before flowers open, when these organs elongate especially quickly (Nagpal et al., 2005; Tabata et al., 2010; Reeves et al., 2012).

As the *arf* mutants could respond to both brassinosteroid and GA, it is likely that the responses to these two hormones are adequate for baseline hypocotyl

growth under diurnal conditions. ARF6 can interact with PIF4 and BZR1 proteins, suggesting that, at many promoters, complexes of BES/BZR, ARF, and PIF proteins together activate genes required for growth (Oh et al., 2014). Indeed, ARF-deficient seedlings responded less to eBL or GA_3 than did wild-type seedlings, suggesting that full BZR/BES and PIF effects may require auxin response (Nemhauser et al., 2004; Zhou et al., 2013). Conversely, ARFs are probably not sufficient on their own to activate maximal hypocotyl elongation in the absence of PIF and BES/BZR factors, because higher order *bes/bzr* and *pif* mutants also have short hypocotyls in darkness and in diurnal conditions (Yin et al., 2005; Leivar et al., 2008, 2012; Shin et al., 2009), and inhibition of brassinosteroid or GA biosynthesis also reduces hypocotyl elongation.

Brassinolide inhibited hypocotyl elongation of wild-type seedlings in darkness but promoted elongation in light. In contrast, it promoted elongation of hypocotyls of ARF-deficient seedlings grown under either condition. Exogenous brassinolide or increased brassinolide signaling also promoted hypocotyl elongation in GA-deficient seedlings (Bai et al., 2012; Gallego-Bartolomé et al., 2012). In each of these cases, when the hypocotyls were short, brassinolide promoted elongation. These results raise the possibility that the brassinolide response may depend on the growth state of the seedling rather than on the light environment per se.

ARFs also were required for the long hypocotyl growth in *elf3-1* seedlings deficient in the evening complex. Epistasis of the *nph4-1 arf6-2 arf8-3* triple mutation combination to the *elf3-1* mutation for hypocotyl elongation is consistent with the model that *elf3-1* acts by increasing auxin response in hypocotyls (Nozue et al., 2011; Nusinow et al., 2011).

Seedlings lacking combinations of just two of the three ARFs and seedlings deficient in multiple *IAA* genes each had longer hypocotyls than did wild-type seedlings in short days. These results support the model that ARF-Aux/IAA protein complexes may normally limit expression of growth-promoting genes under diurnal conditions (Fig. 5). The C-terminal domains of ARF6, NPH4/ARF7, and ARF8 each can interact strongly with Aux/IAA proteins (Vernoux et al., 2011; Piya et al., 2014). Under conditions of increased auxin level, turnover of Aux/IAA proteins would normally convert these repressing ARF-Aux/IAA complexes to activating ARF complexes. Partial ARF deficiency may increase expression of growth-promoting target genes through a combination of decreased ARF-Aux/IAA complex repression and increased recruitment or activity of PIF, BZR/BES, or other activating factors that the missing ARF-Aux/IAA complexes would normally counteract (Fig. 5C). *arf* double mutants with slightly elongated hypocotyls had slightly increased or decreased levels of different *IAA* gene transcripts, suggesting that feedback-regulated *IAA* expression levels may buffer changes in ARF levels. Such feedback also

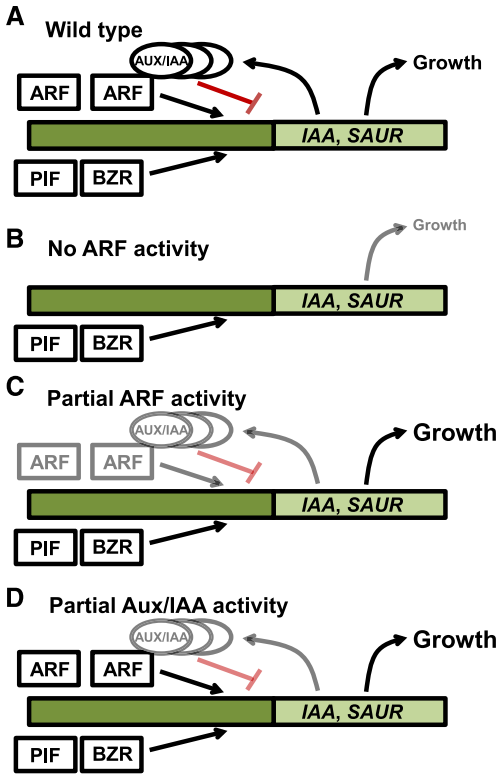

Figure 5. Conceptual model for ARF-Aux/IAA control of hypocotyl growth. A, In wild-type seedlings, ARF, PIF, and BZR/BES transcription factors regulate expression of growth-promoting genes such as *SAUR* genes and of *IAA* genes encoding Aux/IAA repressors of transcription. When the auxin level is high, Aux/IAA proteins are turned over and ARF transcription factors can activate gene expression. When auxin levels are low, ARF-Aux/IAA protein complexes are more persistent and repress gene expression. B, In the absence of ARF6, NPH4/ARF7, and ARF8, elongation depends primarily on PIF and BZR/BES factors and is reduced in darkness and other conditions that require an auxin response. C, An intermediate ARF level, as in *arf* double mutants, may decrease both gene activation by ARF transcription factors and gene repression by ARF-Aux/IAA complexes. Levels of *IAA* gene transcription may be increased or decreased depending on the gene. These combined effects produce an altered regulatory balance. In darkness, this decreases growth, whereas in diurnal conditions, the balance of gene expression favors slightly increased growth. Additional feedbacks and non-cell-autonomous effects such as PIF regulation of auxin biosynthesis genes likely also contribute to the final output. D, A reduced Aux/IAA level, as in *aMIR-IAA* plants, decreases repression by ARF-Aux/IAA complexes, leading to increased ARF activation of *SAUR* genes and slightly increased growth.

may rewire auxin responsiveness under persistent shade conditions, leading to altered steady-state levels of *IAA* gene transcription (Pucciariello et al., 2018). The mechanisms may also involve genes not assayed here, or more subtle changes in the kinetics or tissue-specific expression of relevant target genes. Partial ARF deficiency can cause increased growth in both hypocotyls and fruits (Tian et al., 2004; Goetz et al., 2006; de Jong et al., 2009).

The elongated hypocotyls of *axr2/iaa7 axr3/iaa17* double loss-of-function mutants are consistent with

AXR2/IAA7 and AXR3/IAA17 each regulating ARF activity in hypocotyls (Fig. 5D). However, these seedlings still responded to auxin, so other *IAA* genes may also contribute to hypocotyl growth control. New genome-engineering technologies may make it feasible to knock out more *IAA* genes at once to reveal more fully their roles in growth control and the integration of environmental and stress stimuli (Shani et al., 2017). Mutants of the moss *Physcomitrella patens* lacking multiple *IAA* genes have a constitutively active auxin response (Lavy et al., 2016).

Collectively, ARF6, NPH4/ARF7, ARF8, and ARF19 also regulate growth of inflorescence stems, leaves, and flower organs. Moreover, the phenotypes of higher order *arf* mutants resemble many of those seen for mutants with gain-of-function mutations in *IAA* genes that decrease the turnover of the corresponding Aux/IAA protein (Reed, 2001). Our results here thus validate the notions that those *iaa* mutant phenotypes arose primarily from inhibition of ARF-mediated gene activation, and that use of such mutant *IAA* genes as tools in ectopic expression studies reveals functions of ARFs in affected tissues (Procko et al., 2016). The *MIR167a* transactivation system described here will also be a useful tool for further studies of auxin responses and of brassinolide or GA responses in the absence of confounding auxin responses.

MATERIALS AND METHODS

Plant Genotypes

All Arabidopsis (*Arabidopsis thaliana*) genotypes were in the Columbia background. Mutations *arf6-2*, *arf8-3*, *arf19-4*, *axr2-1*, *elf3-1*, *nph4-1*, and *nph4-6* have been described previously (Wilson et al., 1990; Zagotta et al., 1996; Harper et al., 2000; Nagpal et al., 2000, 2005; Wilmoth et al., 2005). *shy2-2* and *shy2-3* mutations were originally identified in Landsberg *erecta* (Tian and Reed, 1999) and were introgressed for eight and nine generations, respectively, into Columbia for experiments in this work. *axr2/iaa7-7* (SALK_089809) and *axr3/iaa17-7* (SALK_011820; Alonso et al., 2003; http://signal.salk.edu/cgi-bin/tdnaexpress) were obtained from the Arabidopsis Biological Resource Center at Ohio State University. Plants expressing the *GAL4:VP16* fusion gene encoding a strong transcriptional activator behind the P_{RPS5a} promoter were described previously (Weijers et al., 2003). P_{RPS5a}:*GAL4:VP16* driver line 5 conferred stronger phenotypes than line 10 when crossed with *UAS:MIR167a* responder lines. *UAS:MIR167a* was constructed by inserting the *MIR167a* sequence (Wu et al., 2006) into pSDM7006 (Weijers et al., 2003), and the resulting construct was transformed into Columbia plants by the floral dip method (Clough, 2005). We characterized two single-locus *UAS:MIR167a* insertion lines and found that line 9 gave stronger phenotypes than line 2 when crossed with P_{RPS5a}:*GAL4:VP16* driver lines. F1 progeny of crosses between the stronger driver and responder lines were used for the experiments presented. We refer to these F1 plants carrying both transgenes as P_{RPS5a}>*MIR167a*. These transgenes were crossed into *nph4* mutant backgrounds, and *nph4* P_{RPS5a}:*GAL4:VP16* driver and *nph4 UAS:MIR167a* responder lines were then crossed to each other to obtain *nph4* P_{RPS5a}>*MIR167a* seedlings.

The artificial microRNA sequence against *IAA* genes was engineered in the *MIR167a* precursor backbone (Wu et al., 2006) by site-directed mutagenesis using primers listed in Supplemental Table S1 to replace the miR167 and miR167* sequences with amiR-IAA and amiR-IAA* sequences. *aMIR-IAA* then was cloned into pB2GW7 behind the P_{35S} promoter (Karimi et al., 2002), and transgenic lines were generated by floral dip.

Phenotypic Analyses

For hypocotyl length measurements, Arabidopsis seedlings were grown on petri plates containing 0.5× Murashige and Skoog salts (Murashige and Skoog, 1962) and 0.6% (w/v) phytoagar (Research Products International) without Suc. Hormones or inhibitors were diluted from stocks dissolved in DMSO (533), ethanol (GA_3 or brassinazole), or methanol (paclobutrazol) and added to plates when they were poured. Surface-sterilized seeds were given 1 to 2 d of cold treatment before incubation under growth conditions. Plates were oriented vertically in a growth chamber at 22°C or 28°C. For dark growth experiments, seedlings were first exposed to light for 6 to 18 h to induce germination, and hypocotyls were measured after 3 d of growth in darkness at 22°C. For short-day conditions, plates were incubated under an 8-h-light/16-h-dark photoperiod (100–120 µmol m^{-2} s^{-2}), and hypocotyl lengths were measured after 4 d unless indicated otherwise. Under diurnal conditions, most growth was achieved after 4 d (Supplemental Fig. S3). Images of seedlings were scanned, and hypocotyl lengths were then measured using ImageJ (Abramoff et al., 2004). For shade-avoidance assays, seeds were sown directly onto soil (Primasta potting substrate) and stratified for 4 d at 4°C in darkness. Seedlings were then grown for 4 d in a 16/8-h (light/dark) photoperiod at 130 µmol m^{-2} s^{-1} white light (R:FR ratio = 1.8; Philips HPI 400 W) before transfer to low R:FR light (R:FR ratio = 0.15; Philips HPI 400 W supplemented with Philips Green Power FR LED research modules) or control white light conditions and measured after a further 3 d. In cases where segregating populations were assayed, seedling genotypes were assessed subsequently either by diagnostic PCR assays or by the characteristic closed-flower-bud phenotype of *arf6 arf8* mutant plants (Nagpal et al., 2005).

For time-lapse imaging of hypocotyl elongation, seedlings were grown on vertically oriented 1× Murashige and Skoog plates and imaged over time using near-infrared image detection following Brooks et al. (2010) with slight modifications. In particular, images were acquired using an AVT Guppy F-146 Mono CCD Camera (Edmund Optics) and flexible arm-mounted LED lights (LED890-66-60, 890-nm peak wavelength IR LEDs from Roithner Lasertechnick, with heatsinks [47 × 20 TO-66]) instead of back lighting. Hypocotyl lengths were measured for images taken every 36 min using ImageJ, starting with the earliest time point for which hypocotyl length could be measured. Hypocotyl lengths at each time point for six seedlings per genotype (or fewer for the very first time points) were averaged and then smoothed by averaging over a window of five successive time points centered at each time point. Data were converted to rates by subtracting successive smoothed values, normalized to length and time scales, and graphed.

Flower organs and rosettes were measured as described previously (Nagpal et al., 2005; Wilmoth et al., 2005; Reeves et al., 2012). RT-qPCR was performed as described previously (Reeves et al., 2012) using the primers listed in Supplemental Table S1.

Accession Numbers

The Arabidopsis Genome Initiative numbers of featured genes are as follows: *ARF6* (At1g30330), *NPH4/ARF7* (At5g20730), *ARF8* (At5g37020), *ARF19* (At1g19220), *AXR5/IAA1* (At4g14560), *SHY2/IAA3* (At1g04240), *AXR2/IAA7* (At3g23050), *IAA8* (At2g22670), *IAA9* (At5g65670), *AXR3/IAA17* (At1g04250), *MSG2/IAA19* (At3g15540), *IAA26* (At3g16500), *ELF3* (At2g25930), *SAUR19* (At5g18010), *SAUR63* (At1g29440), and *GH3.3* (At2g23170).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Hypocotyl lengths of seedlings with altered auxin response.

Supplemental Figure S2. Adult phenotypes of P_{RPS5a}>*MIR167a* transactivated plants and *arf* mutants.

Supplemental Figure S3. Time-lapse growth rates of seedlings grown in short days.

Supplemental Figure S4. Hormone responses of *arf* mutant seedlings.

Supplemental Figure S5. Rosettes of plants with decreased auxin response.

Supplemental Table S1. Primers used in this work.

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