Specificity in auxin responses is not explained by the promoter preferences of activator ARFs

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One Sentence Summary
The plant growth hormone auxin regulates development via a family of transcription factors that share promoter sequence preferences, despite activating different genetic networks.

Dear Editors,

AUXIN RESPONSE FACTOR (ARF) transcription factors diverged basally in evolution (Mutte et al., 2018) and there is evidence that they provide specificity in auxin-regulated genetic networks (e.g., Krogan et al., 2016; Wilmoth et al., 2005). Studies contrasting the binding behavior of Class A ("activator") and Class B ("repressor") ARFs (Boer et al., 2014; O’Malley et al., 2016; Galli et al., 2018) found distinct promoter preferences between classes; however, there was not strong evidence for differences within each class. This finding might reflect limits of the DAP-sequencing method, such as that caused by the required clustering of many promoters according to investigators’ hypotheses about functional features, or the fact that transcription factor binding and activation are frequently decoupled (Para et al., 2014).

To complement past studies, we tested the activation profiles of Class A ARFs (specifically the AtARF5/ZmARF4/ZmARF29 clade and the AtARF19/ZmARF27 clade) from Arabidopsis thaliana and maize (Zea mays) on standardized synthetic promoter variants in yeast (Saccharomyces cerevisiae) (Figure 1A) (Pierre-Jerome et al., 2014). Promoter variants were
inserted into the pIAA19 promoter with mutated AuxREs. None of the ARFs tested can activate transcription to any appreciable extent on this promoter (Supplemental Figure S1). Through these analyses, we discovered several ARF-activated promoter design rules. First, activation strength was directly proportional to Auxin Response Element (AuxRE) copy number (Figure 1B). Second, ARFs activated more strongly on two AuxREs facing towards each other rather than away from each other (Figures 1C, D). Only AtARF19 showed appreciable activation on two AuxREs facing away from each other. Third, orientation relative to the TSS mattered. AtARF19 activated slightly more strongly on two AuxREs facing towards the TSS as opposed to away from the TSS (Figure 1E). No other tested ARFs activated on two AuxREs facing away from the TSS, though ZmARF27 and ZmARF29 activated on two AuxREs facing towards the TSS (Figure 1F). These design rules suggest that ARFs exhibit conserved preferences for cis-element orientation and number.

In addition to number and orientation, the sequence of AuxREs impacted the magnitude of ARF-mediated activation. The first described AuxRE was the six-mer TGTCTC (Ballas et al., 1993); however, later work revealed flexibility in the fifth and sixth position. AtARF1 and AtARF5 bind more strongly to the AuxRE TGTCGG than to TGTCTC (Boer et al., 2014). Our assays reveal that activation strength follows this binding preference, with each ARF tested activating more strongly on two AuxREs facing towards each other of the sequence TGTCGG (Figure 2A). The nearly nine-fold increase in AtARF5 activity was particularly dramatic, and is of relevance for the design and interpretation of auxin reporters. DR5v2, which uses TGTCGG (Liao et al., 2015), may over-report responses driven by AtARF5 relative to other ARFs.

To define a minimal auxin-responsive promoter, we tested whether any of the ARFs could activate on a single AuxRE of the sequence TGTCGG, and found that only AtARF19 was able to activate on this single AuxRE (Figure 2B). In fact, AtARF19 activated almost as strongly on a
promoter with a single AuxRE of this sequence as that observed on promoters with two
AuxREs. ARFs are thought to require dimerization through their N-terminal dimerization domain
(DD) to bind DNA (Boer et al., 2014). We tested whether dimerization through either the DD or
the C-terminal Phox and Bem1 (PB1) domain (Korasick et al., 2014; Nanao et al., 2014)
impacted AtARF19 activation on a promoter with a single AuxRE (Figure 2C and 2D), as
mutations in either of these domains reduce AtARF19 activity on promoters with multiple
AuxREs (Pierre-Jerome et al., 2016). The DD mutations caused a severe loss of activity
comparable to a mutation in the DNA-binding domain itself (Figure 2E). The effect of the DD
mutations was less severe on promoters with two AuxREs, suggesting a compensatory effect of
multiple binding sites. The mechanism through which an ARF dimer binds to a single-AuxRE
promoter is unknown. One possibility is that a single ARF-AuxRE contact provides sufficient
stability to allow activation and the other ARF in the dimer forms transient interactions with DNA
sequences that serve as low-affinity binding sites. Alternatively, the two ARFs within a dimer
pair could ‘trade places’ on the single AuxRE, providing sufficient occupancy time to allow for
activation.

Sequence alignments revealed a difference within the DD of AtARF19 and its maize homolog
(Figure 2C, D). To test if this single residue difference, so close to the contact residues within
the DD (Figure 2D), could explain AtARF19’s unique ability to activate on a single AuxRE, we
mutated the AtARF19 DD to the ZmARF27 sequence (N256A). This mutation abolished
AtARF19 activity on a single AuxRE, whereas activity on two AuxREs was essentially
unchanged (Figure 2F). The polarity of the asparagine may help stabilize the dimeric form of
AtARF19, leading to higher transcriptional activation and expanding the range of promoters it
can activate. Whereas N256 is necessary for AtARF19’s ability to activate on promoters with a
single AuxRE, it is not sufficient. AtARF7, which shares the same asparagine residue in its DD,
cannot activate on a single AuxRE (Supplemental Figure S2), suggesting that inter-domain
interactions may be critical for overall ARF function.

It has been widely speculated that specificity in ARF-promoter interactions is responsible for the
diversity of auxin responses. Our results suggest that this model is unlikely to be true, and
suggests that factors outside the core auxin response machinery may provide that function.
Closely spaced AuxREs are found only rarely in the Arabidopsis genome (Grigolon et al., 2018),
and frequently are neither of the ideal sequence nor in the ideal orientation relative to the TSS.
The rarity of “ideal” promoters may allow for integration of signals from multiple pathways, a
hypothesis supported by the enrichment of transcription factor binding sites for other proteins in
auxin-responsive promoters (Berendzen et al., 2012; Cherenkov et al., 2018; Mironova et al.,
2014). Future efforts that combine synthetic and native approaches will be needed to pinpoint
the combination of factors that make up the “auxin code”, as well as to make it possible to
retrace the evolutionary path that connected novel auxin response modules to diversity in plant
form and function.

Supplemental Material
Materials and Methods
Acknowledgements
Author contributions
Two supplemental figures:
Supplemental Figure S1. Arabidopsis and maize ARFs do not activate on mpIAA19.
Supplemental Figure S2. Arabidopsis ARF7 does not activate on a single AuxRE.

Figure Legends
Figure 1. Arabidopsis and maize ARFs share promoter preferences. A) Schematic of yeast engineered to constitutively express ARF proteins and promoter variants. The transcription start site (TSS) is to the right and arrowheads indicate the orientation of the AuxRE, starting with 5′-TGTC-3′. Fluorescence was measured by flow cytometry with the results depicted as median values and 95% confidence intervals. Twenty thousand events (cells) were measured for each replicate. B) AtARF19 and AtARF5 show strong activation on promoters with four AuxREs (five base pair spacer of the sequence CCTTT). C) AtARF19 and AtARF5 show stronger activity on promoters with two AuxREs facing towards each other rather than away from each other (seven base pair spacer of the sequence CCAAAGG). D) ZmARF4, ZmARF27, and ZmARF29 show stronger activity on promoters with two AuxREs facing towards each other rather than away from each other (seven base pair spacer of the sequence CCAAAGG). E) AtARF19 and AtARF5 show stronger activity on promoters where the two AuxREs face towards rather than away from the TSS (five base pair spacer of the sequence CCTTT). F) ZmARF4, ZmARF27, and ZmARF29 show stronger activity on promoters where the two AuxREs face towards rather than away from the TSS (five base pair spacer of the sequence CCTTT).

Figure 2. The AuxRE sequence TGTCGG strengthens ARF activity and allows for AtARF19 activity on a single cis-element. Fluorescence was measured by flow cytometry with the results depicted as median values and 95% confidence intervals. Twenty thousand events (cells) were measured for each replicate. A) All ARFs tested activate more strongly on two AuxREs facing each other of the cis-element sequence TGTCTC/GAGACA when compared to two AuxREs facing each other of the cis-element sequence TGTCGG/CCGACA. B) Only AtARF19 of the ARFs tested can induce transcription on a promoter with one AuxRE of the sequence 5′-TGTCGG-3′. (The no ARF control data point is directly underneath the ZmARF4 data point). C) Alignment of the DNA-binding domains and dimerization domains (DDs) of AtARF19 and ZmARF27 with relevant mutations highlighted. D) Structure of ARF5 DNA-binding
domain with mutated residues highlighted. E) AtARF19 must dimerize for full activity, even for a promoter with a single AuxRE. The KO triple mutation (K962A; D1012A; D1016A) disrupts dimerization in the PB1 domain. The A250N and G247I mutations disrupt dimerization at the DD, adjacent to the DNA-binding domain. The H138A mutation disrupts the DNA-binding domain itself. F) An N256A mutation in AtARF19 causes a total loss of activity on a promoter with one AuxRE (5′-TGTCGG-3′), whereas activity on two AuxREs was largely intact.

Supplemental Figure S1. Arabidopsis and maize ARFs do not activate on mpIAA19. A) Fluorescence was measured by flow cytometry with the results depicted as median values and 95% confidence intervals. Twenty thousand events (cells) were measured for each replicate. Activity of AtARF5 and AtARF19 on the mpIAA19 promoter, with all the AuxREs mutated. B) Activity of ZmARF4, ZmARF27, and ZmARF29 on the mpIAA19 promoter, with all the AuxREs mutated.

Supplemental Figure S2. Arabidopsis ARF7 does not activate on a single AuxRE. Fluorescence was measured by flow cytometry with the results depicted as median values and 95% confidence intervals. Twenty thousand events (cells) were measured for each replicate. Despite a conserved asparagine shared with AtARF19 within the dimerization domain, AtARF7 does not activate on a single AuxRE of the sequence 5′-TGTCGG-3′, but activates on two AuxREs of this sequence facing each other.

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Figure 1 Arabidopsis and maize ARFs share promoter preferences. A) Schematic of yeast engineered to constitutively express ARF proteins and promoter variants. All promoter variants were inserted into the A1 site of a pIAA19 promoter with mutated AuxREs. None of the ARFs tested can activate transcription to any appreciable extent on this promoter (Supplemental Figure S1). The transcription start site (TSS) is to the right and arrowheads indicate the orientation of the AuxRE, starting with 5'-TGTC-3'. Fluorescence was measured by flow cytometry with the results depicted as median values and 95% confidence intervals. B) AtARF19 and AtARF5 show strong activation on promoters with four AuxREs (five base pair spacer of the sequence CCTTT). C) AtARF19 and AtARF5 show stronger activity on promoters with two AuxREs facing towards each other rather than away from each other (seven base pair spacer of the sequence CCAAAGG). D) ZmARF4, ZmARF27, and ZmARF29 show stronger activity on promoters with two AuxREs facing towards each other rather than away from each other (seven base pair spacer of the sequence CCAAAGG). E) AtARF19 and AtARF5 show stronger activity on promoters where the two AuxREs face towards rather than away from the TSS (five base pair spacer of the sequence CCTTT). F) ZmARF4, ZmARF27, and ZmARF29 show stronger activity on promoters where the two AuxREs face towards rather than away from the TSS (five base pair spacer of the sequence CCTTT).
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