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SHORT COMMUNICATION

The role of *AUXIN RESPONSE FACTOR*s in the development and differential growth of inflorescence stems

Nicholas S. Carey and Naden T. Krogan

Department of Biology, American University, Washington DC, USA

ABSTRACT

Plants react to environmental cues by altering their growth and development, which can include organ tropic responses. These differential growth responses are triggered by the hormone auxin, and *AUXIN RESPONSE FACTOR*s (*ARF*s) have been implicated in numerous organ tropisms in *Arabidopsis thaliana*. Surprisingly, despite being critical for light capture and overall plant morphology, inflorescence stem tropic responses remain relatively understudied, with presumed direct links to *ARF* function yet to be established. Here, we show that the expression patterns of *ARF5/MONOPTEROS* and *ARF7/NONPHOTOTROPIC HYPOCOTYL4* are consistent with roles in inflorescence stem tropisms. Mutation of these factors does not alter inflorescence stem responses to gravity or unilateral auxin application, meaning their participation in these processes is presumably masked by functional redundancies. Future resolution of these redundancies will likely require higher order *arf* mutant combinations, guided by detailed expression analyses of *ARF*s in the inflorescence stem.

Abbreviations: ARF, Auxin Response Factor; DAG, days after germination; DEX, dexamethasone; GR, glucocorticoid receptor; MP, MONOPTEROS; NPH4, NONPHOTOTROPIC HYPOCOTYL4; PIN, PIN-FORMED

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As sessile organisms, higher plants need to continuously assess their surroundings to optimize their growth and development. This includes tropic (or differential) growth of organs in response to environmental cues such as light and gravity. Plant stems generally exhibit positive phototropism and negative gravitropism (upward growth) to facilitate the harvesting of light for photosynthesis, while roots display positive gravitropism (downward growth) to acquire nutrients and water and to anchor the plant.[1]

Distinct stages of growth tropisms have been identified.[1,2] For instance, gravitropism begins with gravity perception within specific tissues that harbor starch-rich plastids termed amyloplasts. Amyloplasts are assumed to function as statoliths, which sediment in response to gravity and provide information regarding the optimal direction of organ growth.[3] In stems, the endodermis is an amyloplast-containing cell layer believed to be critical in gravitropism. This notion is supported by mutants defective in endodermis formation, which produce stems with abnormal gravitropic responses.[4] The second stage of gravitropism involves the transduction of information provided by statolith sedimentation into a signal, namely the asymmetric lateral distribution of the phytohormone auxin across the stem or root. This asymmetric auxin localization results in the third stage of the tropic response, organ bending, which occurs via differential cell elongation.[3]

Genetic analyses of *Arabidopsis thaliana* have identified various factors that mediate tropic growth, including those involved in the movement of and response to auxin. For instance, mutations of various genes belonging to the *PIN-FORMED* (*PIN*) family of membrane efflux facilitators and the *AUXIN RESPONSE FACTOR* (*ARF*) family of transcriptional regulators result in abnormal tropic responses in hypocotyls and roots.[5] In contrast, roles for these genes in the differential growth of the inflorescence stem have not been well established, reflecting a general lag in our understanding of the tropic responses of this organ. This knowledge gap is surprising given that inflorescence stem tropisms are critical for dictating overall plant shape and for optimizing processes like photosynthesis. Even so, *ARF* participation in this tropic response is to be expected, and is indirectly suggested by dominant mutations of the *Aux/IAA* gene *IAA7/AUXIN RESISTANT2* (*AXR2*), which result in pleiotropic auxin-related defects including abnormal inflorescence stem tropisms.[6] These types of gain-of-function mutations stabilize Aux/IAA protein products, which then antagonize the activity of transcriptional activating ARFs.[7]

We therefore sought to identify *ARF*s that participate in inflorescence stem tropic responses, and reasoned that those known to modulate tropisms in other plant organs would be promising candidates. *ARF5/MONOPTEROS* (*MP*) and *ARF7/NONPHOTOTROPIC HYPOCOTYL4* (*NPH4*) are 2 closely related genes that have been shown to redundantly function in root gravitropism.[8] Furthermore, *nph4* hypocotyls are defective in both phototropic and gravitropic responses.[9,10] Finally, both ARF5 and ARF7 physically interact with IAA7 at the protein level,[11] interactions which could, in part, underlie defective

CONTACT Naden T. Krogan nkrogan@american.edu Department of Biology, American University, 4400 Massachusetts Avenue NW, Washington DC 20016, USA.
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inflorescence stem tropisms in dominant *iaa7/axr2* mutants. Thus, to investigate roles for these *ARF*s in differential stem growth, we determined expression patterns of *MP* and *NPH4* in inflorescence stems and analyzed tropic responses in corresponding mutant backgrounds.

Expression of *ARF*s that facilitate auxin-mediated stem tropisms would be expected in cell layers that contribute to such responses, including auxin-rich source tissues (vasculature), gravity sensing layers (endodermis), or outer cells that undergo differential growth. Cross-sections of inflorescence stems expressing a functional *MP::MP-GUS* translational reporter showed strong MP levels in vascular bundles (Fig. 1A), in agreement with *MP* expression at the RNA level.[12] It is therefore possible that MP facilitates differential organ growth by promoting the lateral distribution of auxin from central tissues to the stem periphery. Interestingly, we have previously shown that *PIN3* is a direct target of MP,[8] raising the possibility that MP-dependent expression of *PIN3*, a gene implicated in both root and hypocotyl gravitropisms,[13,14] mediates auxin redistribution during inflorescence stem tropic responses. Additionally, MP is required for floral bud initiation and vascular strand continuity,[15] indicating that MP may also indirectly contribute to inflorescence stem tropisms by facilitating the production and long-distance transport of auxin from apical source tissues. In contrast to MP expression, an *ARF7::GFP* transcriptional reporter was strongly expressed in the stem periphery, including in the area of the epidermis and outer cortex (Fig. 1B and C). *NPH4* would be a logical factor involved in mediating differential inflorescence stem elongation in peripheral cells, as it is central to auxin-dependent differential growth responses in numerous other tissues.[16]

Since both ARFs are suitably positioned to contribute to inflorescence stem tropisms, we assessed whether mutation of *MP* and *NPH4* affected such processes. We reasoned that testing the inflorescence stems of *mp nph4* double mutants would

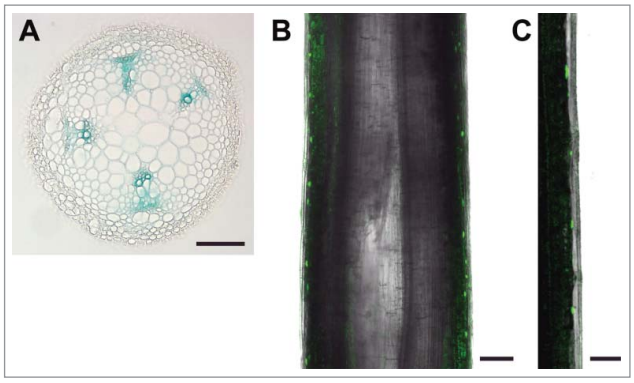

Figure 1. Expression of *ARF5/MP* and *ARF7/NPH4* in *Arabidopsis* inflorescence stems. (A) Stem cross-section showing *MP::MP-GUS* expression localized to vascular bundles. (B and C) Confocal images of lateral hand-sections of stems displaying *ARF7::GFP* expression in peripheral cell layers. GFP confocal images are overlayed on brightfield images. (C) Close-up of peripheral cell layers (epidermis on right) showing distribution of the nuclear-localized GFP reporter. Bars: (A and B) 100 μm; (C) 50 μm.

reveal any defects present in either single mutant. Moreover, although non-overlapping expression patterns of *MP* and *NPH4* argue against functional redundancy in the stem, analysis of *mp nph4* would unmask any synthetic or synergistic interactions between the 2 mutants. Unfortunately, however, *mp nph4* mutants do not form a hypocotyl or embryonic root, and exhibit an enlarged shoot apical meristem initially devoid of lateral organs (Fig. 2A and B).[8,17] With time, these meristems initiate radialized lateral outgrowths (Fig. 2C and D) that ultimately transition into highly disorganized masses of atypical structures, a subset of which loosely resemble pin-like inflorescence stems (Fig. 2E-G). Overall, however, this severely compromised meristem activity precludes the formation of organized inflorescence stems.

To overcome this hurdle, we transiently activated MP using a dexamethasone (DEX)-inducible *MP::MP-GR* transgene

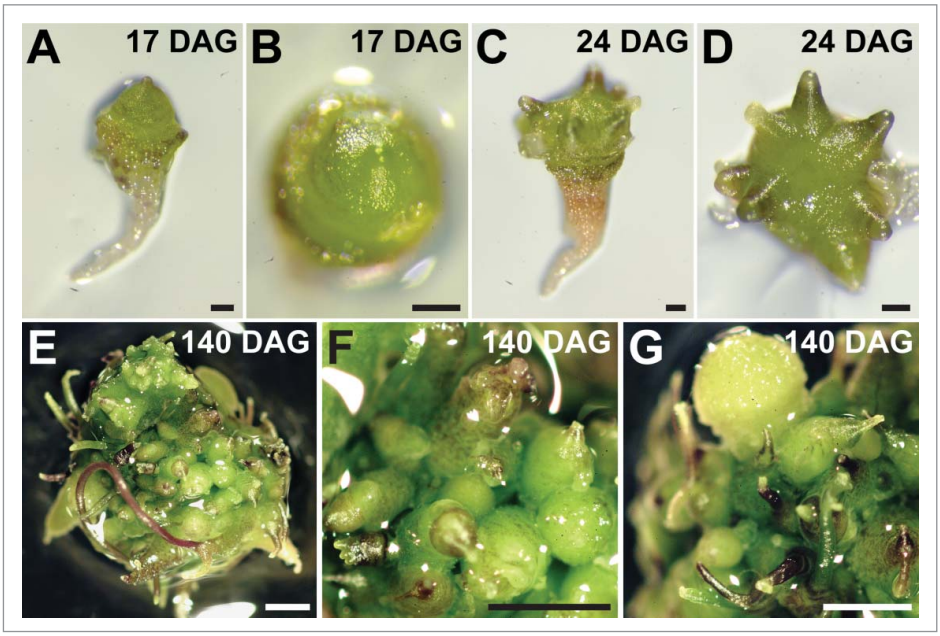

Figure 2. Shoot apical meristem proliferation of untreated *mp nph4 MP-GR* seedlings. (A and C) Lateral and (B and D) apical views of *mp nph4 MP-GR* seedlings. Leaves are absent at 17 d after germination (DAG) (A and B) while lateral outgrowths become apparent by 24 DAG (C and D). (E-G) Prolonged growth of the shoot apical meristem (140 DAG) results in the proliferation of disorganized structures that bear little resemblance to normal plant organs. Bars: 200 μm.

(*MP-GR* in the following) during early *mp nph4* development to obtain normally patterned seedlings.[8] Subsequent growth in the absence of DEX results in the tapering off of *MP-GR* activity and leads to phenotypic reversion to *mp nph4*. This allows the assessment of *ARF* contribution to tropic growth of organs, such as stems, that would otherwise not form in this genetic background. In a similar manner, we recently determined that, unlike either single mutant, *mp nph4* primary roots are agravitropic.[8]

We first performed an assay on *mp MP-GR* individuals to confirm that activation of MP early in development later tapers off upon DEX removal. Plants were germinated and grown on different concentrations of DEX for varying amounts of time, followed by transfer to DEX-free growth conditions (Fig. 3). MP-mediated phenotypic rescue was apparent by the formation of flowers and in turn seed-bearing siliques, which do not form in *mp* mutants due to defects in lateral organ initiation during reproductive growth.[15] The number of siliques produced was positively correlated with both the concentration and duration of DEX treatment (Fig. 3A). Upon DEX removal, inflorescence stems of all treatments invariably lost the ability to produce flowers and seed-bearing siliques, reverting back to the *mp* mutant condition (Fig. 3A and B). We were therefore confident that we had established a protocol that allowed us to trigger the initiation of inflorescence stems that could be used to test the effect of *mp nph4* mutation on tropic responses.

To generate organized *mp nph4* inflorescence stems, *mp nph4 MP-GR* individuals were treated with DEX during embryogenesis and for part of their vegetative growth. This transient treatment sufficiently normalized the overall

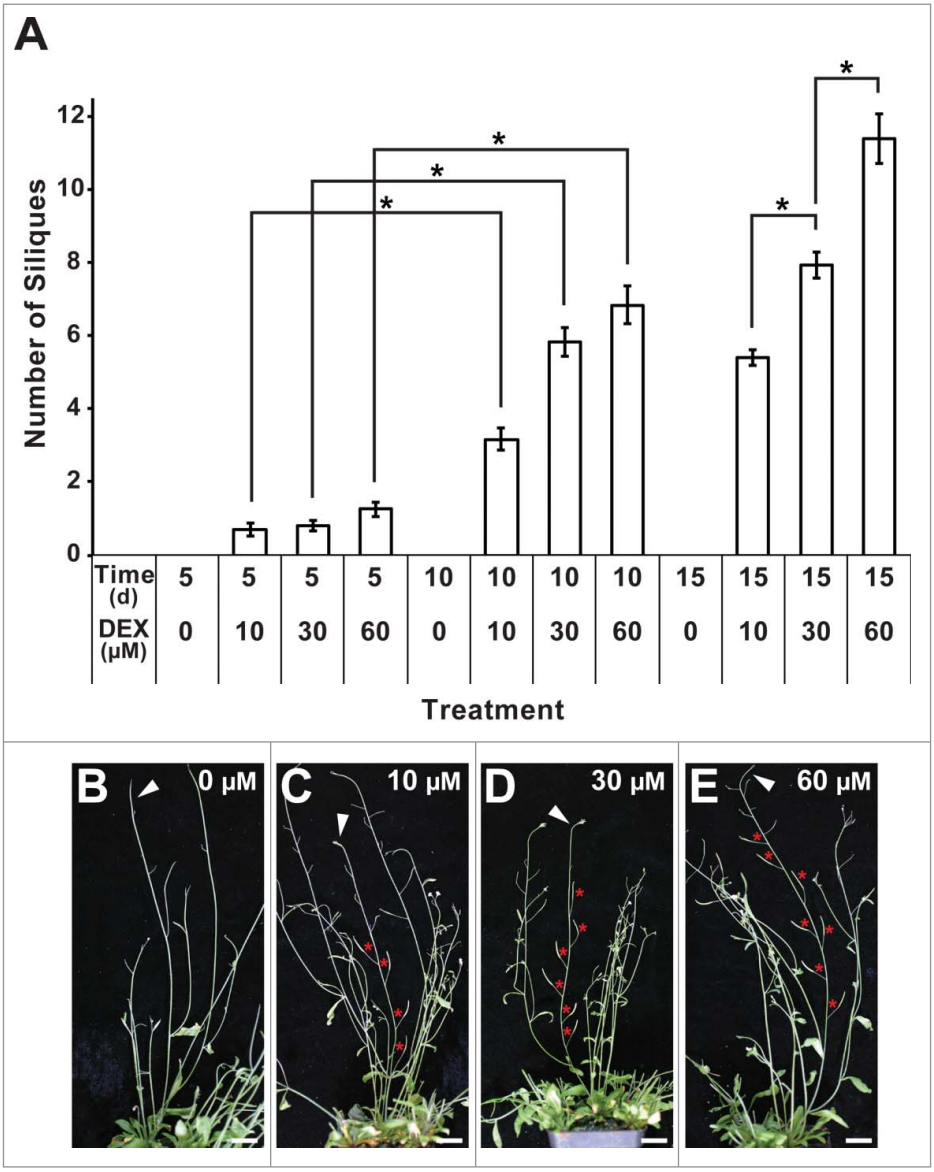

Figure 3. Effect of concentration and duration of DEX treatment on MP activation in *mp MP-GR* inflorescence stems. (A) Seedlings were germinated and grown on 0, 10, 30 or 60 μM DEX for 5, 10 or 15 d, and numbers of seed-bearing siliques on the primary inflorescence stems produced from each treatment were recorded. Columns represent mean values +/− SEM. Statistical significance of treatment comparisons was determined by Student *t* test analysis (*, $P < 0.0005$). The results show an increase in silique number with increasing duration of DEX treatment (see 5 d vs 10 d values) and increasing DEX concentration (see 15 d values). (B-E) Representative plants treated for 10 d on varying concentrations of DEX. The primary inflorescence stem of each plant is indicated (arrowhead), while seed-bearing siliques are designated with asterisks. In each case, at the depicted stage of development, the inflorescence stem has lost the ability to initiate functional flowers/siliques. Bars: 2 cm.

bodyplan to allow inflorescence stem initiation. However, the pin-like nature of these stems indicated that the effect of MP activation had worn off by the time of assessment (Fig. 4). To analyze stem gravitropism, plants of reproductive age were placed horizontally in the dark (to abrogate possible phototropic interference) for 24 h, a time sufficient to assess gravity-induced differential growth.[4] The inflorescence stems of wild type and 2 *mp* alleles (cultivated via adventitious rooting) showed normal negative gravitropism (Fig. 4A-C). Similarly, *mp nph4* double mutant stems also responded properly to the gravity stimulus (Fig. 4D). These results indicate that *MP* and *NPH4* are not strictly required for inflorescence stem gravitropism.

To test for a general role of *ARF*s in auxin-mediated tropic growth, we next unilaterally applied auxin (as a lanolin paste) to the apical regions of inflorescence stems of these same genetic backgrounds. Auxin treatment of wild type stems resulted in differential growth away from the side of application, the extent of which correlated with the concentration of auxin used (Fig. 4E, I, and M). Inflorescence stems of *mp* mutants also bent upon auxin treatment (Fig. 4F, G, J, K, N, and O), consistent with previous observations,[18] indicating that *mp* inflorescence stems are not entirely unresponsive to auxin application. Again, *mp nph4* stems responded to auxin treatment normally, bending to a degree that reflected the amount of auxin applied (Fig. 4H, L, and P), and therefore demonstrating that these 2 *ARF*s are not absolutely necessary for this response.

Since auxin signal transduction is intimately tied to differential organ growth, *ARF* participation in inflorescence stem tropic responses is to be expected, even though such links have yet to be established. Many lines of evidence suggest involve-

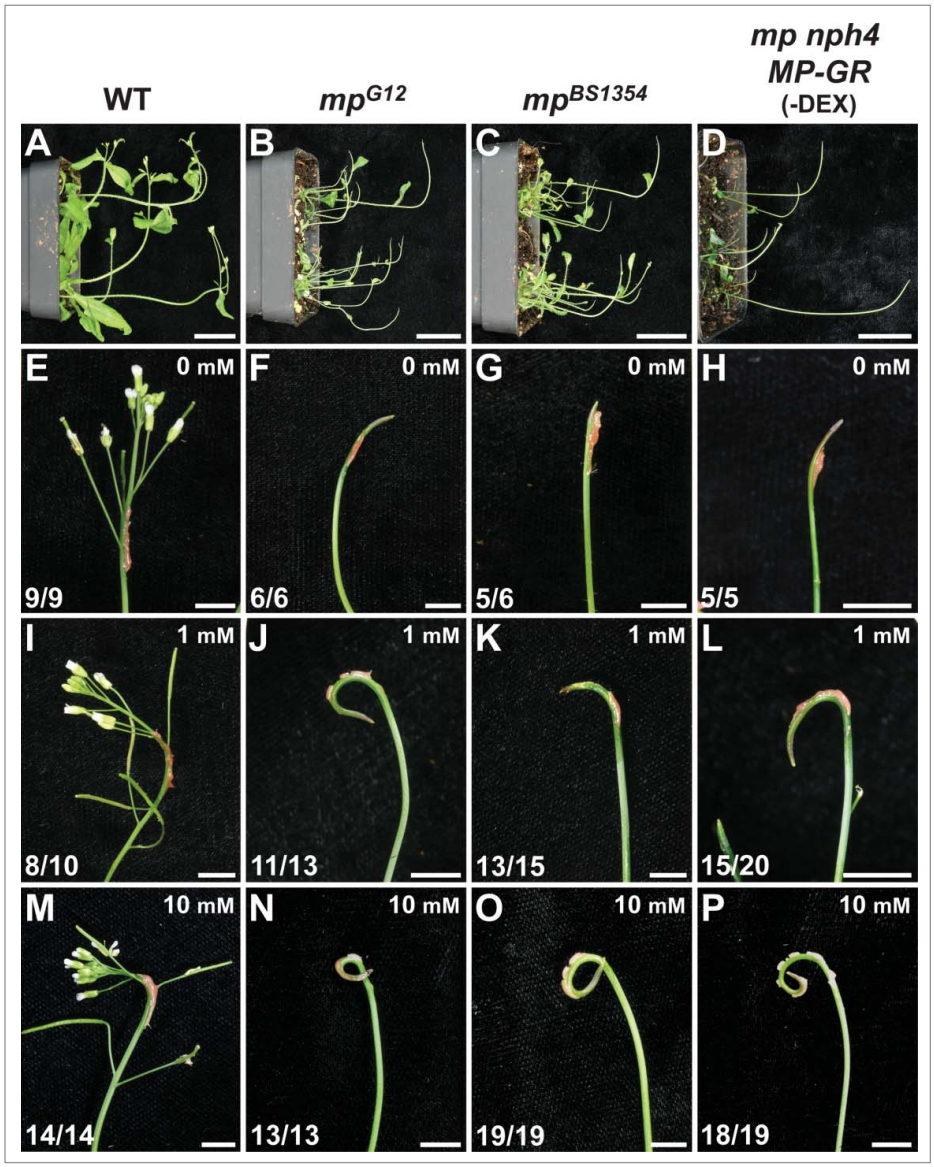

Figure 4. Tropic responses of *Arabidopsis* inflorescence stems. (A, E, I, and M) Wild type (WT), (B, F, J, and N) mp^{G12}, (C, G, K, and O) mp^{BS1354} and (D, H, L, and P) *mp nph4 MP-GR* plants are depicted. (A-D) Inflorescence stem gravitropic responses. Potted plants (with initially upright inflorescence stems) were turned 90° onto their sides and placed in the dark for 24 h. Images depict plants at the conclusion of the assay, with the gravity vector downward. (E-P) Unilateral auxin application. Lanolin paste (pink) containing 0, 1 or 10 mM indole-3-acetic acid was applied near the apices of inflorescence stems. Inflorescence stem bending was assessed 24 h after application. Ratios indicate the fraction of treated inflorescence stems resembling the depicted image with respect to degree of bending. Bars: (A-D) 2 cm; (E-P) 5 mm.

ment of *MP* and *NPH4* specifically, including gene expression patterns in relevant stem tissues, their confirmed roles in the tropic responses of other organs, and their physical interaction with the one canonical auxin-signaling component (IAA7/AXR2) implicated in inflorescence stem tropisms by its mutation. In this context, it may seem surprising that *mp* and *nph4* mutant backgrounds display normal stem tropisms. However, *ARF*s belong to a large multi-gene family with many reported functional redundancies between members.[7] Therefore, it is entirely plausible that overlapping functions between *ARF*s have prevented mutational analyses from establishing a direct link between *ARF*s and inflorescence stem tropisms. Although *MP* and *NPH4* act redundantly in multiple processes (including embryonic and vascular patterning),[17] their complementary expression patterns in inflorescence stems indicate that their participation in stem tropic responses is likely masked by other *ARF* members. This may include *ARF19*, which functions redundantly with *NPH4* in root gravitropism, lateral root formation and auxin-inducible gene regulation.[19-21] More detailed investigations into the expression domains of *ARF19* and other *ARF*s will be required to tease apart these genetic relationships, as the high-resolution stem expression patterns described here for *MP* and *NPH4* are lacking for other members of this gene family. In the future, a complete *ARF* expression map will predict the *arf* mutant combinations most likely to be compromised in inflorescence stem tropisms. Such relationships are completely predictable, given widespread roles for *ARF*s in the differential growth of other organs.

Materials and methods

Plant growth conditions and treatments, as well as *Arabidopsis* mutant and transgenic lines, have been described previously,[8] along with the *ARF7::GFP* line which uses a nuclear localized GFP.[22] Brightfield microscopy of *MP::MP-GUS* sections was performed using an Olympus BX61 compound microscope, while *ARF7::GFP* distribution was visualized with an Olympus Fluoview FV1200 Laser Scanning Confocal microscope.

Disclosure of potential conflicts of interest

The authors declare no conflicts of interest, financial or otherwise.

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