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Auxin perception and downstream events

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Abstract

Auxin responses have been arbitrarily divided into two categories: genomic and non-genomic effects. Genomic effects are largely mediated by $SCF^{TIR1/AFB}$-Aux/IAA auxin receptor complexes whereas it has been postulated that AUXIN BINDING PROTEIN 1 (ABP1) controls the non-genomic effects. However, the roles of ABP1 in auxin signaling and plant development were recently called into question. In this paper, we present recent progress in understanding the $SCF^{TIR1/AFB}$-Aux/IAA pathway. In more detail, we discuss the current understanding of ABP1 research and provide an updated view of ABP1-related genetic materials. Further, we propose a model in which auxin efflux carriers may play a role in auxin perception and we briefly describe recent insight on processes downstream of auxin perception.

Introduction

Auxin plays essential roles in many developmental processes. Much effort has been directed toward understanding the precise molecular mechanisms by which auxin regulates diverse aspects of plant growth and development. A key aspect of the effort is to understand auxin perception. There are two well-studied auxin perception systems: $SCF^{TIR1/AFB}$-Aux/IAA auxin receptor complexes (SCF [(SKP, CULLIN, F-BOX), TIR1/AFB (TRANSPORT INHIBITOR RESPONSE 1/AUXIN-RELATED F-BOX PROTEINS), AUX/IAA (AUXIN/INDOLE-3-ACETIC ACID)] and the AUXIN BINDING PROTEIN 1 (ABP1) system, which were proposed to control auxin-mediated transcription and non-genomic effects, respectively [1,2]. In this paper, we review the recent progress in our understanding of the regulation of the stability and assembly of the $SCF^{TIR1/AFB}$-Aux/IAA auxin receptor complexes. We will also address the controversies surrounding ABP1, which was accepted as a plasma-membrane-associated auxin receptor that forms an auxin receptor complex with a small family of receptor-like kinases (TMKs) to regulate many developmental processes including pavement cell development, cytoskeleton re-organization, and polar auxin transport [1–5]. However, a recent study showed that ABP1 is not required for auxin signaling and plant development [6], prompting re-analyses of the previous *abp1* genetic

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materials that formed the basis for assigning ABP1 functions. We detail the experimental results that have adequately resolved the underlying causes of the conflicting results regarding the roles of ABP1 in auxin signaling and plant development. Finally, we present several recent studies of auxin signaling events downstream of the $SCF^{TIR1/AFB}$-Aux/IAA auxin receptor complexes.

AUXIN BINDING PROTEIN 1 (ABP1)

Since the discovery of ABP1 in 1972, research into this proposed auxin receptor has been surrounded with controversy [7–9]. ABP1 is a 22 kDa protein with a C-terminal KDEL ER-retention signal [10,11]; over 90% of ABP1 is ER-localized [12]. Yet nearly all ABP1 functional studies focused on the small fraction of plasma-membrane-bound ABP1 [1]. ABP1 has been suggested to have essential roles in almost every aspect of plant growth and development including embryogenesis [13], root growth and maintenance of the root meristem [14], hypocotyl elongation [15], pavement cell development [4], vascular patterning [16], cytoskeleton re-organization and polar auxin transport [3,5], control of flowering time [17], and flower development [16].

Many resources have been dedicated to study ABP1 and more than 100 ABP1 papers have been published. During the era of Arabidopsis genetics, researchers generated various knockdown lines and mutants for determining ABP1 functions and the action mechanisms: 1) T-DNA insertion mutants *abp1-1* and *abp1-1s* [13,18,19]; 2) *abp1-5*, carrying a point mutation in the auxin-binding pocket [4]; 3) Three knockdown lines (*abp1-AS, SS12K, SS12S*) [16]; 4) *ABP1* overexpression lines [3,4,20,21]. All of these lines were reported to have obvious phenotypes and were the basis for all functional studies of ABP1 since 2000. Therefore, it was shocking when ABP1 was reported not to be required for either auxin signaling or Arabidopsis development [6]. Making use of the CRISPR/Cas9 (Clustered regularly-interspaced short palindromic repeats/CRISPR associated protein 9) technology [22], Gao et al. generated a true *abp1* null allele (*abp1-c1*), consisting of a 5-bp deletion in the first exon of the *ABP1* gene [6]. This *abp1-c1* allele did not produce detectable full length or truncated ABP1 protein [6]. Both the *abp1-c1* mutant and the *abp1-TD1* mutant, carrying a T-DNA insertion in the first exon of *ABP1*, are indistinguishable from WT in overall morphology, development, and auxin responses [6]. The results presented in the Gao et al. paper were unequivocal whereas previous results appeared solid as well. It has been a difficult task for the plant biology community to reconcile the apparent contradictory findings regarding the roles of ABP1 in auxin signaling and plant development [9,23,24]. Fortunately, the discrepancies between the Gao et al. paper [6] and previous ABP1 papers appeared to have been largely resolved.

The first genetic evidence revealing *ABP1* importance in plant development was the embryo lethality of the *abp1-1* T-DNA insertion mutant [13]. Indeed, *abp1-1* was a cornerstone of ABP1 research. Prior to the *abp1-1* description, skepticism about ABP1 as an auxin receptor was pervasive, and ABP1 was even called a red herring [7,8]. The *abp1-1* mutant contains a T-DNA insertion 51 bp downstream of the ATG start codon and a *35S:ABP1 cDNA* construct was reported to have complemented the *abp1-1* embryo lethal phenotypes [13]. Further, the *abp1-1s* allele harbors a T-DNA in the promoter region of *ABP1* [18] and was

endorsed as a second *abp1* null allele. A cross between *abp1-1* and *abp1-1s* failed to complement the embryo lethal phenotype, suggesting that these two mutants were disrupted in the same gene, although there are no reports on complementation of *abp1-1s* using WT *ABP1* genomic DNA or cDNA. Although both *abp1-1s* and *abp1-1* were cited as null mutants, analyses of *ABP1* mRNA and ABP1 protein levels were precluded by the homozygous lethality of the two mutations [9,19,25]. Recent re-analyses of *abp1-1* and *abp1-1s* have uncovered the underlying basis of their embryo lethality [25,26], which is not from disruption of *ABP1*. The T-DNA insertion in *abp1-1* not only disrupted the *ABP1* gene, but also deleted the entire neighboring *BSM* gene [26], which is an essential gene [27]. In fact, *abp1-1* embryo phenotypes closely resembled to those observed in *bsm* mutants [27]. Introduction of the *BSM* gene under the control of the *CaMV 35S* promoter into the *abp1-1* mutant plants completely rescued the embryo lethal phenotype [26]. Furthermore, *abp1-1* failed to accumulated detectable ABP1 protein, but the *abp1-1*$^{-/-}$ *CaMV 35S:BSM* plants did not display obvious developmental defects [26]. These results unequivocally demonstrated that the *abp1-1* embryo lethal phenotype is not caused by the *ABP1* disruption, but rather by the deletion of the neighboring *BSM* gene [26]. Analyses of reciprocal crosses between *abp1-1*, *abp1-1s*, *abp1-c1*, *abp1-TD1*, and *bsm-1* [25] further support the conclusion that the embryo lethality observed in *abp1-1* and *abp1-1s* is caused by disruption of the *BSM* gene. A caveat to the latter study is the possibility that *ABP1* function may have been compromised in the *bsm-1* mutant, which was not examined. This point is significant because T-DNA insertions can profoundly affect expression levels of adjacent genes [28]. Nevertheless, the data in Gao et al. paper [6], combined with results from the re-analyses of the *abp1-1* and *abp1-1s* [25,26], allow for the confident conclusion that Arabidopsis plants do not require *ABP1* for normal growth and development under laboratory conditions.

The *abp1-5* mutant also played a major role in a series of ABP1 studies. It was reported that the development of pavement cell and polar auxin transport were affected in *abp1-5* [1,3–5]. Moreover, exogenous auxin failed to rescue *abp1-5* developmental defects [1,3–5]. However, recent whole genome sequencing of the *abp1-5* mutant revealed that there are more than 8000 polymorphisms between *abp1-5* and the parental line Arabidopsis Col-0 [29], suggesting that the mutant has not been properly backcrossed as previously reported [4]. Some of the phenotypes associated with *abp1-5* are likely not caused by the *abp1* lesion. For example, the longer hypocotyl phenotype observed in light-grown *abp1-5* seedlings [15] is linked to a secondary mutation in the *PHYTOCHROME B* gene present in the *abp1-5* background, and is not linked to the *abp1-5* mutation itself [29]. Because *abp1-5* contains numerous background mutations, conclusions derived from this allele should be treated with caution.

Three *abp1* knockdown lines (SS12K, SS12S, *abp1-AS*) played instrumental roles in connecting ABP1 to many developmental processes [16]. The SS12K and SS12S lines, often referred as ABP1 antibody lines, were generated by expressing the single chain fragment variable regions (scFv12) of ABP1 monoclonal antibody under the control of an ethanol-inducible promoter [16]. The SS12K contains the KDEL ER retention signal whereas SS12S lacks the KDEL motif [16]. The SS12K and SS12S lines behaved very similarly, even though one was targeted to the ER and the other was targeted to the apoplast region [16].

Another knockdown line is *abp1-AS,* which expresses an *ABP1* antisense RNA under the control of the aforementioned ethanol inducible promoter [16]. Although the initial study using the *ABP1* knockdown lines reported that multiple lines from each construct produced similar phenotypes [16], subsequent studies almost exclusively used SS12K6, SS12S9, and *abp1-AS* [1,3–5,14,30,31]. Recently, the three knockdown lines were crossed to *abp1-c1* and *abp1-TD1* null alleles [32]. Analysis of the segregation patterns resulting from the crosses clearly demonstrated that the phenotypes observed in these lines are not caused by affecting *ABP1* and are likely caused by off-target effects [32].

Results from overexpressing *ABP1* in various systems including tobacco leaves, BY2 cells, and Arabidopsis have been used to support that ABP1 plays roles in cell expansion and other developmental processes [3,33]. Results from *ABP1* overexpression should be treated with caution as the different lines did not always produce consistent results. For example, overexpression of full length *ABP1* in Arabidopsis did not result in obvious developmental phenotypes, but overexpression of *ABP1* lacking the KDEL ER-retention signal led to auxin-related phenotypes including three cotyledons, shorter roots, reduced apical dominance, sterility, and seedling lethality [3]. Interestingly, the $ABP1^{\Delta KDEL}$ overexpression phenotypes are consistent with a decrease in auxin signaling in plants. The shorter roots and three cotyledon phenotypes were also observed in *abp1-5* and the antibody lines [1,3,16].

A key argument supporting the importance and requirement of ABP1 is the presence of homologues in all plant species [32,34]. This argument was based on available genomic sequences at that time, and recently has been refuted by the absence of ABP1 in *Marchantia polymorpha*, a liverwort species [35], indicating that ABP1 is dispensable, at least in some plant species.

The contradictory results between Gao et al paper and the previous studies have been reasonably resolved; however, the physiological functions of ABP1 remain unknown because all of the available *abp1* knockouts display no aberrant phenotypes under normal laboratory conditions. The simplest explanation is that ABP1 is not required for normal plant development. However, two competing hypotheses addressing the lack of phenotypes in the known *abp1* null mutants are worth discussing: 1) The *abp1-c1*, *abp1-TD1*, and *abp1-1* may not be null [9]; 2) Proteins with functions overlapping/redundant with ABP1 compensate for the loss of ABP1 [32,36,37]. All of the available *abp1* null alleles (*abp1-1*, *abp1-c1*, and *abp1-TD1*) have the mutations in the first exon of *ABP1* [6,26]. Although ABP1 protein was undetectable by Western analysis in these mutants, it remains possible that the ABP1 antibody used in these experiments might be specific to the ABP1 N-terminal region and thus might have failed to detect potentially functional truncated ABP1 proteins. No full-length or partial *ABP1* mRNAs could be identified in *abp1-TD1* [6]. However, transcript coverage by the primer pairs used for the RT-PCR reactions might have been insufficient or mRNA quantities may have been below the detection limit in these assays [9]. Therefore, it is still possible that the reported *abp1* null mutants make functional truncated ABP1 proteins. The only unequivocal means of guaranteeing an *abp1* null mutant is to delete the entire *ABP1* coding region from the genome. Advancements in CRISPR/Cas9 gene editing technology have made it feasible to obtain such mutants [22]. Indeed, we successfully generated two new *abp1* alleles, which contained 1141 bp and 711 bp deletions

in the *ABP1* gene, respectively (Zhao, unpublished results). These two new *abp1* mutants conclusively demonstrated that ABP1 is not required for normal Arabidopsis development.

The hypothesis that proteins with functions overlapping/redundant with ABP1 compensate for the loss of ABP1 has been raised repeatedly [32,36,37]. *ABP1* is a single copy gene in Arabidopsis, and there are no cryptic copies of *ABP1* in the Arabidopsis genome [25]. Using ABP1 protein sequence as a query to BLASTP the Arabidopsis proteins failed to yield any significant hits other than ABP1 itself (using an E-value ≤ 1 as the cutoff). Therefore, there are no detectable ABP1 homologs in the Arabidopsis genome. ABP1 is a member of the Cupin superfamily, which is a functionally diverse protein superfamily with little homology amongst family members [38,39]. Further, GLP4, a Golgi-located Cupin family protein, has been reported to bind auxin [40]. Thus, it was proposed that a lack of phenotypes in *abp1* null mutants might be due to redundant functions of Cupin proteins such as GLP4 [32,36,37]. However, the lack of homology between GLP4 and ABP1 beyond the two short Cupin motifs and their distinct sub-cellular locations suggest that it is very unlikely that ABP1 and Cupin family proteins have overlapping or redundant functions.

In conclusion, it appears that ABP1 research field has to be reset back to the 20th century. This is based on the Gao et al. findings showing no observable phenotypes in the *abp1* null mutants, the recent re-analysis showing problems of the previously used genetic materials, and that ABP1 is dispensable in some plant species.

Auxin efflux carriers as auxin receptors

Because ABP1 is likely not the auxin receptor for auxin-mediated non-genomic effects, it is worth revisiting the hypothesis that auxin efflux carriers may serve as auxin receptors. Such a hypothesis was first proposed in the 1960s, modified in the 1980s, and re-proposed in the 1990s, but it failed to gain traction [7]. PIN (PIN-FORMED) proteins are well-characterized auxin efflux carriers, playing essential roles in many developmental processes [41,42]. Can the PIN proteins also serve as auxin receptors? PIN proteins binds auxins and therefore meet one of the key criteria as auxin receptors. More importantly, genetic studies suggest that members of several gene families such as *PID (PINOID)*, *NPY (NAKED PINS IN YUC MUTANTS*), and *ARF* (*AUXIN RESPONSE FACTOR*) can potentially function downstream of *PIN1*. Disruption of *PIN1* in Arabidopsis leads to the development of pin-like inflorescences [41]. The same pin-like phenotypes have been observed in *pid* [43], *npy1 npy3 npy5* triple mutants [44,45], and *arf5/mp (auxin response factor 5/monopteros)* [46]. Genetic studies clearly place *PID* and *NPY* genes in the same pathway regulating auxin-mediated organogenesis [44,45]. Furthermore, the PID/NPY-mediated signaling pathway is strikingly analogous to the blue-light mediated phototropic pathway (Figure 1). In phototropic response, the phototropins have the N-terminal light-perception LOV domains and the C-terminal Ser/Thr protein kinase domain [47]. Upon receiving light signals by the LOV domains, the phototropin kinase is activated, which leads to a change in phosphorylation status of NPH3 (NONPHOTOTROPIC HYPOCOTYL 3) and phototropic responses [47,48]. The phototropin kinase domain is similar to the protein kinase PID. Further, NPH3 is homologous to the NPYs whereas ARF5/MP is very similar to ARF7/NPH4 (Figure 1). These two pathways differ in that phototropins contain both light-

perceiving LOV domains and a kinase domain while PID only has a kinase domain (Figure 1). PID may have a partner that perceives a signal such that PID combined with its partner is analogous to the phototropins.

PIN proteins serve as good candidates as PID partners. First, both *pid* and *pin1* develop the same pin-like inflorescences, suggesting that they participate in the same pathway [41,43]. Second, PID interacts and phosphorylates PIN1 [49]. Third, the genetic interactions among *pin1*, *pid*, and *npy* suggest that they participate in the same pathway [44,45,50,51]. The hypothesis that PIN proteins partner with PID to form auxin perception complexes can be tested experimentally. For example, determining whether NPYs are phosphorylated in a PIN1 or PID-dependent manner will strengthen or weaken this hypothesis. Previous studies focused on the capacity and directionality of PIN-mediated auxin transport. This new model suggests that auxin transport may be coupled with a signal transduction pathway, which can reasonably account for the observed pin-like phenotypes in various Arabidopsis mutants.

$SCF^{TIR1/AFB}$-Aux/IAA auxin receptor complexes

Auxin perception by $SCF^{TIR1/AFB}$-Aux/IAA complexes and their essential roles in auxin-mediated transcription regulation and plant development have been well established [2]. The $SCF^{TIR1/AFB}$-mediated auxin signaling pathway requires a TIR1/AFB F-box protein, an Aux/IAA transcriptional repressor, and an ARF transcription factor. Auxin-binding brings Aux/IAA proteins to the $SCF^{TIR1/AFB}$ ubiquitin E3-ligase complex and results in degradation of the Aux/IAA repressors and activation of the ARFs. Recent progress in auxin perception and transcription regulation by $SCF^{TIR1/AFB}$ have been reviewed elsewhere [2]. Here we focus on several recent studies on mechanisms by which $SCF^{TIR1/AFB}$-Aux/IAA auxin receptor complexes may be regulated.

TIR1 protein abundance appeared to be controlled by an autocatalytic mechanism. Yu et al. uncovered a series of mutations in the first helix of TIR1 that disrupt TIR1 association with CULLIN 1 [52]. Consequently, the mutated proteins were stabilized, indicating that un-tethering TIR1 from the SCF complex affects the TIR1 stability and that TIR1 itself is likely a substrate for the SCF^{TIR1} ubiquitin E3 ligase.

Aux/IAA recruitment to the $SCF^{TIR1/AFB}$ ubiquitin E3 ligase is a key step in auxin signaling and is dependent on the presence of auxin and the domain II degron [2]. Recently, it was shown that the configuration of a conserved proline residue in the degron dictates Aux/IAA recruitment to the $SCF^{TIR1/AFB}$ complex, adding another layer of complexity to auxin signaling [53]. Mutations in rice *LATERAL ROOTLESS2* (*LRT2*), which encodes a cyclophilin-type peptidyl-prolyl cis/trans isomerase, result in auxin resistance and lateral root development defects [53]. LRT2 catalyzes the *cis/trans* isomerization of the Trp^{104}–Pro^{105} peptide bond of OsIAA11, promoting the formation of the Pro^{105}-*cis* configuration, which is preferred during the assembly of a $SCF^{TIR1/AFB}$Aux/IAA auxin receptor complex [53]. These new insights into $SCF^{TIR1/AFB}$ regulation may provide additional mechanisms for auxin response regulation.

Auxin signaling downstream of auxin perception

ARF proteins activate or repress gene expression by binding cis-regulatory auxin response elements (AuxREs) in the promoters of auxin-responsive genes and serve as key mediators of auxin transcriptional outputs [54,55]. Recent advances in understanding ARF function and regulation include insights provided by ARF recruitment of chromatin remodeling factors and roles for tasiRNA regulation of ARFs to make the auxin-regulated gene expression network robust.

Chromatin remodeling as a mechanism of auxin-responsive gene regulation is only beginning to be understood [56]. Aux/IAA repressors are recruited to specific chromosome regions by interaction with ARF proteins, and in turn recruit TOPLESS and TOPLESS-related co-repressors (Figure 2) [57,58]. These corepressors recruit histone deacetylases that act to condense DNA to prevent gene transcription. A long-standing model of auxin signaling involves de-repression of ARF proteins by Aux/IAA proteolysis. After Aux/IAA disappearance, how then, is transcription to occur if the chromatin is still in a condensed state? An answer to this question may come from a recent study that identified roles for the SWI/SNF nucleosome-remodeling complex in promoting transcription of ARF targets (Figure 2) [59]. The BRAHMA and SPLAYED subunits of the SWI/SNF complex directly interact with ARF5 in a manner dependent on Aux/IAA removal [59]; because SWI/SNF complexes act to de-condense DNA, its recruitment likely acts to reverse the gene repression previously imposed by Aux/IAAs (Figure 2). Although this has yet to be demonstrated as a widespread mechanism of promoting auxin-regulated transcriptional responses, it provides an attractive mechanism to counteract the effects of Aux/IAA proteins and to allow for recruitment of the transcriptional machinery.

In addition to protein regulation by interaction with repressors and chromatin remodeling factors, *ARFs* can be regulated posttranscriptionally. Several repressing *ARFs* have been shown to be regulated by miRNAs [60]. In addition, tasiRNAs have been demonstrated as important regulators of repressing *ARFs* that serve to increase the robustness of auxin response. Working with the bryophyte model plant *Physcomitrella patens*, Plavskin et al. [61] showed that loss of the tasiRNA system in Physcomitrella resulted not only in a decreased auxin response, caused by an increased accumulation of repressor ARF, but also resulted in greater stochasticity in auxin responses. With the use of an elegant combination of experimental data and modeling [61], the authors created a model in which tasiRNAs act to buffer the noise in the auxin gene response network; this role may explain why tasiRNAs have been co-opted frequently into various gene response networks.

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Highlights

- Genetic evidence supporting the proposed roles for ABP1 in auxin signaling has been invalidated
- The PIN auxin efflux carriers may be the missing auxin receptors for non-genomic auxin responses
- Several mechanisms control $SCF^{TIR1/AFB}$-Aux/IAA auxin receptor complex assembly and stability
- Chromatin-remodeling and small RNAs participate in regulating auxin-mediated transcription

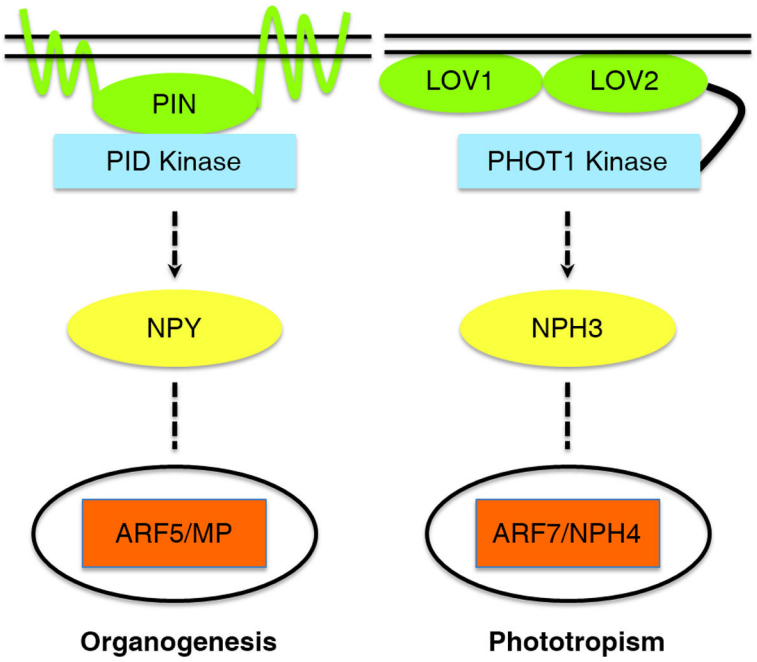

Figure 1. A schematic presentation of the hypothesis that PIN and PID may form a plasma-membrane localized auxin receptor complex important for auxin-mediated Arabidopsis organogenesis

The organogenesis pathway is strikingly analogous to the blue light-mediated phototropic response. Arabidopsis plants with compromised PIN1 or PID or NPY or ARF5/MP fail to produce flowers whereas mutations in PHOT1 or NPH3 or ARF7/NPH4 cause defects in normal phototropic responses to blue light. PID is homologous to PHOT1 and NPYs are homologous to NPH3. Moreover, ARF5/MP is very similar to ARF7/NPH4. The starting point for phototropic response is the perception of light by the LOV domains in PHOT1. Light activates the kinase activity in PHOT1 and consequently phosphorylation status of NPH3 is altered. The role of ARF7/NPH4 in phototropism is not understood because phototropic response is generally believed to be non-genomic. PID lacks a receptor domain and we hypothesize that PIN proteins may function as an auxin receptor based on genetic evidences. The function of PIN/PID complex in the auxin-mediated organogenesis pathway is equivalent to PHOT1 in the phototropism pathway.

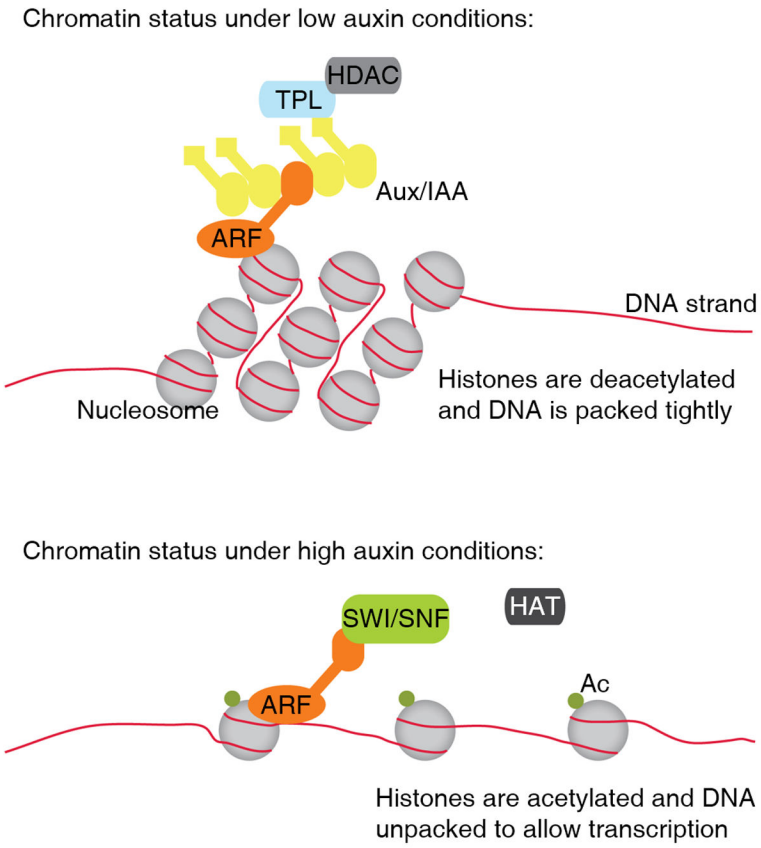

Figure 2. Roles for chromatin packing in auxin-mediated transcriptional response

Under low auxin conditions, Aux/IAA proteins are abundant and interact with ARF proteins. Aux/IAAs recruit the TOPLESS (TPL) family of corepressors, which in turn recruit Histone deacetylases (HDACs), resulting in tightly packed chromatin. Furthermore, Aux/IAA proteins likely block the interaction between ARF and SWI/SNF chromatin remodeling complexes. When auxin levels are elevated, Aux/IAA proteins are targeted for proteasomal degradation, allowing for SWI/SNF interaction with ARF proteins. SWI/SNF-enabled chromatin remodeling, through histone acetyltransferase (HAT) activity, results in chromatin unpacking to allow gene target access by additional transcription factors.