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Yokonolide B, a Novel Inhibitor of Auxin Action, Blocks Degradation of AUX/IAA Factors*[S]

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Yokonolide B (YkB; also known as A82548A), a spiroketal-macrolide, was isolated from *Streptomyces diastatochromogenes* B59 in a screen for inhibitors of β-glucoronidase expression under the control of an auxin-responsive promoter in *Arabidopsis*. YkB inhibits the expression of auxin-inducible genes as shown using native and synthetic auxin promoters as well as using expression profiling of 8,300 *Arabidopsis* gene probes but does not affect expression of an abscisic acid- and a gibberellin A_3-inducible gene. The mechanism of action of YkB is to block AUX/IAA protein degradation; however, YkB is not a general proteasome inhibitor. YkB blocks auxin-dependent cell division and auxin-regulated epinastic growth mediated by auxin-binding protein 1. Gain of function mutants such as *shy2-2*, *slr1*, and *axr2-1* encoding AUX/IAA transcriptional repressors and loss of function mutants encoding components of the ubiquitin-proteolytic pathway such as *axr1-3 and tir1-1*, which display increased AUX/IAAs protein stability, are less sensitive to YkB, although *axr1* and *tir1* mutants were sensitive to MG132, a general proteasome inhibitor, consistent with a site of action downstream of AXR1 and TIR. YkB-treated seedlings displayed similar phenotypes as dominant *AUX/IAA* mutants. Taken together, these results indicate that YkB acts to block AUX/IAA protein degradation upstream of AXR and TIR, links a shared element upstream of AUX/IAA protein stability to auxin-induced cell division/elongation and to auxin-binding protein 1, and provides a new tool to dissect auxin signal transduction.

Auxin controls cell division, elongation, and differentiation and therefore, through its action at the level of the cell, exerts profound effects on growth and development throughout the life of the plant (1). Consistent with the diverse effects of auxin on growth and development is that the expression patterns of a number of genes are dramatically and rapidly altered by auxin application (2), suggesting that auxin ultimately regulates cell growth by controlling the profile of expressed genes. *AUX/IAA* genes comprise a 34-member gene family in *Arabidopsis* that is one of three known gene families that are regulated by auxin and implicated to play essential roles in auxin signaling (3, 4).

The molecular and genetic studies on auxin signaling have revealed that auxin specifically enhances the transcription of many *AUX/IAA* genes within minutes without requiring *de novo* protein synthesis, suggesting that *AUX/IAA* genes are primary auxin-response genes (5, 6). *AUX/IAA* genes encode short lived nuclear proteins capable of heterodimerization with auxin-responsive factors (7) and are thought to act by negatively regulating the expression of early auxin-responsive genes including other members of the *AUX/IAA* family (3).

Studies on *Arabidopsis* mutants with altered responses to auxin such as *iaa3*/*shy2-2*, *iaa7*/*axr2-1*, *iaa17*/*axr3*, *iaa14*/*slr1*, *tir1*, and *axr1* revealed that the turnover rate of AUX/IAA proteins is in some way important in various developmental processes including lateral root growth, hypocotyl elongation, gravitropism, and photomorphogenesis (8, 9). Dominant mutations in domain II of AUX/IAA as defined by *iaa3*/*shy2-2*, *iaa7*/*axr2-1*, *iaa14*/*slr1*, and *iaa17*/*axr3* alleles confer resistance to ubiquitin-mediated degradation (10, 11). *AXR1* is a subunit of the heterodimeric Nedd8/RUB1-activating enzyme that mediates the first step in the conjugation of the ubiquitin-like modification Nedd8/RUB1 to the cullin subunit of Skp1/Cullin/F-box (SCF)[1]-type E3s (12, 13), and *TIR1* encodes an F-box protein interacting with the Skp1 and Cdc53 (Cullin) proteins to form ubiquitin ligase complexes called SCFs (14, 15). This SCF^{TIR} complex mediates a proteolytic pathway responsible for the degradation of AUX/IAA repressors and ultimately impinges on gene expression including its own. Auxin increases the degradation rate of AUX/IAA proteins (16) and promotes the interaction between AUX/IAA and TIR proteins (9). However, probably due to gene redundancy and complex feedback control of *AUX/IAA* expression, loss of function of any specific *AUX/IAA* gene has little, if any, phenotype. The molecular mechanism by which auxin activates the expression of primary auxin-regulated genes and then elicits physiological responses is not fully understood. Therefore, the approach to this problem

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[S] The on-line version of this article (available at http://www.jbc.org) contains an additional table and figure.

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[1] The abbreviations used are: SCF, Skp1/Cullin/F-box; ABA, abscisic acid; ABP1, auxin-binding protein 1; AhTet, anhydrotetracycline; 2,4-D, 2,4-dichlorophenoxyacetic acid; FC, fusicoccin; GA, gibberellin A_3; GST, glutathione *S*-transferase; GUS, β-glucuronidase; NAA, naphthalene-1-acetic acid; YkB, yokonolide B (A82548A); MS, Murashige-Skoog; MES, 2-(*N*-morpholino)ethanesulfonic acid; NPA, 1-naphthylphthalamic acid; E3, ubiquitin-protein isopeptide ligase; MAPK, mitogen-activated protein kinase; RT, reverse transcriptase.

with loss of function for multiple AUX/IAA proteins will need to be accomplished either genetically or biochemically using specific inhibitors.

There are a number of candidate signaling components residing between auxin perception and the action of the AUX/IAA proteins. For example, the MAPK cascade pathway may link auxin-responsive gene expression with apical signaling component(s) (17), because MAPK kinase inhibitors inhibit the expression of a reporter gene driven by an auxin-responsive promoter (18). Phospholipase A_2 is rapidly activated by auxin and could act apically to gene expression (19). Finally, auxin regulation of cell division involves a heterotrimeric G protein (20).

Clearly auxin action is complex with multiple molecular pathways that probably interact via negative and positive feedback. One reason for our incomplete understanding of auxin action is the lack of bioprobes to dissect this complexity. Previously, we isolated yokonolide A and a known related compound, A82548A (designated here as yokonolide B (YkB)) (Fig. 1*A*), from *Streptomyces diastatochromogenes* B59 as inhibitors of auxin-responsive gene expression using a GUS reporter line under the control of an auxin-inducible promoter (21, 22). We report here the biological activities of YkB, demonstrate its specificity, and with it, connect the auxin signaling components to cell division and elongation.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions—For all experiments, *Arabidopsis thaliana* ecotype Columbia, Landsberg *erecta*, and tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR-1) seeds were used as controls. The available *Arabidopsis* mutants were obtained from the *Arabidopsis* Biological Resource Center. Transgenic *Arabidopsis* β-glucoronidase (GUS) reporter lines P_{IAA7}::*GUS*, P_{IAA3}::*GUS*, and *ARR5*::*GUS* and *shy2-2* were provided by Drs. J. Reed and J. Kieber (University of North Carolina). The transgenic *P-IAA 4/5* promoter (Δ-2309)::*GUS* and *parA*::*GUS* lines were provided by Drs. A. Theologis (United States Department of Agriculture, Albany, CA) and Y. Takahashi (University of Tokyo, Japan). The *slr1* mutant was a gift from Dr. H. Fukaki (Nara Institute of Science and Technology, Japan). Tobacco BY2 and *Arabidopsis* T-87 cells were obtained from the RIKEN plant cell bank (Japan). The *HS*::*AXR3NT-GUS* line was provided by Dr. M. Estelle (University of Indiana).

Suspension-cultured tobacco cells (*N. tabacum* cv. BY-2) were maintained in a modified MS medium supplemented with 1 μM 2,4-dichlorophenoxyacetic acid (2,4-D) as described in Ref. 23 on a rotary shaker (100 rpm) at 25 °C in the dark. Auxin deprivation was carried out by washing a 7-day culture twice with the same medium lacking 2,4-D and then cultured in auxin-free medium for 24 h before auxin addition and determination of mitotic indices over time. Tobacco lines MJ10B carrying a tetracycline-inducible ABP1 transgene and the corresponding empty vector control line, R7, were described in Ref. 24. Tobacco plants were grown in soil under continuous light at 23 °C.

Chemicals—MG132 was purchased from The Peptide Institute (Osaka, Japan). YkB (A82548A), shown in Fig. 1*A*, was isolated from culture broth of *S. diastatochromogenes* B59 as previously described (21). Pure samples of YkB (<200 μg/researcher) are available for noncommercial research. The pUB23 plasmid used in the yeast assays was provided by Dr. D. Finley (Harvard Medical School).

Hormone Induction—Seedlings (n = 10–15) were transferred to a 12- or 24-well microtiter plate containing 1 ml of germination medium (0.5× Murashige and Skoog salts (Invitrogen), 1% sucrose, 1× B_5 vitamins, and 0.2 g/liter MES, pH 5.8) containing the indicated hormone and/or chemicals and then incubated for the indicated time to induce each responsive gene. For the *P-IAA 4/5*::*GUS* reporter line, we used the experimental methods previously described (25).

Histochemical GUS Staining and Quantitative Fluorometric GUS Assays—Whole seedlings (n = 10–20) or the roots were homogenized in an extraction buffer as described in Ref. 26. After centrifugation to remove cell debris, GUS activity was measured with 1 mM 4-methyl umbelliferyl β-D-glucuronide as a substrate at 37 °C. For the histochemical GUS assay, the seedlings were washed three times with buffer A (100 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.5 mM $K_4Fe(CN)_6$, 0.5 mM $K_3Fe(CN)_6$, and 0.1% Triton X-100) and then incubated in a staining buffer (buffer A containing 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide, the substrate for histochemical staining (X-gluc)) at 37 °C until sufficient staining developed.

RT-PCR—mRNA was extracted from 100 mg of treated tissue using the QuickPrep Micro mRNA purification kit (Amersham Biosciences) according to the manufacturer's instructions. The corresponding cDNAs were synthesized and amplified by the PCR using primers for the indicated genes as follows: *IAA1*, 5′-ggattacccggagcacaag and 5′-ggagctccgtccatactcac; *IAA5*, 5′-agatatcgtcgtctccggtg and 5′-gccgaagcaagatcttggta; *SAUR-AC1*, 5′-ttgaggagtttcttgggtgc and 5′-catggtattgttaagccgcc; *RAB18*, 5′-ttgggaggaatgcttcacc and 5′-ttgttcgaagcttaacggc; *ACTIN* (*ACT2*), 5′-aacattgtgctcagtggtgg and 5′-tcatcatactcggccttgg. The amplified products (*IAA1*, 208 bp after 27 cycles; *IAA5*, 251 bp after 25 cycles; *SAUR-AC1*, 220 bp after 27 cycles; *RAB18*, 269 bp after 27 cycles; *ACT2*, 206 bp after 25 cycles) were analyzed by 3% agarose gel electrophoresis.

Assays—Aleurone layers were prepared from de-embryonated barley seeds (*Hordeum vulgare* L. cv. Kinuyutaka) as previously described (27). Briefly, isolated aleurone layers were incubated in 5 mM $CaCl_2$ solution containing 0 or 5 μM gibberellin A_3 (GA) and the indicated concentrations of YkB at 25 °C with reciprocal shaking (80 rpm) for 24 h. Aleurone layers were homogenized in 50 mM acetate buffer (pH 5.4, 20 mM $CaCl_2$) and centrifuged. After centrifugation, the α-amylase activity was measured using RBB-starch (Remazol Brilliant Blue R-dyed starch, Sigma) as previously described (28).

BY2 tobacco cells were used for the auxin transport assay exactly as previously described (29). Cells (0.5 g, fresh weight) were resuspended in transport buffer (29) to a final density of 0.1 g/ml and aliquoted into 25-ml flasks containing [^{3}H]NAA with or without 10 μM YkB and/or 10 μM 1-naphthylphthalamic acid (NPA). Flasks were rotated (80 rpm), and for the indicated durations, aliquots of cells were collected by filtration and washed with water (5 ml). Fresh weight of cells was measured, and the radioactivity was counted on filters by liquid scintillation. Experiments were repeated twice.

Seedlings (7 days old, n = 15) harboring the *HS*::*AXR3NT-GUS* transgene were heat-shocked at 37 °C in liquid germination medium containing 1.5% sucrose for 2 h. After 20 min at 23 °C, the indicated inhibitors and auxin were added to the medium. After the indicated times, degradation was stopped by immersing the seedlings into ice-cold 70% acetone for 20 s. Seedlings were immediately washed with distilled water and stored frozen prior to the GUS activity measurement described above. The experiment was performed in triplicate.

The yeast *ise1* deletion mutant (*Saccharomyces cerevisiae* Hansen BY4742 *mat alpha his3D1 leu2D0 lys2D0 ura3D0*, ResGen, record number:10568), harboring a pUB23 plasmid encoding the galactose-inducible ubiquitin-β-galactosidase fusion protein, was cultured on uracil dropout medium (0.67% yeast nitrogen base without amino acids, 0.5% casamino acids, 20 μg/ml L-tryptophan, 20 μg/ml adenine) supplemented with 2% galactose until midlog phase. Cells were washed two times with uracil dropout medium supplemented with 2% glucose to stop further transcription of the fusion protein and resuspended in the same medium containing YkB or MG132 and cultured at 28 °C. Galactosidase activity was measured as previously described (30). ATP-dependent 20 S core unit activity of 26 S proteasome in *Arabidopsis* suspension T-87 cells was measured by peptide-hydrolysis using succinyl-Leu-Leu-Val-Tyr-4-methyl-coumaryl-7-amide as the substrate in the presence or absence of ATP and Mg^{2+}, as previously described (31).

Measurement of NAA-induced tobacco leaf curvature was as described in Ref. 32 with slight modification. The leaf strips were prepared from the sixth leaf of the seven-leaf staged transgenic tobacco MJ10B (tetracycline-inducible ABP1) or R7 (empty vector control) lines. One end of each strip was clamped by a small rubber block. Blocks of 12 interveinal leaf strips were placed in Petri dishes containing 5 ml of buffer (10 mM sucrose, 10 mM KCl, and 0.5 mM MES, pH 6.0) with or without the indicated concentrations of the test compounds. To induce ectopic expression of ABP1, MJ10B and R7 strips were incubated for 4 h in solution containing 4 μg/ml anhydrotetracycline (AhTet) prior to NAA and YkB addition. Photographs of the strips were taken, and the degree of curvature for each strip was measured with NIH Image software (National Institutes of Health, Bethesda, MD).

For the root growth assay, surface-sterilized, stratified seeds were germinated on plates and then transferred onto new plates containing the indicated concentration of chemicals. Seedlings were cultivated vertically under continuous light condition at 23 °C. For dark-grown cultures, seeds were exposed to light for 8–12 h after stratification then treated as above except in darkness.

For the hypocotyls growth assay, the surface-sterilized, stratified seeds were cultured in liquid medium with rotation (80 rpm) until germinated under continuous light or darkness for 2 days before the indicated concentrations of chemicals were added to the medium. Dig-

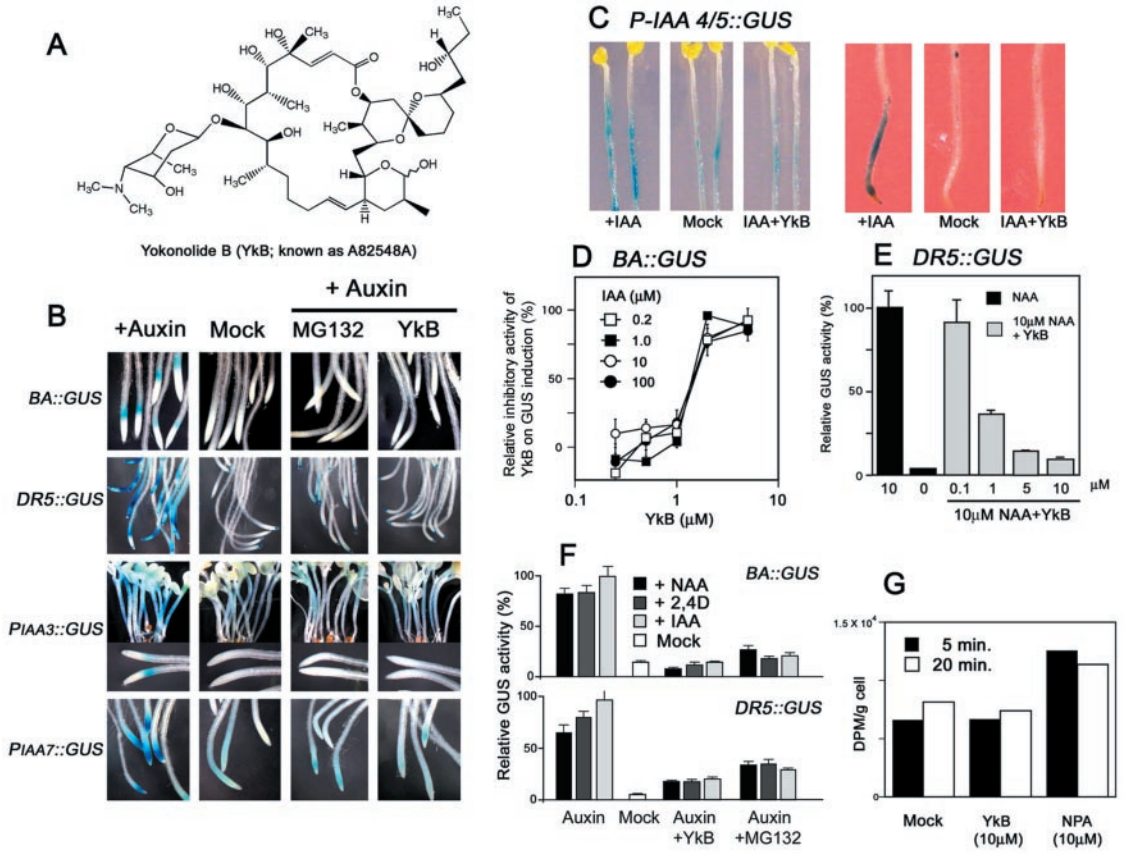

FIG. 1. **Effects on YkB and MG132 on primary auxin-responsive reporter gene expression.** *A*, the chemical structure of YkB (known as A82548A). *B*, effects of YkB and MG132, a 26 S proteasome inhibitor (20 S core protease), on auxin-inducible *BA*::*GUS*, *DR5*::*GUS*, *IAA3*::*GUS*, and *IAA7*::*GUS* reporter gene expression. *Arabidopsis* transgenic lines were treated with 5 μM YkB or 50 μM MG132 in the presence of 10 μM auxin for the following times: *BA*::*GUS* line with IAA and *DR5*::*GUS* with NAA for 3 h, P_{IAA3}::*GUS* and P_{IAA7}::*GUS* with IAA for 7 h. *C*, effect of YkB on IAA-inducible *P-IAA 4/5*::*GUS* reporter gene expression. Transgenic tobacco seedlings (7 days old) were treated with IAA for 8 h. The *gray* and *red background panels* show treated, etiolated hypocotyls and roots, respectively. *Left*, 10 μM IAA alone; *center*, mock; *right*, 10 μM IAA and 5 μM YkB. *D*, dose-response curve for inhibitory activity of YkB on IAA-induced reporter gene expression in *Arabidopsis BA*::*GUS* line. The roots (5 days old) were incubated with YkB and IAA for 5 h. Induced GUS activity was measured fluorometrically as described under "Experimental Procedures." *Error bars*, S.E. *E*, effect of YkB on auxin-inducible reporter gene expression in *DR5*::*GUS* line. Roots (6 days old) were incubated with YkB and NAA for 10 h. The induced GUS activity by NAA is adjusted to 100% value. *F*, comparison of the YkB and MG132 effects on auxin-induced *BA* and *DR5* reporter gene expression using three diagnostic auxins. Seedlings (5 days old) were incubated with 5 μM YkB or 50 μM MG132 together with 10 μM auxin for 5 h. The induced GUS activity by IAA is adjusted to 100% value. *G*, auxin efflux transport assays. BY2 tobacco cells were suspended in the presence of [^{3}H]NAA with or without 10 μM YkB or 10 μM NPA.

ital photographs of roots and hypocotyls were taken and analyzed by NIH Image software to measure the length of roots and hypocotyls.

The lateral root promotion assay was carried out as described in Ref. 33. The seedlings were vertically grown in light on the 1/2 MS plate (1% sucrose) containing 0.5 μM NPA for 9 days to inhibit auxin transport. The seedlings were transferred to 1/2 MS plates containing YkB with or without 0.1 μM NAA. Lateral roots were counted after an additional 4 days of vertical cultivation in light.

Gene Expression Profiling—Etiolated *Arabidopsis* seedlings (4 days old), cultured in 1/2 MS liquid medium containing 1% sucrose, were treated with the indicated concentration of compounds for 20 min in the dark. The seedlings were then rapidly frozen in liquid nitrogen, and total RNA was isolated per the manufacturer's instructions (Plant RNAeasy kit; Qiagen, Tokyo, Japan). In order to average out variability but to minimize the number of chips needed, treatments were done in triplicate, and frozen seedlings were pooled followed by three RNA isolations, which were then pooled after a quality control check of the RNA. The raw intensity data of each condition obtained from imaging the hybridized microarrays (Affymetrix, Santa Clara, CA) were normalized using the global normalization method and further analyzed by MAExplorer freeware developed by the National Cancer Institute. The obtained profiles of regulated genes were evaluated individually with Student's *t* test. All profiles were statistically significant ($p < 0.01$).

RESULTS

YkB Blocks Primary Auxin-responsive Gene Expression—*BA*::*GUS* plants contain an auxin-inducible promoter composed of two auxin-responsive elements derived from the pea *AUX/IAA* promoter, *P-IAA 4/5* (26). YkB (5 μM) completely inhibited IAA-induced GUS reporter gene expression in the *BA*::*GUS* line (Fig. 1, *B* and *D*). The activity of the synthetic *DR5* promoter, which is composed of tandem elements taken from the primary auxin-responsive *GH3* promoter (34), was also inhibited by 5 μM YkB (Fig. 1, *B* and *E*). In addition to synthetic promoters, YkB also inhibited GUS driven by the native auxin-inducible promoters, P_{IAA3}::*GUS*, P_{IAA7}::*GUS*, and pea *P-IAA 4/5*::*GUS* (Fig. 1, *B* and *C*) in roots and hypocotyls (25, 35). YkB does not alter GUS activity *in vitro* (data not shown) and does not compete with auxin at an auxin-binding site because YkB inhibition was independent of auxin concentration (Fig. 1*D*). The effects of YkB on the steady-state levels of primary auxin-responsive *AUX/IAAs* (*IAA1*, *IAA5*) and *SAUR-AC1* (36) messages were directly confirmed using RT-PCR (Fig. 2*A*). These results suggest that YkB is an effective inhibitor of expression of auxin-responsive genes.

Two lines of evidence indicate that YkB does not act by altering the level of auxin in cells. Auxin influx carriers transport IAA and 2,4-D into the cell, whereas NAA entry is unfacilitated. In contrast, auxin efflux carriers transport IAA and NAA from the cell, but the movement of 2,4-D is unfacilitated;

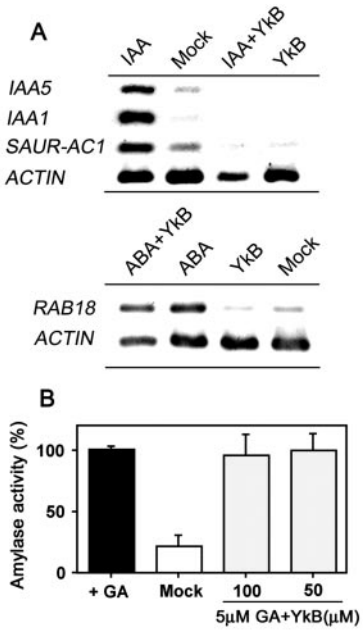

FIG. 2. **Effects of YkB on gene expression.** *A*, YkB effect on steady-state levels of IAA-induced *IAA1*, *IAA5*, *SAUR-AC1*, and of ABA-induced *RAB18* transcripts in *Arabidopsis*. For *IAA1*, *IAA5*, and *SAUR-AC1* induction, 5-day-old, etiolated seedlings were treated with 10 μM IAA for 45 min after preincubation with or without 5 μM YkB for 10 min. The steady state mRNA level was analyzed by RT-PCR. For ABA-inducible *RAB18* transcripts, 13-day-old, light-grown plants were incubated in 50 μM ABA with or without 10 μM YkB for 18 h. *B*, the effects of YkB on GA-induced α-amylase expression. Barley aleurone layers were treated with 5 μM GA for 24 h together with or without YkB. *Error bars*, S.E.

thus, any difference in the activity profiles of these three auxins is diagnostic for a role in auxin transport (29). As shown in Fig. 1*F*, YkB was equally effective at blocking auxin-induced gene expression by these three auxins, suggesting that YkB does not alter auxin influx. Furthermore, as shown in Fig. 1*G*, YkB had no effect on [^{3}H]NAA efflux through BY2 cells as measured directly in standard accumulation experiments. In addition, YkB showed no effects on 2,4-D influx in BY2 cells, indicating that YkB does not alter auxin uptake (data not shown).

To assess whether this inhibitory activity is specific to primary auxin-responsive genes, the effects of YkB were assayed using the *Arabidopsis* cytokinin-inducible ARR5 reporter line (37), *Arabidopsis* ABA-inducible gene (*RAB18*) transcription (38), and barley GA-inducible α-amylase expression (Fig. 2, *A* and *B*). YkB had no effect on ABA-inducible *RAB18* gene expression or GA-inducible α-amylase expression. At relatively high concentrations of YkB, cytokinin-induced GUS expression in the *ARR5*::*GUS* line was partially reduced (data not shown).

YkB Inhibits Degradation of an AUX/IAA Repressor, but Not 26 S Proteasome Activity—Because auxin alters the stability of AUX/IAA repressors, which in turn could explain the results on reporter gene expression above, we measured the effect of YkB on auxin-induced degradation of an AUX/IAA protein *in vivo* (9, 11). The *Arabidopsis HS*::*AXR3NT-GUS* transgenic line strongly expresses an AUX/IAA-GUS (IAA17/AXR3-GUS) translational fusion protein under the heat shock promoter (9). The degradation rate of the AUX/IAA fusion protein is rapid and is enhanced by auxin, but it is inhibited by the 20 S core protease inhibitor, MG132. Both MG132 and YkB inhibited degradation of the fusion protein even in the presence of auxin (Fig. 3, *A* and *B*). This inhibitory activity of YkB on AUX/IAA-GUS degradation suggests that the inhibition of primary auxin-responsive gene expression is achieved by AUX/IAA repressor stabilization. The effect of MG132 on auxin-responsive reporter gene induction confirmed this regulatory model (Fig. 1*B*).

To test the possibility that YkB is a general proteasome inhibitor, we examined the effects of YkB on ATP-dependent proteasome activity using *Arabidopsis* T-87 cells and an *S. cerevisiae ise1* (*erg6*) strain expressing ubiquitin-β-galactosidase fusion protein as proteasome substrate under the control of the GAL4 promoter (39, 40). The *ise1* yeast strain was chosen to preclude detoxification of the drug by pumping via multidrug resistance channels (41). As shown in Fig. 3*C*, β-galactosidase activity was decreased within 3 h in the untreated *ise1* yeast cells. MG132 prevented the β-galactosidase fusion protein from degradation indicated by unchanged β-galactosidase activity over time. In contrast, YkB had no effect on the rate of degradation of the fusion protein, illustrating that, at a concentration that is supraoptimal to inhibit AUX/IAA degradation in plants, YkB does not alter proteasome activity in yeast. Moreover, MG132 and YkB did not affect the growth rate of yeast, suggesting that the distinct effect between MG132 and YkB on the degradation of the β-galactosidase fusion protein was not due to the effect on yeast growth (Fig. 3*C*). The 20 S core unit proteolytic activity of the 26 S proteasome in *Arabidopsis* T-87 cells was directly measured with a fluorogenic peptide substrate. As shown in Fig. 3*D*, YkB did not inhibit plant proteasome activity in contrast to complete inhibition by MG132. Furthermore, YkB (100 μM) did not inhibit GA-induced barley α-amylase expression, which is known to be repressed by MG132, since it requires the degradation of SLN1 repressor via the ubiquitin-proteolytic pathway (42). Taken together, these results suggest that the inhibition of primary auxin-responsive genes by YkB is due to AUX/IAA stabilization but not general proteasome inhibition and that this site of action of YkB is specific to auxin.

YkB Blocks Auxin-regulated Gene Expression Globally—The effect of auxin and YkB on gene expression was performed with the hybridized microarray using an 8,300-gene probe set. Several interesting patterns were identified supporting the conclusion that YkB, in concert with auxin, regulates the expression of many genes including primary auxin-responsive genes. Four-day-old, etiolated *Arabidopsis* seedlings were treated with or without auxin in the presence or absence of YkB for 20 min. Approximately 4,500 gene sets hybridized detectable cRNA in all five conditions as indicated in Fig. 4*A* and were used for further expression analysis. Representative clustered genes among the 74 up-regulated and 113 down-regulated gene sets are shown (the entire sets are provided in supplemental data). Gene profiling used strict *p* value and change criteria as well as triplicates to assure that the differences are statistically significant. Four major types of expression profiles (designated a–d) were clustered into up-regulated gene sets (Fig. 4*A*). The classes of up-regulated genes (profiles a, b, and c) included *ACS* (ethylene biosynthesis gene), defense/stress-related proteins, and transcription factors. Type a profiled genes required both auxin and YkB for up-regulation, and the genes in type b are up-regulated by auxin, which is further enhanced by YkB. Consistent with the results obtained through reporter lines and direct measurement of mRNA steady-state levels, half of the IAA up-regulated genes were inhibited by YkB (profile c). Profile c comprised *AUX/IAA*, *SAUR*, *GH3*, and *ACS* genes. However, no *SAUR*, *GH3*, *AUX/IAA*, or *ACS* genes were up-regulated by YkB alone. Four expression profiles (H–K) define the down-regulated genes. The down-regulated genes (types H and I) contained defense/stress-related, metabolic enzyme, cell wall-related, and IAA biosynthesis genes. The largest gene set is illustrated in profile K (82% of down-regulated genes) and

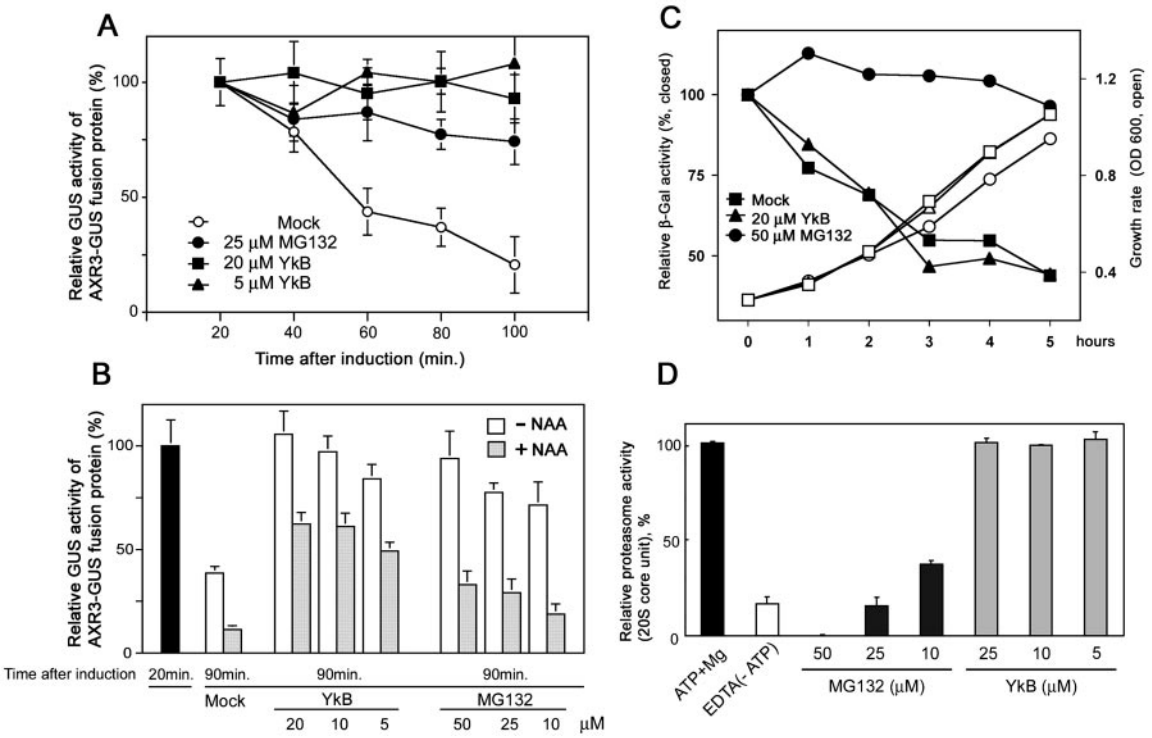

FIG. 3. **Effects of YkB and MG132 on AUX/IAA protein stability.** *A*, inhibition of YkB and MG132 on IAA17/AXR3-GUS fusion protein degradation was determined using 7-day-old, light-grown *HS*::*AXR3NT-GUS Arabidopsis*, which were heat-shocked (37 °C) for 2 h to induce expression of the fusion protein. The seedlings ($n = 15$) were treated with YkB or MG132 for the indicated periods after incubation for 20 min at 23 °C. The retained GUS activity was measured as described under "Experimental Procedures." *B*, inhibition of YkB and MG132 on auxin destabilization of IAA17/AXR3-GUS fusion protein. After induction, the retained GUS activity was measured at the indicated time. *C*, effect of YkB on the degradation of ubiquitinated β-galactosidase in *S. cerevisiae* (*ise1*). *Closed* and *open symbols* indicate relative β-galactosidase activity at time after the exchange for medium containing glucose to repress *GAL4* promoter activity and growth rate (A_{600}), respectively. *Squares*, control; *triangles*, 20 μM YkB-treated; *circles*, 50 μM MG132. *D*, *Arabidopsis* ATP-dependent proteasome inhibition by YkB and MG132 in T-87 suspension cells. 20 S core unit activity in 26 S proteasome was assayed as described under "Experimental Procedures." *Error bars*, S.E.

required both auxin and YkB for down-regulation. Major classes of genes in profile K are ribosomal, light-responsive, ubiquitin-pathway related, auxin-responsive genes; auxin synthesis genes, including tryptophan synthetase; and stress/defense-related genes, including those encoding heat shock proteins and glutathione *S*-transferase (GST).

The quantitative change by YkB in gene chip on auxin-responsive gene expression, such as *AUX/IAAs* and *SAURs*, appears to be less than as determined for *IAA1*, *IAA5*, and *SAUR-AC1* genes by RT-PCR because of differences in experimental protocol. For the RT-PCR experiment, seedlings were preincubated with YkB (10 min) before the addition of auxin and treated for a longer induction period (45 min), whereas for the gene profiling experiment, a shorter induction period (20 min) and the simultaneous addition of auxin and YkB were used in order to detect the earliest changes in gene expression. The higher sensitivity of the gene profiling technique over the GUS reporter system enabled us to detect these differences at an earlier point without preincubation.

Evidence suggests that the stress-activated MAPK pathway negatively regulates auxin-responsive gene expression (43–45), and our gene chip data indicated that YkB, in concert with auxin, modulates the expression of stress/defense-related genes. To assess a possible linkage of YkB action between stress/defense with auxin signaling, we examined the effect of YkB on auxin and stress-inducible *parA* promoter (encoding GST-like protein) (46). In long term treatments (12 h), *parA*::*GUS* promoter activity was enhanced by YkB alone much effectively than 2,4-D (Fig. 4*B*), implying that, by itself and together with auxin, YkB modulates stress and auxin shared signal, leading to regulation of auxin-responsive gene expression.

YkB Inhibition Links AUX/IAA Stability to Auxin-regulated Cell Elongation and Division and Auxin-binding Protein 1 (ABP1)—Auxin is essential for the growth of suspension-cultured tobacco BY2 cells. Deprivation of auxin stops cell division and reveals the underlying cell elongation. Readdition of auxin stimulates division in a semisynchronous manner (23). As shown in Fig. 5*A*, the addition of 2,4-D to auxin-starved cells induced division within 3 h, confirming the timing and the absolute values of increase in mitotic index as previously described (23). YkB (1 μM) completely inhibited cell division induced by 2,4-D, and cells entered stasis (Fig. 5*A*) without any effect on viability as determined by vital staining (data not shown). A visual effect of YkB on auxin cell division can be illustrated by cell size in this system. Auxin-starved BY2 cells have variable but larger cell sizes, compared with chains of small cells when they are exposed to auxin. However, when cells are treated with both 2,4-D and YkB, many BY2 cells expanded similarly as if auxin-starved, although this is an incomplete inhibition of cell division, since some chain-like structures were observed (Fig. 5*B*). The effect of YkB was also determined by examination of auxin-induced lateral root formation. Interestingly, a low concentration of YkB (<1 μM) enhanced lateral root promotion in the absence of auxin, but above this concentration YkB repressed NAA-induced lateral root formation in a dose-dependent manner (Fig. 5, *C* and *D*), suggesting that YkB abolishes auxin-induced cell division in roots.

An effect of YkB on auxin-dependent expansion mediated by ABP1 was revealed using tobacco leaves inducibly expressing *Arabidopsis ABP1* (24, 32). The tip region of young tobacco leaves expressing ABP1 display a developmentally acquired,

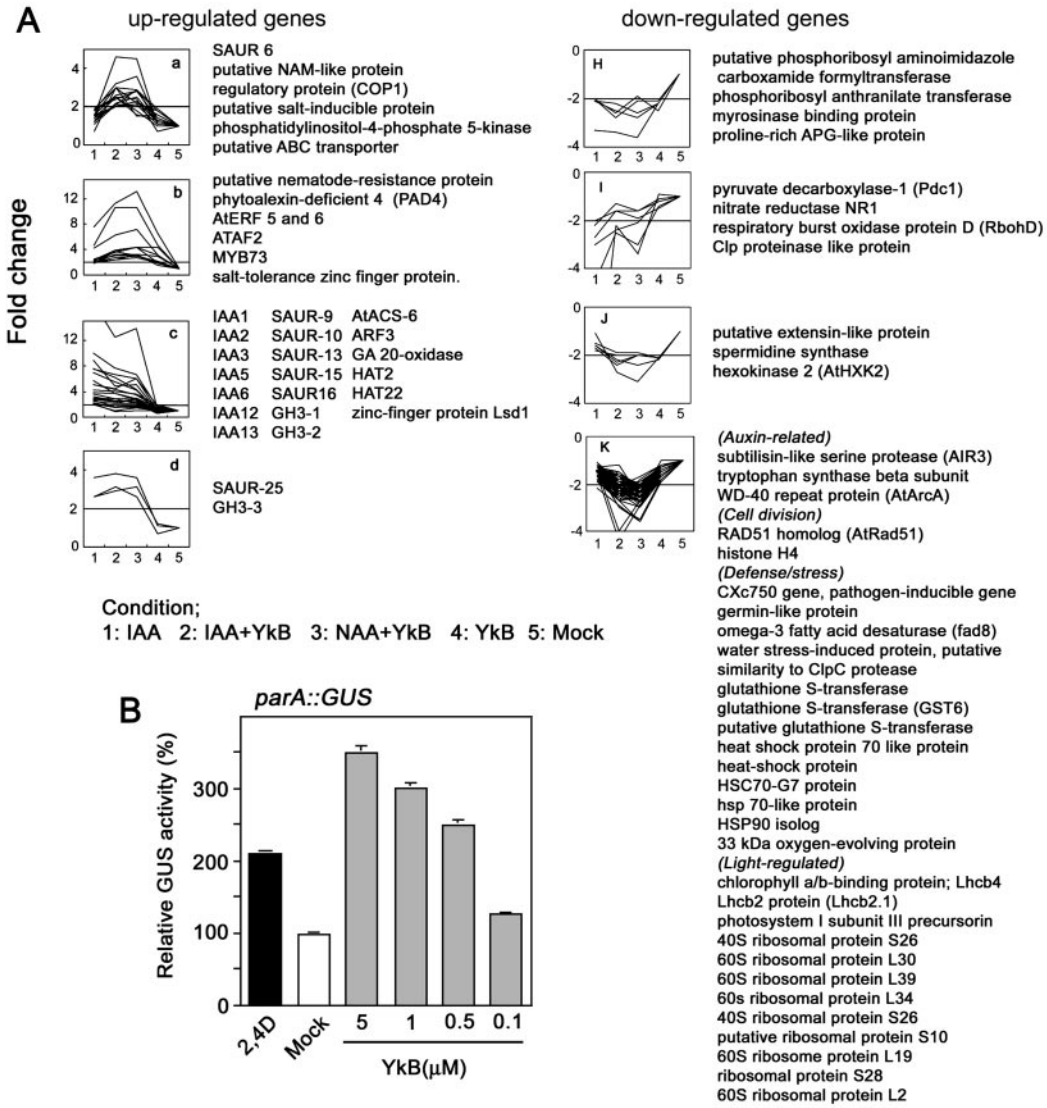

FIG. 4. *A*, the effect of auxin and YkB on global gene expression. The expression profiles of genes regulated by YkB and auxin using microarrays containing 8,300 gene probe sets. Four-day-old, etiolated seedlings were treated with 10 μM IAA (condition 1), 10 μM IAA with 5 μM YkB (condition 2), 10 μM NAA with 5 μM YkB (condition 3), 5 μM YkB alone (condition 4), or mock control (condition 5) for 20 min. Selected genes in each profile are indicated as up-regulated genes (*a–d*) or down-regulated genes (*H–I*). Fold change represents relative expression ratio of each condition to mock control (condition 5). The *horizontal line* in each *panel* shows the 2-fold change line. *B*, dose-dependent activation of auxin and stress-inducible *parA*::*GUS* reporter gene expression in transgenic tobacco. Seedlings (8 days old) were incubated with 5 μM 2,4-D or YkB for 12 h, and GUS activity was measured as described under "Experimental Procedures."

auxin-specific, epinastic-growth response, whereas the base of young leaves does not respond to auxin (24, 47). Young leaves lack expression of ABP1 in the base, and the developmental acquisition of auxin-inducible growth in leaves strongly correlates with the accumulating levels of ABP1 (32). If ABP1 is prematurely expressed in cells at the base of young leaves, expansion occurs in an auxin-dependent manner. We utilized a transgenic tobacco line (MJ10B) expressing ABP1 under the control of an AhTet-inducible promoter (24). Interveinal strips excised from the base of young MJ10B leaves expressing ABP1 (plus AhTet) exhibited NAA-dependent curvature resulting from epinastic cell growth. In contrast, the control R7 line harboring the empty vector did not respond to NAA in the presence of AhTet (Fig. 6*A*). YkB (2 μM) completely inhibited NAA-induced curvature in AhTet-treated MJ10B leaf strips (Fig. 6*A*). In contrast, MG132 did not inhibit NAA-induced curvature in MJ10B leaf strips. To confirm whether this type of expansion is independent of proton secretion, the effect of YkB on fussiccocin (FC)-induced growth was examined. FC-induced cell growth involves a vanadate-sensitive, plasma membrane ATPase. Leaf strip elongation induced by FC was completely inhibited by vanadate but not inhibited by YkB (Fig. 6*B*), suggesting that YkB acts specifically on auxin signaling leading to cell elongation and is not a general growth inhibitor.

YkB Acts on Upstream Components of AXR1 and TIR1—If YkB acts on a target that controls AUX/IAA stability, several predictions on the effect of YkB on seedling development must be met. These predictions are based on the known phenotypes of dominant, stabilizing mutations in AUX/IAA and other non-auxin pathway proteins requiring degradation and loss of function mutations in components of the AUX/IAA degradation machinery. Specifically, these predictions are as follows. 1) Severe phenotypes of auxin-insensitive mutants such as *iaa3/shy2-2*, *iaa7/axr2-1*, and *axr1-3* are shared with phenotypes of YkB-treated wild-type seedlings, whereas MG132 shows non-specific inhibition of seeding development resulting from general proteasome inhibition. 2) In the dominant AUX/IAA mutants, the expression of many *AUX/IAA* genes would be

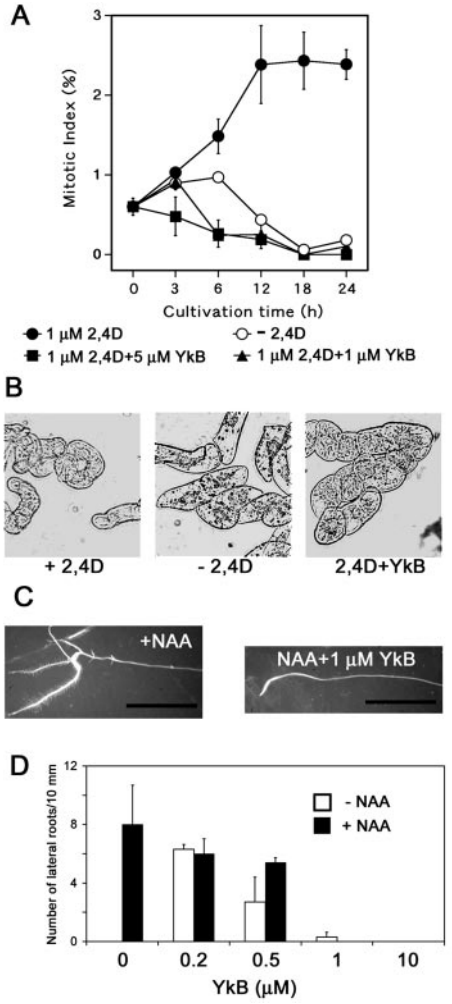

FIG. 5. **Effect of YkB on auxin-dependent cell division and expansion.** *A*, inhibition of auxin-induced cell division of auxin-deprived cells by YkB. Auxin-starved cells cultured without 2,4-D for 24 h were incubated with 2,4-D and/or YkB. *B*, auxin-starved cells were treated with or without 1 μM 2,4-D and with 0.5 μM YkB plus 1 μM 2,4-D for 3 days. The *photographs* are of the same magnification. *C*, roots treated with 0.1 μM NAA (*left*) or with 0.1 μM NAA plus 1 μM YkB (*right*). *Bar*, 5 mm. *D*, effects of YkB on NAA-induced lateral roots formation. The number of lateral root was counted after the treatment with YkB in the presence and absence of 0.1 μM NAA as described under "Experimental Procedures."

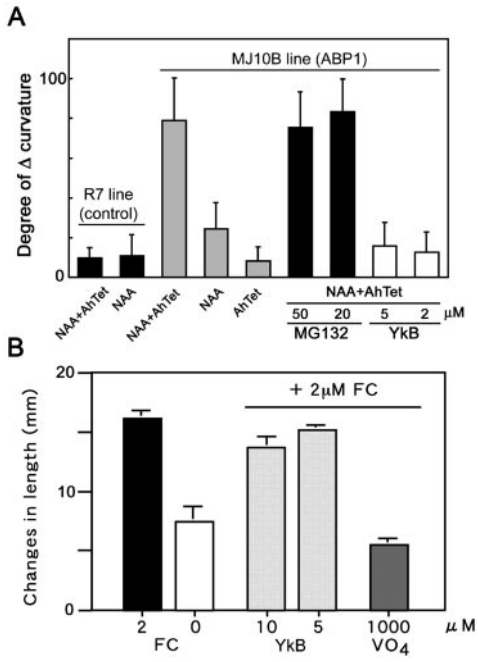

FIG. 6. **Effect of YkB on auxin-responsive epinastic elongation mediated by ABP1 and FC-induced cell elongation.** *A*, inhibition of YkB on NAA-induced epinastic elongation mediated by ABP1. The MJ10B line expresses ABP1 under the control of the AhTet-inducible promoter. The R7 line harbors the AhTet-inducible promoter without the ABP1 open reading frame. Leaf strips from each line were incubated with auxin and/or inhibitors together with AhTet (inducer) for 6 h after preincubation with AhTet for 4 h. Leaf strips were digitally recorded, and the degree of Δ curvature was determined as previously described (47). *B*, effects of YkB and H^+-ATPase inhibitor on FC-induced leaf strip elongation. The leaf strips from 3-month-old tobacco were treated with 2 μM FC with/without YkB or VO_4, ATPase inhibitor. The length of leaf strips was measured after 20 h of incubation.

unaffected by YkB and MG132, because expression is already repressed by the cognate mutated stable repressor. Therefore, dominant AUX/IAA mutants would be insensitive to both inhibitors. 3) The *axr1-3* and *tir1-1* mutations in ubiquitin E3 complexes are sensitive to MG132 due to its action on the proteasome downstream of AXR1 and TIR action but would be less sensitive to YkB if this inhibitor acts on apical to protein degradation. 4) ABA- and GA-insensitive mutants would display the same sensitivity to YkB as wild-type if YkB action is specific to auxin.

As shown in Fig. 8*G*, light-grown, wild-type seedlings treated with YkB displayed short hypocotyls and small, curled leaves, characteristic of the severe dominant *AUX/IAA* mutants such as *iaa3/shy2–2* and *iaa7/axr2–1*, whereas nonspecific developmental inhibition was observed after MG132 treatment (Fig. 8*D*) (prediction 1). As shown in Fig. 7*A*, hypocotyl elongation of *iaa3/shy2–2* and *iaa7/axr2–1* was less sensitive to MG132 (prediction 2), whereas *axr1–3 and tir1–1* hypocotyl elongation was sensitive to MG132 (prediction 3). All of the examined auxin-insensitive mutants were less sensitive to YkB in hypocotyl growth (predictions 2 and 3), especially the *iaa7/axr2–1* mutant, which was the least sensitive to YkB when grown in both light and dark conditions (Fig. 7, *A–C*; Fig. 8, *cf. B* and *C* with *E* and *F*). In contrast, the hypocotyl elongation in *abi1–1* (48) and *gai* (49) displayed the same sensitivity to YkB as wild-type (prediction 4) (Fig. 7*A*).

Hormonal control of hypocotyl elongation is complex, but it involves both auxin and gibberellin. It is also known that auxin regulates the expression of a GA biosynthetic gene (50). To exclude the possibility that YkB inhibits hypocotyl elongation by inhibiting GA response, the hypocotyl length was measured after the treatment of seedlings with various concentrations of GA and YkB. As shown in Fig. 7*D*, hypocotyl growth was suppressed by YkB; however, the relative hypocotyl elongation response to GA in the presence of YkB was not significantly different to controls, suggesting that the inhibition of hypocotyl elongation by YkB (Fig. 7*C*) does not occur through GA signaling (Fig. 7*D*; see values in parentheses).

YkB and MG132 have different effects on root growth, suggesting different target sites of action. Whereas both YkB and MG132 exhibited almost the same inhibition of primary root growth between all tested mutants and wild type (data not shown, except for *iaa7/axr2–1* in Fig. 7*B*), the two inhibitors showed strikingly different effect on root hair and lateral root formation. As shown in Figs. 5*D* and 8*A*, YkB promoted lateral root formation, but MG132 did not (data not shown). Furthermore, YkB did not alter root hair formation, but MG132 strongly inhibited it (Fig. 8, *H–J*).

DISCUSSION

Several lines of evidence listed below indicate that YkB is an auxin-specific inhibitor that stabilizes AUX/IAA repressors

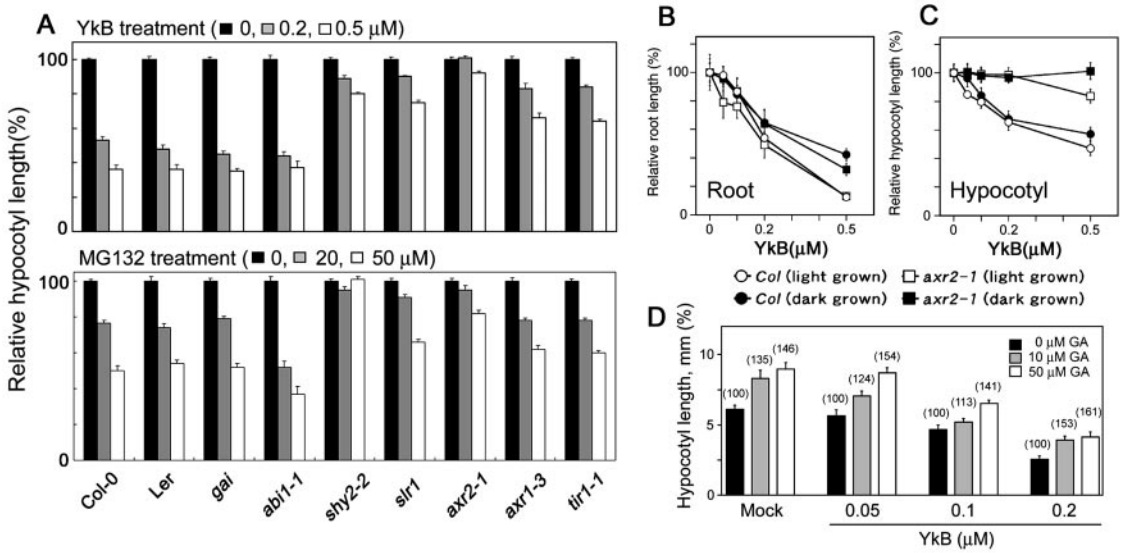

FIG. 7. **The effects of YkB and MG132 on the growth of *Arabidopsis* wild type and auxin-, GA-, and ABA-insensitive mutants.** *A*, seedlings were treated with YkB (*upper*) or MG132 (*lower*) for 7 days in light. *Black bars* (mock), *gray bars* (0.2 μM YkB or 20 μM MG132), and open bars (0.5 μM YkB or 50 μM MG132) represent relative hypocotyl length. *Error bars*, S.E. *B* and *C*, dose-response curve of YkB on primary roots (*B*) and hypocotyls (*C*) of 9-day-old, wild-type, and *iaa7/axr2–1* seedlings (*open circle*, light-grown wild-type; *closed circle*, etiolated wild-type; *open square*, light-grown *axr 2-1*; *closed square*, etiolated *axr 2-1*). *D*, effect of YkB on GA-induced hypocotyl elongation. Two-day-old *Arabidopsis* seedlings were cultured under light in the presence of GA. The hypocotyl length of 8-day-old seedlings was measured, and the percentage increase in growth due to GA treatment is shown in *parentheses above* the corresponding *bars*. The percentage increase in hypocotyl growth induced by GA is statistically the same at all YkB and mock treatments ($p < 0.05$).

without inhibiting proteasome activity, and its target site lies upstream to AXR1- and TIR-mediated degradation of AUX/IAA proteins. 1) YkB blocks the expression of both native and synthetic auxin reporter genes but does not alter ABA- or GA-induced gene expression. Whereas YkB does affect cytokinin reporter gene expression, it does so inefficiently, which is not surprising given the known cross-talk between auxin and cytokinin. The induction of *ARR5* was also inhibited by MG132, consistent with the reported role of the RPN12 proteasome subunit in cytokinin signaling (51). 2) YkB does not alter GA-inducible α-amylase expression, which is known to be controlled by an unstable repressor via the ubiquitin-proteolytic pathway (42). 3) YkB blocks the degradation of IAA7/AXR3, an AUX/IAA repressor, *in vivo* and is slightly more effective at stabilizing AXR3 than the general proteasome inhibitor, MG132. 4) Unlike MG132, YkB has no effect on either *Arabidopsis* or yeast ATP-dependent proteasome activity as determined by direct measurement. 5) *iaa3/shy2-2* and *iaa7/axr2-1*, dominant *AUX/IAA* mutants are less sensitive to YkB and MG132 than wild-type. In contrast, mutants of the ubiquitin E3 complex, *axr1-3* and *tir1-1*, are less sensitive to YkB but as sensitive to MG132 as wild type. Based on the auxin mutant seedling responses, YkB acts on a signaling component prior to the point of action of AXR1 and TIR1 (AXR1/TIR1 E3 complex), whereas MG132 acts after this point, consistent with its known inhibitor activity on the proteasome. 6) YkB treatment of wild-type seedlings phenocopies the dominant auxin response mutant, *axr2-1*, whereas MG132 treatment causes a pleiotropic, apparently nonspecific inhibition of seeding development. 7) MG132 and YkB affect differently on lateral root and root hair formation. YkB induces lateral roots, whereas MG132 does not. MG132 blocks root hair formation, whereas YkB has no obvious effect. 8) YkB blocks ABP1-mediated, auxin-dependent growth, whereas MG132 does not.

Our gene expression profile, in combination with previous reports, also suggests a mode of action whereby YkB modulates the MAPK cascade shared with auxin and stress signaling. In our gene chip data, YkB together with auxin down-regulated many defense/stress-related genes. Mockaitis *et al.* (18) demonstrated that auxin activated MAPK within 5 min in *Arabidopsis* roots. The MAPK kinase inhibitor, U0126, also was shown to block auxin-induced *BA*::*GUS* expression, but activates MAPK only when auxin was present (18). Because YkB also repressed auxin-responsive genes but activated auxin/stress-responsive *parA* (GST-like) promoter, YkB may modulate MAPK activity involved in both stress and auxin signaling. Defense/stress-related genes such as GST and heat shock proteins are up-regulated by oxidative stress, pathogen infection, wounding, and auxin (45), resulting from MAPK activation. The MAPK cascade involving the *ANPs*, *Arabidopsis* MAPK kinase kinases, is activated by oxidative stress (44). The activation of ANPs repressed *GH3* promoter activity but induced the GST6 promoter, suggesting that MAPK cascades negatively regulate auxin gene-responsive gene expression via MAPKs and cross-talking with oxidative stress and auxin signal (17). Gene expression profiling of a wounding response revealed that wounding enhanced transcription of the *NPK*-like kinase (MAPK kinase kinase) and repressed auxin-responsive genes such as *SAUR*, *GH3*, and *IAA* genes (43).

YkB inhibited an ABP1-mediated cell elongation response, whereas MG132 had no effect, suggesting that the YkB site of action lies at a junction in the auxin signaling network where one branch leads to altered AUX/IAA stability and is MG132-sensitive whereas the other branch leads toward steps involved in cell elongation and division and is insensitive to MG132 in the short term. Alternatively, the type of cell expansion mediated by ABP1 leads to stabilization of a specific subset or individual *AUX/IAA* gene(s); further stabilization by YkB or MG132 has no effect.

Repressor activity of AUX/IAA factors on auxin-responsive promoters was demonstrated to be dependent upon different degradation rates of individual AUX/IAA proteins (52). *axr1-12*, *iaa7/axr2-1*, and *iaa3/shy2-2* mutants showed distinct repression patterns of many primary auxin-responsive genes indicating that auxin-induced gene activation and/or repression, including the AUX/IAA genes, is dependent on the profile of AUX/IAA proteins in a particular cell (5, 35, 53). Our gene expression profiles also demonstrated that the activity of YkB

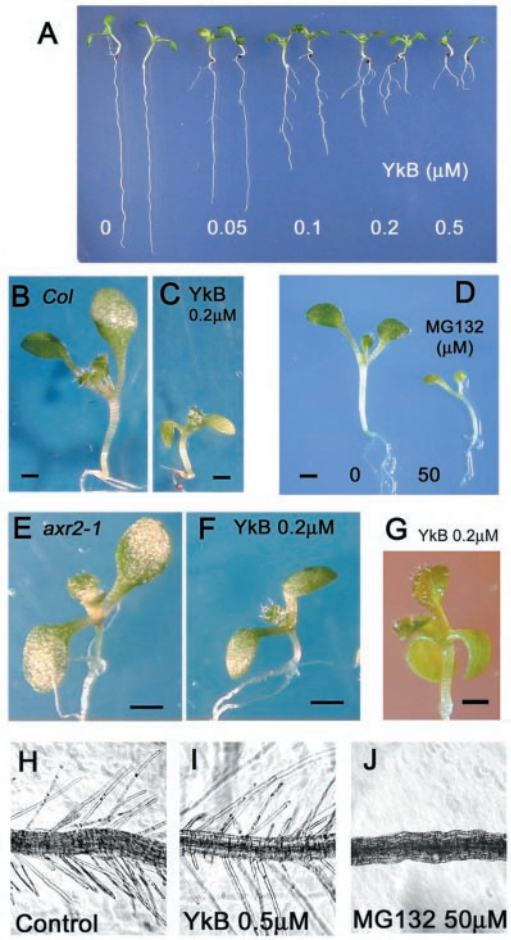

FIG. 8. *A*, pairs of 7-day-old seedlings (*Col*) grown vertically on agar containing the indicated concentrations of YkB. *B* and *C*, wild-type seedlings (9 days old) grown in liquid medium (*B*, mock control; *C*, 0.2 μM YkB). *Bar*, 1 mm. *D*, wild-type seedlings (7 days old) grown in liquid medium (*left*, mock control; *right*, 50 μM MG132). *E* and *F*, *iaa7/axr2-1* seedlings (9 days old) grown in liquid medium (*E*, mock control; *F*, 0.2 μM YkB). *G*, higher magnification of 0.2 μM YkB-treated seedling at 10 days. *Bar*, 1 mm. *H–J*, photograph of wild-type roots treated with inhibitors for 7 days (*H*, mock control; *I*, 0.5 μM YkB; *J*, 50 μM MG132).

for individual auxin primary responsive genes can be either positive or negative. These different activation/repression patterns of primary auxin-responsive genes would thus reflect the different phenotype manifested in auxin-insensitive mutants and YkB treatment.

Treatment of wild-type seedlings with YkB stabilizes AUX/IAA proteins and phenocopies the *shy2–2* dominant mutant. Null mutants of individual *AUX/IAA* genes typically lack an obvious phenotype, whereas dominant mutations in individual genes cause phenotypes that are difficult to interpret or predict including activation and repression of other *AUX/IAA* genes. At present, the working hypothesis is that the profile of AUX/IAA proteins present in the cell is the basis for cellular decisions. This is consistent with a range of observations; many auxin-insensitive mutants have distinct aerial phenotypes, whereas their primary roots, as well as their growth rates, are nearly wild-type (54–56). Recent reports demonstrated that *BA*::*GUS* expression was repressed in the root tip of *iaa3/shy2-2* and *iaa14/slr1* mutants, which display normal primary root growth rate, whereas GUS expression in *iaa3/shy2-2* was enhanced in the hypocotyls even in the absence of auxin (53, 54). Auxin treatment of roots stimulates *AUX/IAA* expression but inhibits root growth. Similarly, *iaa7/axr2-1* and *iaa17/axr3* hypocotyls have an ectopic pattern of *BA*::*GUS* expression (26).

These differences in root and shoot phenotypes for the auxin-insensitive mutants point to the reason for the tissue-specific sensitivity of YkB in hypocotyls and primary roots. In contrast to primary root growth rate, the lateral root formation was greatly affected by dominant mutations in individual AUX/IAA factors and by YkB in a dose-dependent manner. Low concentrations of YkB stimulate lateral roots, whereas higher concentrations inhibit root formation. Similarly, the phenotype of *axr1-12*, *tir1*, *iaa14/slr1*, *iaa19/msg2*, *iaa28*, and *iaa6/shy1* have fewer lateral roots, whereas *iaa7/axr2-1* and *iaa17/axr3* have more lateral roots than wild type (3, 8). Stabilization of individual AUX/IAA proteins with these dominant mutations confers distinct repression profiles in primary auxin-responsive genes, which is presumably the basis for the different phenotypes among mutants (*i.e.* the working hypothesis). Thus, lateral root promotion by low concentration of YkB may be a consequence of different sensitivity to the inhibition on individual AUX/IAA stabilities.

An explanation for the lack of a YkB effect on root hair phenotype also rests upon this working hypothesis. The mechanism underlying root hair formation is unknown; however, individual AUX/IAA proteins differently contribute to root hair formation. For example, *iaa3/shy2-2* roots have normal root hair, whereas *iaa14/slr1*, *iaa7/axr2–1*, *axr1-3*, and *tir1-1* roots (8, 54, 57) show abnormal root hair, suggesting that IAA/SHY2 protein is less important for root hair formation. The lack of a YkB effect on root hair could be explained by preferential inhibition of degradation of a specific AUX/IAA protein that is less contributive to root hair development.

In conclusion, we have described a YkB mechanism of inhibition of auxin signaling controlling plant growth using wild-type and auxin mutants. YkB stabilizes AUX/IAA repressors by targeting an upstream component to AXR1 and TIR. YkB provides a new probe, not only for dissecting auxin signal transduction but also for analyzing the protein degradation system in plants. Finally, we anticipate that YkB will be instrumental in forward screens to identify new genetic components of the auxin signaling pathway.

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