ARF1, a Transcription Factor That Binds to Auxin Response Elements

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The plant hormone auxin regulates plant physiology by modulating the interaction of transcription factors with auxin response elements (AuxREs) of the affected genes. A transcription factor, Auxin Response Factor 1 (ARF1), that binds to the sequence TGTCTC in AuxREs was cloned from *Arabidopsis* by using a yeast one-hybrid system. ARF1 has an amino-terminal DNA-binding domain related to the carboxyl terminus of the maize transactivator Viviparous-1. Sequence requirements for ARF1 binding in vitro are identical to those that confer auxin responsiveness in vivo. The carboxyl terminus of ARF1 contains two motifs found in the Aux/IAA class of proteins and appears to mediate protein-protein interactions.

Hormones such as auxin regulate growth and development of plants by altering expression of early response genes (*1*). We investigated the transcription factors that regulate these genes. The soybean gene *GH3* responds rapidly and specifically to auxin in a tissue-specific manner (*2*, *3*). The *GH3* promoter contains two auxin response elements (AuxREs), D1 and D4, each of which is composed of a constitutive element (an element that confers constitutive expression when fused to a minimal promoter) adjacent to or overlapping with a TGTCTC sequence element (*3*). The TGTCTC element confers repression and activation of the constitutive element when auxin quantities are low and high, respectively. These composite AuxREs share some similarity with composite glucocorticoid response elements (GREs) in animal cells. GRE TGTTCT half-sites may overlap with other DNA-binding sites in composite GREs (*4*), and TGTCTC sites may overlap with constitutive elements in composite AuxREs (*3*). Because the preferred DNA-binding site for the glucocorticoid receptor is a palindrome, AGAACAnnnTGTTCT, we conducted tests to determine whether palindromic repeats of TGTCTC functioned as AuxREs (Fig. 1). The P3(4×) construct, consisting of four tandem copies of inverted repeats (IRs) of the TGTCTC element, conferred auxin responsiveness in carrot protoplasts to a β-glucuronidase (GUS) reporter gene driven by the minimal (−1 to −46) promoter of the cauliflower mosaic virus (CaMV) 35*S* gene. Mutated AuxREs were inactive. Because of its high activity, the P3(4×) construct was used as bait in a yeast one-hybrid system to screen an *Arabidopsis* cDNA expression library (*5*). Five cDNA clones of two size classes, 2.3 and 2.4 kb, were isolated that encoded the same protein (Auxin Response Factor 1 or ARF1) (Fig. 2A).

The NH_2-terminal sequence of ARF1 is similar to that in two *Arabidopsis* expressed sequence tags (GenBank accession numbers Z37232 and R30405), IAA24 (GenBank accession number U79556) and ARF3 (*6*) and the COOH-terminal region of the maize transcriptional activator Viviparous-1 (VP1) (*7*) and its *Arabidopsis* homolog ABI3 (*8*) (Fig. 2B). The COOH-terminal sequence of ARF1 is similar to the COOH-terminal regions of Aux/IAA proteins, including IAA24 (Fig. 2C). The Aux/IAA proteins contain four islands of amino acid sequence similarity (boxes I to IV, Fig. 2A) (*1*). ARF1 and IAA24 differ from most Aux/IAA proteins in being larger and containing boxes III and IV only. Box III is part of a motif related to the amphipathic βαα-fold found in β-ribbon DNA-binding domains of prokaryotic Arc and MetJ repressor proteins, and Aux/IAA proteins are hypothesized to be transcription factors (*9*). Analyses with both Garnier-Osguthorpe-Robson and Chou-Fasman algorithms predict that box III in ARF1 conforms to an amphipathic βαα-motif. ARF3 differs from ARF1 and IAA24 in that it lacks boxes III and IV (Fig. 2A).

The middle region of the ARF1 protein contains a putative nuclear localization sequence (NLS) and is rich in proline, serine, and threonine. The latter features correspond to activation or repression domains in other transcription factors (*10*). Because ARF1 cDNA clones recovered in the yeast one-hybrid screen were either out-of-frame with or in reverse orientation to the yeast GAL4 activation domain and NLS, ARF1 must contain a NLS that targets it to the yeast nucleus where it functions as a transcriptional activator. Southern (DNA) blot analysis suggested that ARF1 is a single-copy gene, and Northern (RNA) blot analysis indicated that ARF1 mRNA (2.4 kb) is a low-abundance transcript in all organs tested and is not induced by exogenous auxin (*11*).

We used gel-shift experiments (Fig. 3A) to test interactions of ARF1 with the P3(4×) DNA sequence. Two complexes were observed that may represent different numbers of ARF1 proteins bound to P3(4×). ARF1 also bound to the natural D0 AuxRE sequence, although with lower affinity. ARF1 binding to composite elements like D0 may be facilitated by a different factor that binds to the constitutive element (*3*). We tested truncated proteins lacking boxes III and IV to determine whether the βαα-motif in ARF1 functioned as a DNA-binding domain. These truncated proteins bound the P3(2×) probe as efficiently as full-length ARF1 protein. The P3(2×) probe contains two tandem copies of the P3 palindrome and forms only one ARF1 complex. COOH-terminal truncations up to amino acid 359 bound P3(2×), but truncations that extended into the VP1-like motif (amino acid 286) did not bind. NH_2-terminal truncations at amino acid 63 or 154 failed to bind the P3(2×) probe (Fig. 3A). These results suggested that a region in ARF1 that includes the VP1-like motif is a DNA-binding domain.

To determine whether in vitro binding of ARF1 to P3(4×) was consistent with in vivo AuxRE activity, we tested mutant variants of the P3 AuxRE for ARF1 binding in gel-shift assays (Fig. 3B) and for auxin inducibility in carrot protoplasts (Fig. 3C). Nucleotide positions within the TGTCTC element are defined as +1 to +6, and the

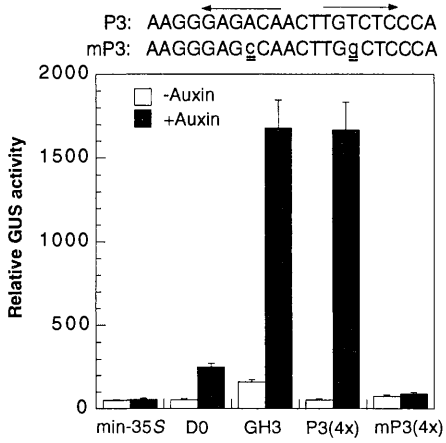

Fig. 1. Palindromic copies of the TGTCTC function as AuxREs. Constructs were tested in transfected carrot protoplasts with or without auxin (*3*). min-35*S*, −46 CaMV 35*S* promoter; D0, one copy of the 74-base pair (bp) D0 AuxRE from the GH3 promoter; GH3, 592-bp GH3 promoter; P3(4×), four tandem copies of the P3 element; mP3(4×), four tandem copies of a mutated P3 element.

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corresponding mutations are defined as m1 to m6; m1 through m4 abolished auxin responsiveness and ARF1 binding. Results with m5 and m6 and the double-mutation m5,6 suggested that positions +5 and +6 contribute to ARF1 binding and auxin inducibility but to a lesser extent than positions +1 through +4. Mutations outside the TGTCTC element had little effect on auxin inducibility or ARF1 binding. IAA24 binding specificity was identical to that of ARF1 (Fig. 3B). These results indicate that auxin inducibility and ARF1/IAA24 binding depended on the TGTCTC element and not on flanking or spacer sequences in P3(4×).

To further define the interaction of ARF1 with P3 AuxREs, we used a P3(2×) probe for ARF1 deoxyribonuclease (DNase) I footprinting. The P3(2×) probe contains a central everted repeat (ER) (convergent arrows) and flanking IRs (divergent arrows) (Fig. 4A). The DNase I footprint showed that ARF1 binds to the ER in P3(2×) and that the footprint extends into the +1 and +2 positions of the flanking IRs. DNA methylation interference experiments indicated that the G residues at the +4 position (opposite strand) in one half-site and the +2 position in the second half-site of the TGTCTC ER were critical for ARF1 binding (Fig. 4A).

To confirm that ARF1 prefers TGTCTC ERs as binding sites, we tested single copies of IRs and ERs in gel mobility-shift assays (Fig. 4B). A single-copy P3 IR failed to bind ARF1, but two-copy P3(2×) and four-copy P3(4×) palindromic repeats produced one and two ARF1 complexes, respectively (Fig. 4B, lanes 1 to 3). It is likely that two rather than three ARF1 complexes are observed with the P3(4×) probe, because the spacing between the neighboring ERs is inadequate to allow unobstructed binding to all three ERs at the same time (Fig. 4A). A single copy of ER7 bound to ARF1 and produced a single complex. A site-specific mutation in the TGTCTC sequence of one half-site within mER7 resulted in loss of ARF1 binding and auxin inducibility (Fig. 4, B and C). We identified a natural ER (ER9-IAA4/5) (Fig. 4B) in an auxin-responsive region of the PsIAA4/5 gene (*12*). One copy of ER9-IAA4/5 produced a single ARF1 complex

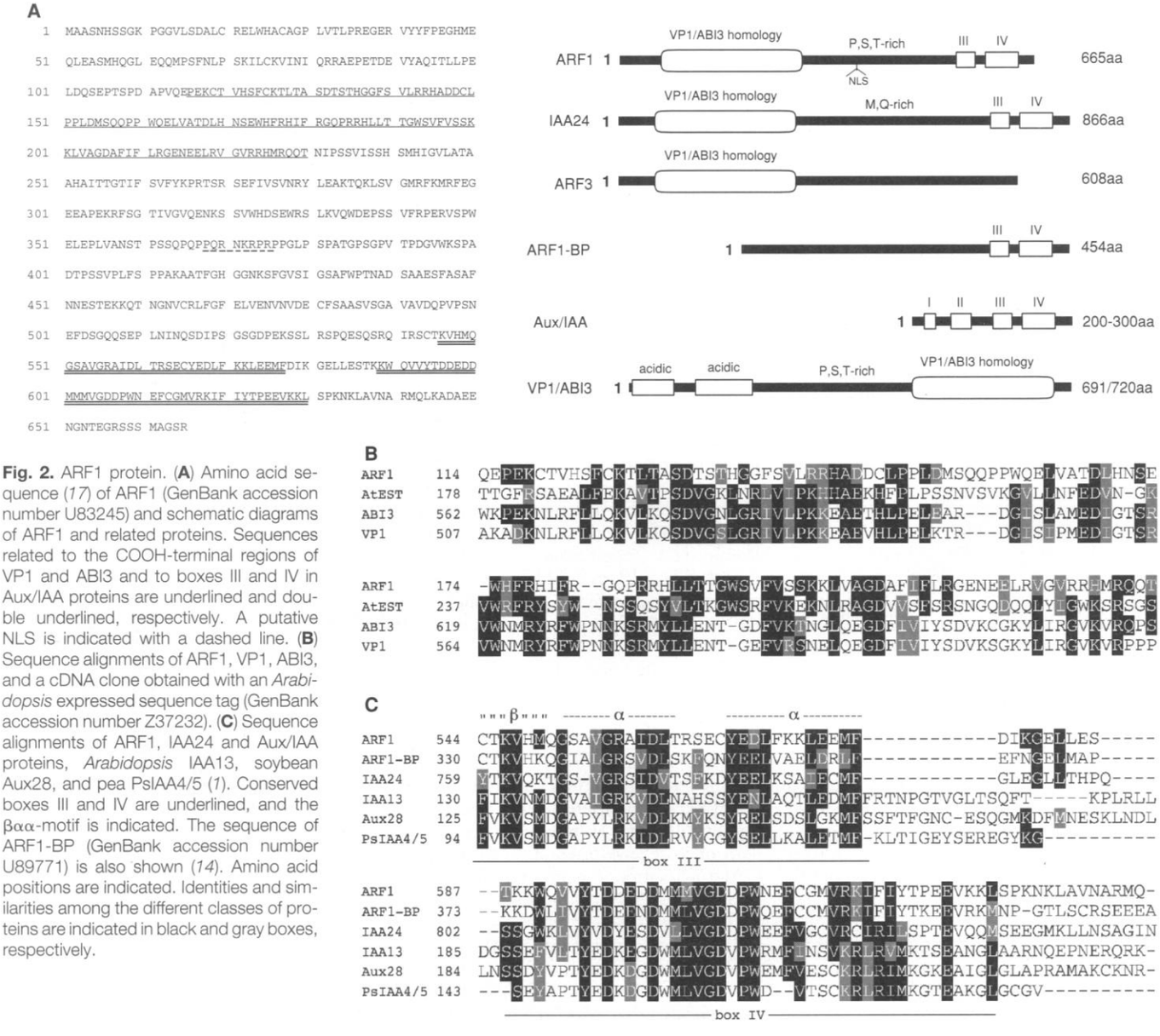

Fig. 2. ARF1 protein. (**A**) Amino acid sequence (*17*) of ARF1 (GenBank accession number U83245) and schematic diagrams of ARF1 and related proteins. Sequences related to the COOH-terminal regions of VP1 and ABI3 and to boxes III and IV in Aux/IAA proteins are underlined and double underlined, respectively. A putative NLS is indicated with a dashed line. (**B**) Sequence alignments of ARF1, VP1, ABI3, and a cDNA clone obtained with an *Arabidopsis* expressed sequence tag (GenBank accession number Z37232). (**C**) Sequence alignments of ARF1, IAA24 and Aux/IAA proteins, *Arabidopsis* IAA13, soybean Aux28, and pea PsIAA4/5 (*1*). Conserved boxes III and IV are underlined, and the βαα-motif is indicated. The sequence of ARF1-BP (GenBank accession number U89771) is also shown (*14*). Amino acid positions are indicated. Identities and similarities among the different classes of proteins are indicated in black and gray boxes, respectively.

(Fig. 4B, lanes 10 to 12) and functioned as an AuxRE in carrot protoplasts (*11*). Because spacing between half-sites in GREs can affect binding of the receptor (*4*), we altered the spacing between TGTCTC half-sites (*13*). The optimal spacing for auxin responsiveness in vivo and for ARF1 (and IAA24) (*11*) binding in vitro is 7 or 8 nucleotides (Fig. 4C). Thus, ARF1 and

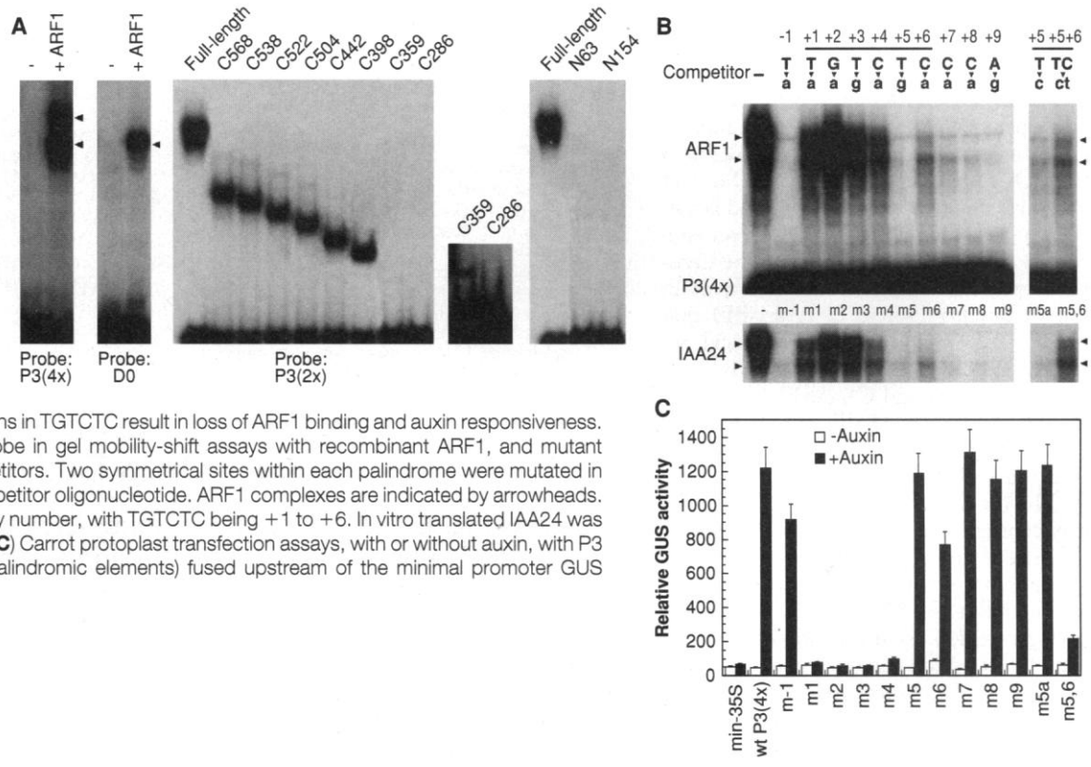

Fig. 3. ARF1 DNA-binding domain and its DNA target. (**A**) Two left panels show recombinant ARF1 complexes with P3(4×) and natural promoter GH3 (D0) probes. Other panels show gel mobility-shift assays with in vitro translated, full-length and truncated forms of ARF1 (*18*). Amino acid positions in ARF1 for COOH-terminal (C) and NH_2-terminal (N) truncations are shown above each lane. Longer exposures are shown for the COOH-terminal truncations (C359 and C286). (**B**) Mutations in TGTCTC result in loss of ARF1 binding and auxin responsiveness. P3(4×) was used as the probe in gel mobility-shift assays with recombinant ARF1, and mutant variants were used as competitors. Two symmetrical sites within each palindrome were mutated in the P3 element for each competitor oligonucleotide. ARF1 complexes are indicated by arrowheads. Mutated sites are indicated by number, with TGTCTC being +1 to +6. In vitro translated IAA24 was used in the lower panel (*19*). (**C**) Carrot protoplast transfection assays, with or without auxin, with P3 and mutant elements (4× palindromic elements) fused upstream of the minimal promoter GUS reporter gene.

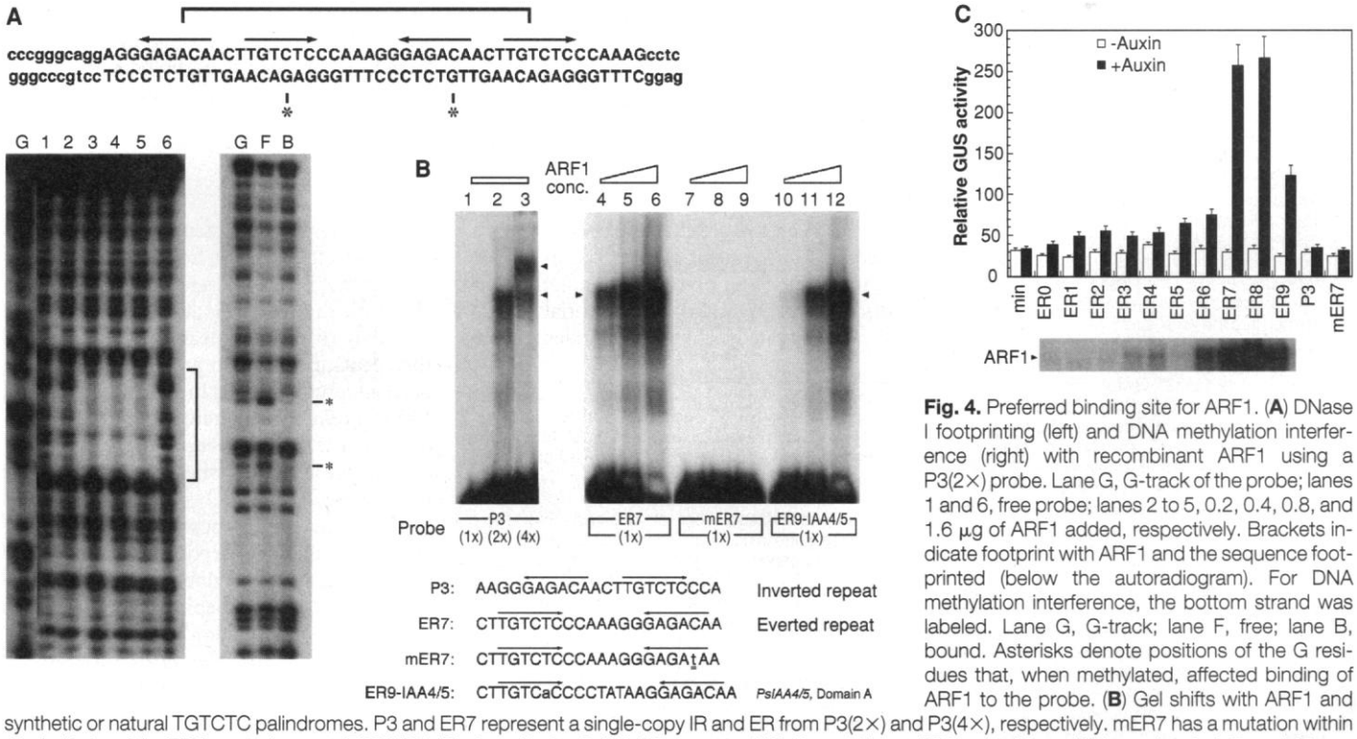

Fig. 4. Preferred binding site for ARF1. (**A**) DNase I footprinting (left) and DNA methylation interference (right) with recombinant ARF1 using a P3(2×) probe. Lane G, G-track of the probe; lanes 1 and 6, free probe; lanes 2 to 5, 0.2, 0.4, 0.8, and 1.6 μg of ARF1 added, respectively. Brackets indicate footprint with ARF1 and the sequence footprinted (below the autoradiogram). For DNA methylation interference, the bottom strand was labeled. Lane G, G-track; lane F, free; lane B, bound. Asterisks denote positions of the G residues that, when methylated, affected binding of ARF1 to the probe. (**B**) Gel shifts with ARF1 and synthetic or natural TGTCTC palindromes. P3 and ER7 represent a single-copy IR and ER from P3(2×) and P3(4×), respectively. mER7 has a mutation within one half-site of the ER7 everted repeat. ER9-IAA4/5 is found in the PsIAA4/5 promoter (*12*). Recombinant ARF1 protein used in lanes 1 to 3, 4, 7, and 10 was 100 ng; in lanes 5, 8, and 11 it was 200 ng; and in lanes 6, 9, and 12 it was 400 ng. (**C**) Transfection and gel mobility-shift assays, with or without auxin, with single-copy spacing constructs.

IAA24 are likely participants in auxin gene regulation through the TGTCTC elements. A single copy of ER8 was a more active AuxRE than other constructs that contained two copies of TGTCTC (*3*, *11*) and could represent the perfect palindromic AuxRE, similar to the perfect palindromic GRE (*4*).

As the COOH-terminal βαα-motif has no apparent effect on ARF1 binding to DNA, what might be its function? We used the COOH-terminal region of ARF1 as bait in a yeast two-hybrid screen (*14*) and isolated two identical cDNA clones from an *Arabidopsis* cDNA expression library. The translated open reading frame encoded a protein (ARF1-Binding Protein or ARF1-BP) that contained a region with amino acid sequence similarity to boxes III and IV of ARF1 (Fig. 2, A and C). ARF1-BP showed less similarity to boxes III and IV in Aux/IAA and IAA24 proteins. Thus, boxes III and IV in ARF1 may facilitate interaction of ARF1 with ARF1-BP, and these interactions may contribute to auxin responsiveness.

Genetic approaches to dissect the auxin signal transduction pathway have resulted in the cloning of *AXR1*, *AUX1*, and *hookless1* genes (*15*). Identification of the relevant transcription factors should facilitate elucidation of the mechanisms involved in auxin-regulated gene expression.

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5. The P3(4×) AuxRE was placed upstream of the minimal promoter in pHISi-1 and pLacZi vectors (MATCHMAKER One-Hybrid System; Clontech). These vectors were linearized and sequentially transformed into the yeast strain Y4271 (Clontech). Background LacZ activity in late logarithmic-phase cells for the engineered strain Y4271::P3(4×) was higher than for the pLacZi vector (no AuxREs) but low enough to distinguish from a positive interaction. Low plating density (10^8 cells per 150-mm plate) and 3-aminotriazole (3-AT; 45 mM) were used to suppress growth on histidine-deficient (−His) plates resulting from the low amount of P3(4×)-HIS3 reporter gene expression. An *Arabidopsis* cDNA expression library cloned into the GAL4 activation domain vector pGAD10 (Clontech) was amplified in *Escherichia coli*. Purified library DNA (500 μg) was used to transform the Y4271::P3(4×) strain. Of 1.2×10^8 transformants, 500 colonies grew on −His plates containing 3-AT, and these were screened for lacZ activity. LacZ-positive colonies (212) were selected, and library plasmids were isolated. Sizes of the cDNA inserts were determined by polymerase chain reaction (PCR) with primers that flanked the cDNA inserts. Plasmids harboring different-sized inserts were rescued by transformation into *E. coli* and retransformed into the Y4271::P3(4×) strain. Clones that restored lacZ activity were sequenced, and five clones encoding ARF1 were recovered.
6. An ARF3 cDNA (GenBank accession number U89926) was isolated from the MATCHMAKER cDNA library by using an unannotated *Arabidopsis* sequence (GenBank accession number U78721) related to the NH_2-terminal region of ARF1.
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13. The ER0 spacing construct was actTGTCTCGAGACAac. ER1 to ER9 contained spacers between actTGTCTC and GAGACAac of one to nine nucleotides: a, aa, cag, caag, ccagg, ccaagg, ccaaagg, ccattagg, and ccatttagg.
14. The COOH-terminus of ARF1 [amino acids (aa) 533 to 665] served as bait to screen the library used in the one-hybrid screen. The interacting clone encoded the COOH-terminal portion of ARF1-BP (aa 273 to 410). Complementary DNA clones encoding NH_2-terminal regions of ARF1-BP cDNA were isolated by PCR.
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17. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
18. Histidine-tagged ARF1 was expressed and purified from *E. coli*, full-length and truncated forms of ARF1 were synthesized in vitro in FlexiRabbit reticulocyte lysates (Promega), and gel-shift assays were done as described (*16*).
19. The IAA24 ORF was isolated from the MATCHMAKER cDNA library by PCR. IAA24 protein was produced by in vitro translation.

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Large Porous Particles for Pulmonary Drug Delivery

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A new type of inhalation aerosol, characterized by particles of small mass density and large size, permitted the highly efficient delivery of inhaled therapeutics into the systemic circulation. Particles with mass densities less than 0.4 gram per cubic centimeter and mean diameters exceeding 5 micrometers were inspired deep into the lungs and escaped the lungs' natural clearance mechanisms until the inhaled particles delivered their therapeutic payload. Inhalation of large porous insulin particles resulted in elevated systemic levels of insulin and suppressed systemic glucose levels for 96 hours, whereas small nonporous insulin particles had this effect for only 4 hours. High systemic bioavailability of testosterone was also achieved by inhalation delivery of porous particles with a mean diameter (20 micrometers) approximately 10 times that of conventional inhaled therapeutic particles.

Inhaled aerosols are effective therapeutic carriers for the treatment of respiratory inflammation (*1*), cystic fibrosis (*2*), and other lung disorders (*3*); they also offer potential for noninvasive systemic delivery of peptides and proteins (*4*). Local and systemic inhalation therapies can often benefit from a controlled release of the therapeutic agent (*5*), as is achievable with the use of biodegradable polymeric materials (*6*). Slow release from an inhaled therapeutic particle can prolong the residence of an administered drug in the airways or acini and can diminish the rate of a drug's appearance in the bloodstream (*7*). Also, patient compliance increases when dosage frequency is reduced (*7*).

The human lungs, however, have efficient means of removing deposited particles over periods ranging from minutes to hours. In the upper airways, ciliated epithelia contribute to the "mucociliary escalator" (*8*), by which particles are swept from the airways toward the mouth. In the deep lungs, an army of alveolar macrophages is capable of phagocytosing particles soon after their deposition (*9*). An effective slow-release inhalation therapy therefore requires a means of avoiding or suspend-

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