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The role of auxin-binding protein 1 in the expansion of tobacco leaf cells

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Summary

Tobacco leaf was used to investigate the mechanism of action of auxin-binding protein 1 (ABP1). The distributions of free auxin, ABP1, percentage of leaf nuclei in G2 and the amount of auxin-inducible growth were each determined in control tobacco leaves and leaves over-expressing *Arabidopsis ABP1*. These parameters were compared with growth of tobacco leaves, measured both spatially and temporally throughout the entire expansion phase. Within a defined window of leaf development, juvenile leaf cells that inducibly expressed *Arabidopsis ABP1* prematurely advanced nuclei to the G2 phase. The ABP1-induced increase in cell expansion occured before the advance to the G2 phase, indicating that the ABP1-induced G2 phase advance is an indirect effect of cell expansion. The level of ABP1 was highest at the position of maximum cell expansion, maximum auxin-inducible growth and where the free auxin level was the lowest. In contrast, the position of maximum cell division correlated with higher auxin levels and lower ABP1 levels. Consistent with the correlations observed in leaves, tobacco cells (BY-2) in culture displayed two dose-dependent responses to auxin. At a low auxin concentration, cells expanded, while at a relatively higher concentration, cells divided and incorporated [^{3}H]-thymidine. Antisense suppression of *ABP1* in these cells dramatically reduced cell expansion with negligible effect on cell division. Taken together, the data suggest that ABP1 acts at a relatively low level of auxin to mediate cell expansion, whereas high auxin levels stimulate cell division via an unidentified receptor.

Keywords: ABP1, auxin, cell division, cell expansion, leaf growth, nuclear cycle.

Introduction

Auxin regulates both division and expansion of plant cells. The action of auxin is mediated, in part, by an unusual putative receptor called auxin-binding protein 1 (ABP1). ABP1 has a binding affinity profile consistent with the biological activity of auxins (Jones, 1994). ABP1 is predominantly an endoplasmic reticulum lumenal protein (Jones, 1994; Napier, 1995), but, as shown by various single cell assays, can mediate auxin effects at the plasma membrane (Barbier-Brygoo *et al.*, 1989; Barbier-Brygoo *et al.*, 1991; Bauly *et al.*, 2000; LeBlanc *et al.*, 1999; Ruck *et al.*, 1993; Thiel *et al.*, 1993). Ectopic and inducible expression of *ABP1* confers auxin-dependent cell expansion in cells normally lacking auxin responsiveness (Jones *et al.*, 1998). *abp1* mutants arrest at a time during embryogenesis when cell expansion normally drives the transition from globular to heart-stage embryos, the point when the embryo visibly obtains its axiality (Chen *et al.*, 2001). These results all point to a role for ABP1 mediating auxin-induced expansion. However, it is unclear how ABP1 accomplishes this.

Because cell division and expansion both require movement of cell wall material through the endomembrane lumen where ABP1 resides, it is possible that ABP1 controls the direction and/or the flux of materials to the

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growing wall or newly laid down cell plate. Previous results (Jones *et al.*, 1998) suggest that over-expression of *ABP1* in the presence of auxin sacrifices a division wall for an expanding wall. If cell volume and the nuclear cycle are not tightly coupled, one would expect that those cells over-expressing *ABP1* and having failed to lay down the division wall would have an increased nuclear DNA content. In the first part of this work, we tested this hypothesis by measuring the percentage of nuclei in the G1 and G2 phases of the nuclear cycle. We report here that cells over-expressing *ABP1* have a single nucleus but this nucleus is more likely to be in the G2 phase. This effect is developmentally regulated and transient. This illustrates the complexity in control of cell division and expansion in the developing leaf. Additional growth-limiting elements must exist and it is reasonable to presume that auxin gradients within leaves are a part of this control. Therefore, in the second part of this work, we compared leaf growth with auxin and ABP1 levels in an attempt to unravel this complexity. A hypothesis is presented where ABP1 acts at a relatively low level of auxin to mediate cell expansion, whereas high auxin levels stimulate cell division via an unidentified pathway. We conclude with an initial test of this hypothesis using leaf-derived tobacco cells in culture.

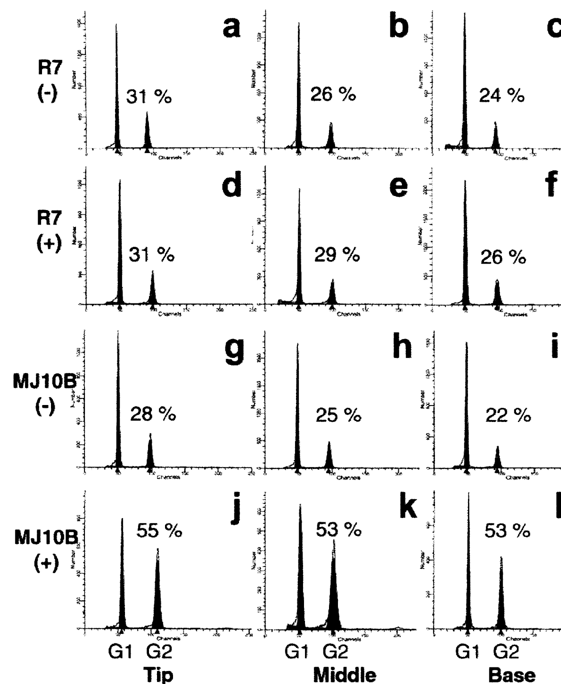

Figure 1. Nuclear stage of cells in developing leaves of control and tobacco plants inducibly expressing the *Arabidopsis ABP1* transgene.
At the five-leaf stage, R7 and MJ10B were hydroponically fed with 4 μg ml^{-1} of the non-toxic tetracycline analog, anhydrotetracycline (AhTet), via the roots for 8 days. Strips of interveinal leaf tissue taken from the tip (a, d, g, j), middle (b, e, h, k) and base (c, f, i, l) regions of the 6th leaf were used to isolate nuclei as described in the Experimental procedures section. Nuclear stages were measured by FACScan. Shown are representative results from one of three independent experiments.
(a–c), R7 without AhTet addition; (d–f), R7 with AhTet addition; (g–i), MJ10B without AhTet addition; (j–l), MJ10B with AhTet addition.

Results

Tobacco juvenile leaf cells inducibly expressing Arabidopsis ABP1 have nuclei in the G2 phase

Because tobacco plants over-expressing *Arabidopsis ABP1* have a normal morphology and volume but are comprised of larger and fewer cells (Jones *et al.*, 1998), we hypothesize that ABP1 may be involved in cell cycling, or in the cellular decision to place the wall either toward the outside leading to cell expansion or to the cell plate via the phragmoplast. Thus, in accordance with this hypothesis, cells over-expressing *ABP1* would either be binucleate or contain nuclei in the G2 phase because wall precursors are being redirected to the extracellular matrix rather than the cell plate during karyokinesis. Therefore, we measured the nuclear stage of cells in developing leaves of control (R7) and of anhydrotetracycline (AhTet)-inducible, *ABP1* over-expressing tobacco leaves (MJ10B) by flow cytometry. We found that all regions (tip, middle and base) of the developing 6th leaf of MJ10B plants have an increased percentage of nuclei in the G2 phase 8 days after AhTet induction of *ABP1* (Figure 1). The percentage of nuclei in the G2 phase doubled at all tested positions in MJ10B leaves, whereas no significant increase was observed in the R7 control leaves. Epidermal cells taken from the same leaves contained a single, but distinctly larger DAPI-stained nucleus (data not shown), consistent with the percentage of nuclei in the G2 phase shown by flow cytometry.

Tobacco leaf cell sizes correlate with nuclei phases

To refine the developmental relationship between the increase in cell size and the advance in nuclei to the G2 phase observed in the *ABP1* expressing plants, it was necessary first to measure these two parameters at all positions (tip, middle and base) in all leaves of mature control tobacco plants. Figure 2 reports the data obtained from the middle region of each control leaf. As tobacco leaves aged, both the percentage of nuclei in the G2 phase and cell size increased, but neither cell area nor the percentage of nuclei in the G2 phase correlated with leaf area. We also prepared protoplasts from the middle section of leaves and found that protoplast volume increased progressively with the age of the leaf (data not shown), consistent with the cell area increase determined using epidermal cell area alone (Figure 2). The results on the percentage of nuclei in the G2 phase and leaf age are in agreement with and extend the work of Galbraith *et al.* (1983). Since control leaf cells normally advance to G2 phase as they age (Figure 2), we interpret the increased

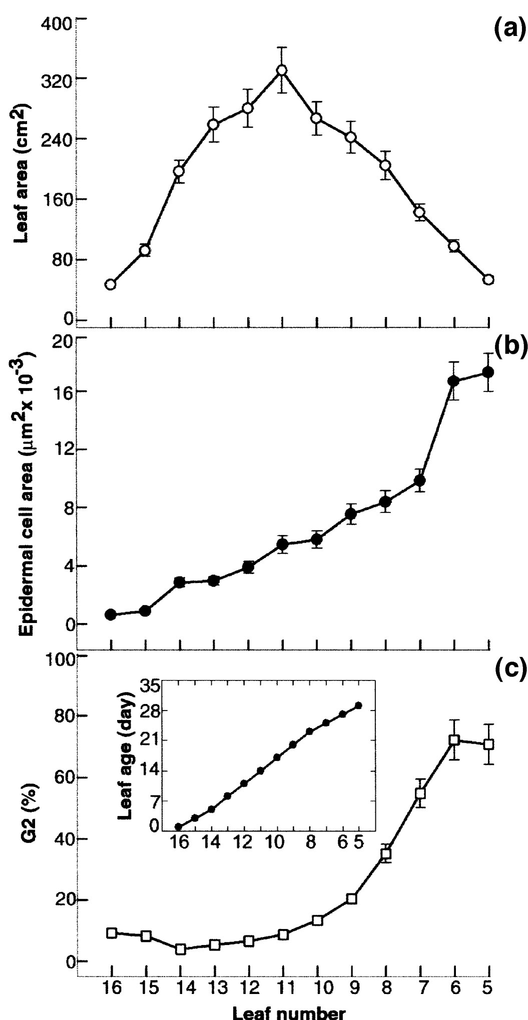

Figure 2. Relationship between tobacco leaf epidermal cell size and leaf cell nuclear stage.
(a) The leaf size is indicated as the mean ± SE of three individual plants.
(b) The epidermal cell size of cells in the leaf middle region is indicated as the mean ± SEM of three individual plants (at least 50 epidermal cells were measured each plant).
(c) The nuclear stage of cells in the middle leaf region is indicated as the mean ± SE of three individual plants. Inset in (c) indicates the ages of individual leaves at time of analysis.

percentage of nuclei in the G2 phase in plants over-expressing *ABP1* (Figure 1) as a premature advance in the nuclear cycle.

The effect of ABP1 on the premature advance to G2 is developmentally regulated

Since control cells normally advance to G2 phase as they age (Figure 2), it was necessary to determine whether the advance to the G2 phase associated with *ABP1* expression was maintained in older plants or whether this effect became obscured by the natural advance to the G2 phase

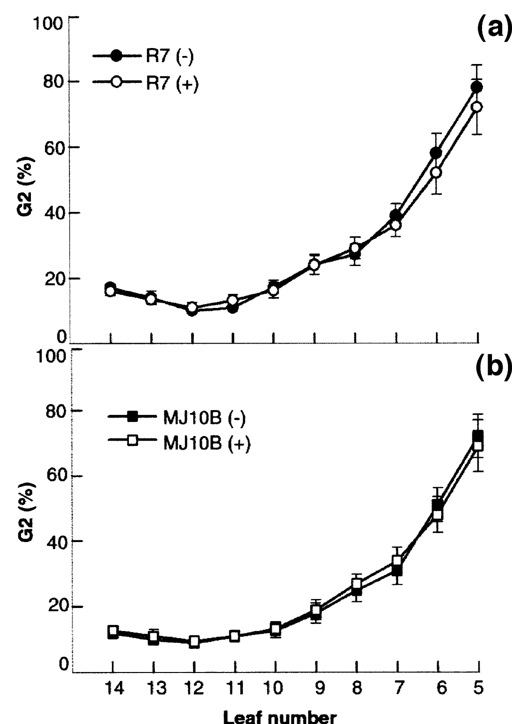

Figure 3. Nuclear stage of cells in leaves of R7 and MJ10B mature plants. R7 and MJ10B plants were hydroponically fed with 4 μg ml^{-1} of AhTet via the roots starting at the five-leaf stage. Strips of interveinal leaf tissue taken from the middle region were used to isolate nuclei. Nuclear stages were measured by FACScan. Data shown are mean ± SE of three individual plants.
(a) R7 without AhTet addition (●); R7 with AhTet addition (○).
(b) MJ10B without AhTet addition (■); MJ10B with AhTet addition (□).

as the cells aged. Therefore, the nuclear stage of cells was determined at the tip, middle and basal interveinal positions in all available leaves of control (R7) and in *ABP1* over-expressing tobacco plants (MJ10B). Because of the large volume of this data set, only the data of the middle region of each leaf are shown (Figure 3). No statistical difference was found between R7 and MJ10B leaves or by treatment with AhTet (Figure 3). This indicates that the effect of ABP1 on the percentage of nuclei in the G2 phase is developmentally regulated and/or transient; it is observable only during a specific developmental window of time. This is consistent with the hypothesis that the ABP1 effect is to advance the nuclear cycle of these young cells prematurely rather than to block cells at the G2 phase or evoke a neomorph-related response.

We therefore focused on the difference observed in juvenile plants (5–8 leaf stage) to pinpoint when cells are susceptible to the effect of *ABP1* over-expression. We measured the nuclear stage of cells in leaves of R7 and MJ10B plants at the juvenile stage. We found that 8 days after induction, significant increments in the percentage of nuclei in G2 were found in the 6th and the 7th leaves,

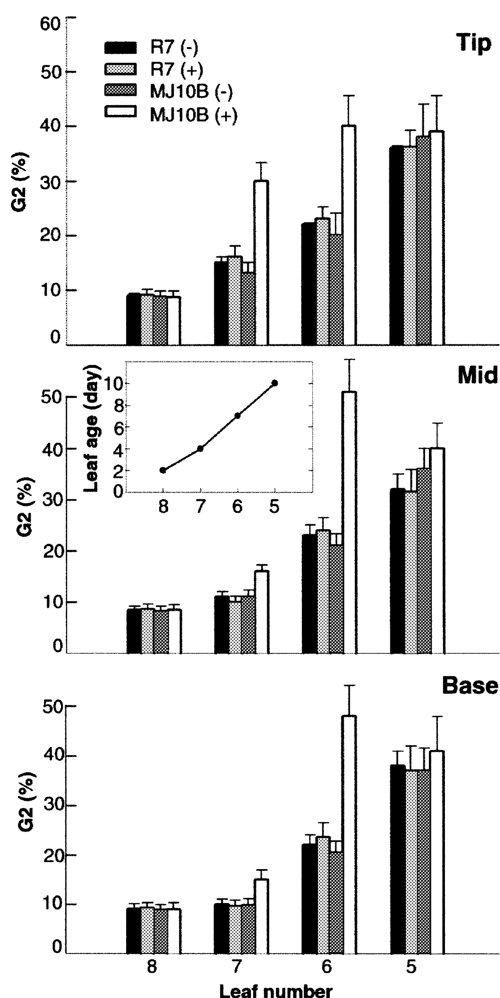

Figure 4. Nuclear stage of cells in leaves of R7 and MJ10B juvenile plants.
R7 and MJ10B plants were hydroponically fed with 4 μg ml^{-1} of AhTet via the roots starting at the five-leaf stage. Strips of interveinal leaf tissue taken from the tip, middle and base regions were used to isolate nuclei. The nuclear stages were measured by FACScan. The inset indicates the age of individual leaves at time of analysis. Data shown are mean ± SEM of three independent experiments. Black bars, R7 plants without AhTet addition. Light gray bars, R7 plants with AhTet addition. Dark gray bars, MJ10B plants without AhTet addition. White bars, MJ10B plants with AhTet addition.

whereas no significant differences were found in the younger leaf (8th leaf, 2-day-old) or the relatively older leaf (5th leaf, 10-day-old) in MJ10B plants (Figure 4). No change in the percentage of nuclei in the G2 phase was found in control (R7) leaves (Figure 4), therefore the increment in the percentage of nuclei in the G2 phase correlates with *ABP1* over-expression.

We also checked the *Arabidopsis ABP1* expression levels in the 5th, 6th, 7th and 8th leaves. RT–PCR revealed that *ABP1* was induced in all of these leaves (Figure 5). The lack of an effect of ABP1 in the 5th and 8th leaves indicates that *ABP1* expression is not limiting. Because ABP1 effects are

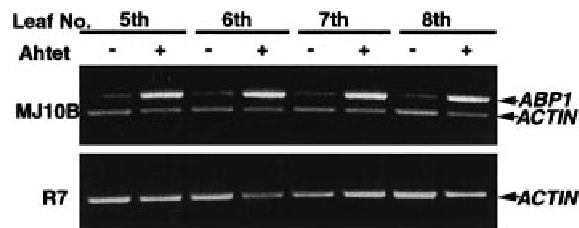

Figure 5. RT–PCR analysis of inducibly expression of *Arabidopsis ABP1* in tobacco leaves.
R7 and MJ10B plants were hydroponically fed with 4 μg ml^{-1} of AhTet via the roots starting at the five-leaf stage for 8 days. Strips of interveinal leaf tissue taken from the middle regions of the 5th, 6th, 7th and 8th leaves were used to isolate RNA. Five micrograms of total RNA was used to synthesize cDNA. The synthesized cDNA was used to amplify *Arabidopsis ABP1* (554 bp) and tobacco *ACTIN* (469 bp) gene transcripts. Each reaction contains both *Arabidopsis ABP1* and tobacco *ACTIN* primers.

auxin dependent (Jones *et al.*, 1998), it is reasonable to assume that the limiting component is auxin.

The effect of ABP1 on the increment of the percentage of nuclei in the G2 phase is transient

The effect of ABP1 on the premature advance to the G2 phase appears to be transient because the increment of the percentage of nuclei in the G2 phase observed in cells of leaves in juvenile plants over-expressing *ABP1* was lost in older plants (Figure 3). Therefore, to determine precisely when this effect occurs, we monitored the percentage of nuclei in G2 in the 6th leaf at various times after AhTet application. Data shown in Figure 6 were obtained from the middle region of the leaf. We found that the ABP1-induced increment in the percentage of nuclei in the G2 phase could be observed as early as 4 days after AhTet induction in MJ10B plants with the maximum occuring 8 days after induction. Eighteen days after application, no significant difference was found between R7 and MJ10B leaves or by treatment with AhTet.

Auxin-induced cell expansion mediated by ABP1 occurs before the G2 advance

Tissues over-expressing *ABP1* have larger cells and have more nuclei in the G2 phase. Consequently, we were interested in determining the relationship between ABP1-induced increases in cell size and ABP1-induced increases in the percentage of nuclei in the G2 phase. To test this, we used a leaf strip assay (Keller and van Volkenburgh, 1997), in which cell expansion is auxin-dependent and shown to be mediated by ABP1 (Jones *et al.*, 1998). Leaf strips from three positions (tip, middle and base) of a leaf were analyzed. Over-expression of ABP1 induces auxin response in all regions of developing leaves of MJ10B, whereas in R7 plants, only cells in the tip region responded

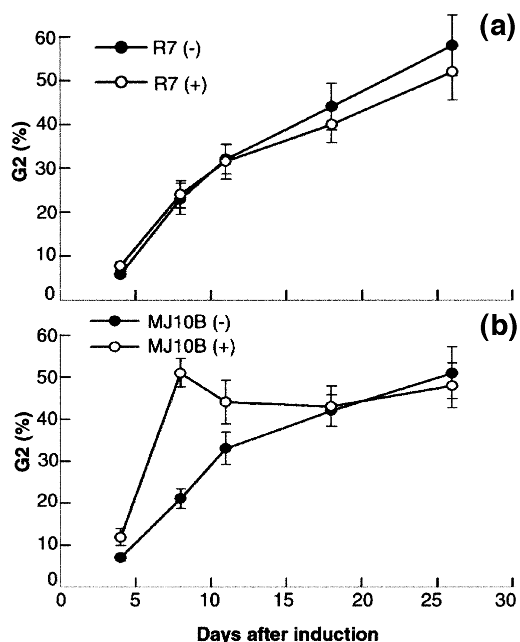

Figure 6. Developmental change of the nuclear stage of cells in the 6th leaf of R7 and MJ10B plants.
R7 and MJ10B plants were hydroponically fed with 4 μg/ml of AhTet via the roots starting at the five-leaf stage. Strips of interveinal leaf tissue taken from the middle region of the 6th leaf were used to isolate nuclei. Nuclear stages were measured by FACScan. Data shown are mean ± SE of three individual plants.
(a) R7 plants without AhTet addition (●); R7 plants with AhTet addition (○).
(b) MJ10B plants without AhTet addition (●); MJ10B with AhTet addition (○).

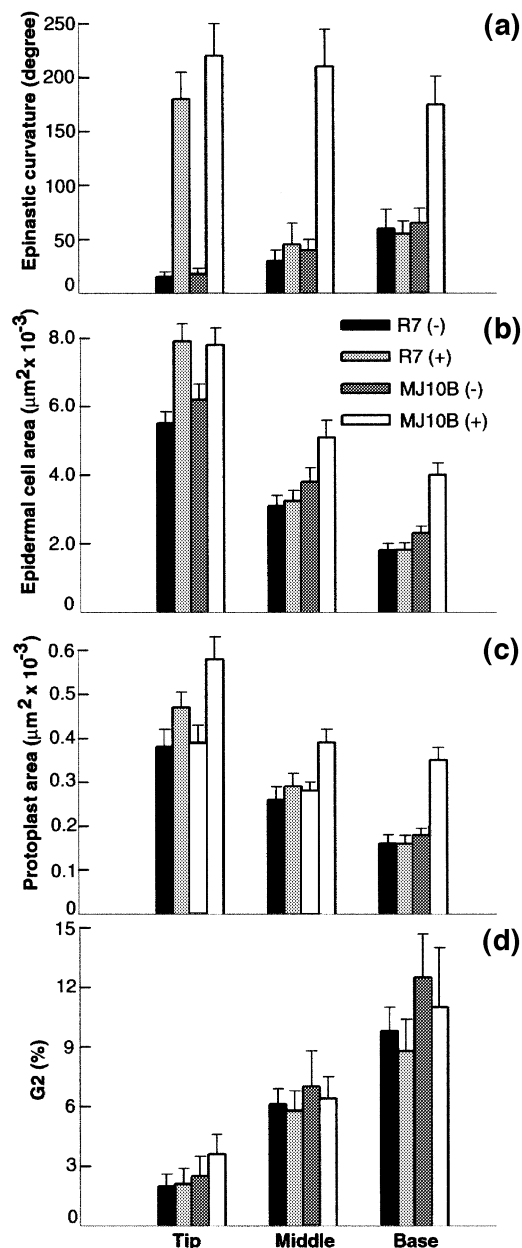

Figure 7. Epinastic curvature and nuclear stage of cells in isolated leaf strips of R7 and MJ10B plants.
Strips of interveinal leaf tissue taken from the tip, middle and base regions of the 6th leaf of a seven-leaf staged plant were floated on a solution containing 4 μg ml^{-1} AhTet plus 10 μM 1-NAA or 10 μM 1-NAA only. Black bars, R7 strips with 10 μM 1-NAA only. Light gray bars, R7 strips with 10 μM 1-NAA plus 4 μg ml^{-1} AhTet. Dark gray bars, MJ10B strips with 10 μM 1-NAA only. White bars, MJ10B strips with 10 μM 1-NAA plus 4 μg ml^{-1} AhTet.
(a) The epinastic curvature is represented as the mean ± SEM of three individual plants.
(b) The epidermal cell size is represented as the mean ± SEM of three individual plants.
(c) Cells in the leaf strips used in (a) and (b) were digested with 0.5% macerase and 1.0% cellulase and the protoplast areas were measured. At least 100 protoplasts from each plant were used to determine the mean ± SEM as shown.
(d) The nuclear stage measured as the percentage of nuclei in G2 is indicated as the mean ± SE of three individual plants.

to auxin (Figure 7). We therefore measured the epidermal cell size, protoplast area and the percentage of nuclei in the G2 phase in these strips. We found that the changes in epidermal cell size and protoplast correlated well with the degree of epinastic curvature (Figure 7). However, no significant change in the percentage of nuclei in the G2 phase occured in the cells over-expressing *ABP1*. Because the auxin-induced cell expansion mediated by ABP1 occurs before the G2 phase advance, the ABP1-induced advance in the nuclear cycle must be an indirect effect or a consequence of the cell volume increase.

Tobacco leaf expansion moves basipetally

The work described above suggests that auxin and ABP1 regulate auxin-induced expansion and, indirectly, the nuclear cycle. Therefore, we investigated the correlative relationship between multiple parameters in the intact expanding leaf. Tobacco leaf expansion has already been well characterized (Avery, 1933; Poethig and Sussex, 1985). Approximately 100 cells initiate a leaf primordium and a subset of these expand first to produce the tip region of the

leaf. Subsequently, cell expansion moves basipetally to expand the middle then basal regions of the leaf. A wave of cell division precedes the cell expansion front to provide cells needed to fill this cell expansion-driven increase in leaf area. In addition, cell division occurs at intercalary positions within the leaf. To determine whether ABP1 abundance in the leaf precisely correlated with expansion, it was necessary first to map spatial and temporal changes in growth of the leaves of control (R7) and of *Arabidopsis ABP1* over-expressing tobacco plants (MJ10B). Figure 8(a) illustrates the previously described wave of expansion moving basipetally. The changes in area over time at each position in the expanding leaves from R7 and MJ10B plants were compared. Neither the overall leaf expansion rate nor local expansion (Figure 8b) differed between R7 and MJ10B plants treated with AhTet at any time during development. To simplify the presentation of this result, only the growth increments for the central region flanking the midribs are shown (Figure 8b).

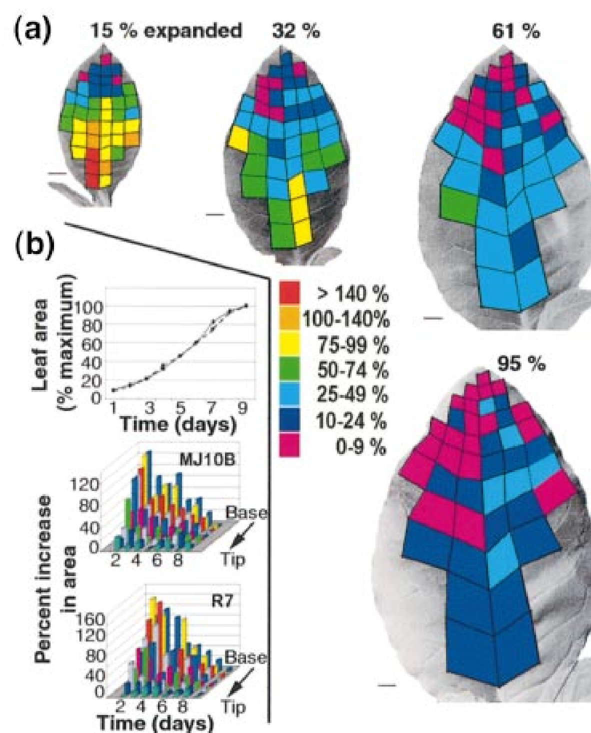

Figure 8. Growth kinetics of tobacco leaf cells.
(a) A grid pattern was placed on young leaves as described in the Experimental procedures section and the increase in local areas was expressed as percentage increase over a 24-h period. The percent increment intervals were color coded and mapped along the leaf. Bar = 1 cm.
(b) The top panel shows the increase over time in area of a leaf from R7 (dashed line) and MJ10B (solid line) plants. Middle and lower panels, the average percent increase in growth determined from both positions straddling and along the midrib from tip to base are plotted for control R7 and MJ10B plants.

ABP1 distribution correlates with the spatial distribution of auxin-inducible cell expansion capacity and cell size

We tested the correlation between the developmental acquisition of auxin sensitivity and the abundance of ABP1 in young and old leaves. In a young leaf, only cells in the tip region are able to respond to auxin in terms of epinastic curvature (Jones *et al.*, 1998; Keller and van Volkenburgh, 1997), and cells in the tip are larger than the cells in the middle and base regions (Figure 9). In a mature leaf, cells in the tip, middle and base regions respond maximally to auxin, and no significant differences in epidermal cell sizes were found among these regions (Figure 9). We found that cells in the tip region of the young leaf, where cells have the highest capacity for auxin-induced growth, also have the highest amount of ABP1 (Figure 9). In a mature leaf, higher and equal amounts of ABP1 were detected among the tip, middle and base regions correlating with the higher and equal amounts of auxin-induced growth capacity (Figure 9). Therefore, ABP1 abundance and distribution correlates with the extent and distribution of auxin-induced cell expansion and cell size.

High auxin levels correlate with the spatial distribution of cell division

In addition to a difference in ABP1 abundance and distribution between young and mature leaves, we also found differences in auxin levels and distribution. Figure 10 shows the general trends in auxin levels in expanding leaves. In leaves where cell division occured at all positions along the lamina but with minimal cell expansion (5–10% of full size), the IAA content was high (approximately 40 ng g^{-1} DW) all along the length of the lamina with no significant difference between the tip and the base. Leaves supporting both expansion and division (10–20% of full size) showed a basipetally increasing longitudinal gradient of free IAA with the lowest level at the tip (6.3 $\pm$ 3.3 ng g^{-1} DW) where elongation occured and highest at the base (21.5 $\pm$ 2.3 ng g^{-1} DW), where cells were actively dividing. By the time leaves reached 60–75% expansion, this gradient collapsed and the overall levels were intermediate to the lowest and highest auxin levels found in the previous comparison. Leaves that were nearly fully expanded had low (approximately 11 ng g^{-1} DW) but equal amounts of IAA levels at all intraleaf positions. These results confirm and extend those of Edlund *et al.* (1995).

Cell expansion and cell division pathways are separable in tobacco cells

The results with developing tobacco leaves indicate that low levels of auxin are associated with cell expansion while high levels are associated with cell division (Figure 8

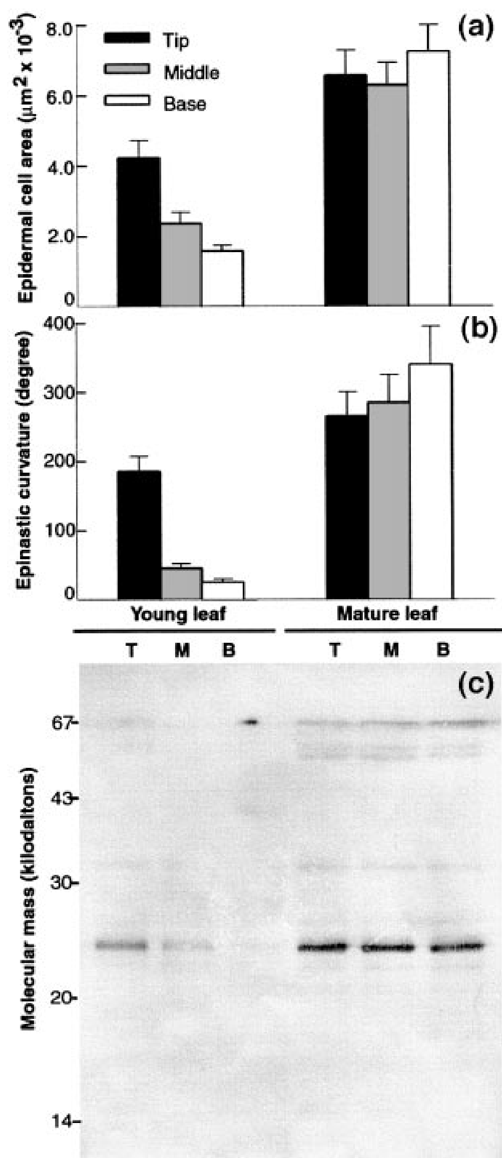

Figure 9. Developmental expression of ABP1 in tobacco leaves.
Black bars, tip; light gray bars, middle; white bars, base.
(a) Epidermal cell sizes in young and mature leaves, mean ± SEM three individual plants.
(b) Epinastic curvature, mean ± SEM of three individual plants.
(c) Amount of ABP1. T, M and B represent the tip, middle and base region, respectively.

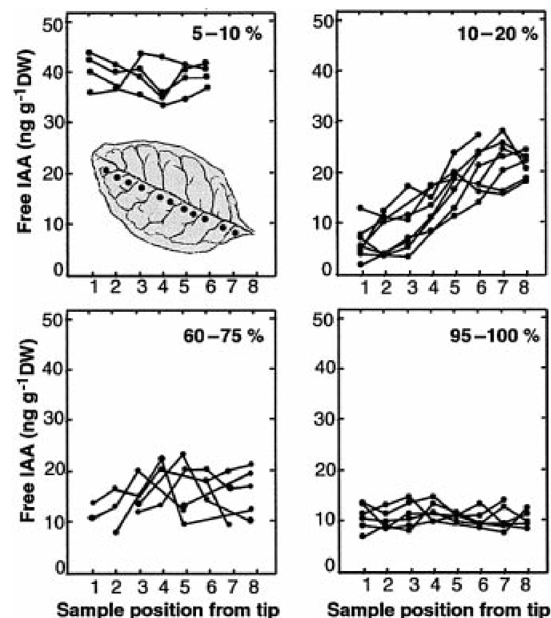

Figure 10. Distribution of auxin in leaves during leaf expansion.
Microanalyzes of IAA were performed as described in the Experimental procedures. The size of the leaf is indicated in each panel as the percent of full expansion. The positions where tissue was sampled are indicated in the leaf inset with #1 at the tip. Replicates are shown and those values for each leaf are grouped by connecting lines.

and Figure 10). To test this further, we utilized BY-2 cells grown in culture. BY-2 cells are derived from tobacco leaf mesophyll and require auxin for proliferation in culture (Hasezawa and Syono, 1983; Nagata *et al.*, 1992). As shown in Figure 11(a), cell expansion was maximum at 0.1 μM NAA while cell division and [^{3}H]-thymidine incorporation were maximal at 30 μM. These separable responses suggest that auxin-regulated cell expansion and division are mediated by a high and a low affinity auxin receptor, respectively.

To test the role of ABP1 in these two responses (cell division and expansion), we measured auxin-induced expansion in a cell line (designated NAS1) antisense suppressed for *ABP1* (Chen *et al.*, 2001). The level of ABP1 in these cells was previously shown to be undetectable by immunoblot analysis using three different antibodies directed against ABP1 (Chen *et al.*, 2001). Figure 11(b) shows that, compared with control cells, NAS1 cells lack auxin-induced expansion, although they retain auxin-induced cell division (Chen *et al.*, 2001). These results demonstrate that ABP1 functions directly in the cell expansion response.

Discussion

ABP1 has been shown to mediate auxin-regulated cell expansion. Tobacco plants over-expressing *Arabidopsis ABP1* have a normal morphology but are comprised of larger cells (Jones *et al.*, 1998). It is therefore possible that ABP1 is involved in the cellular decision to direct wall precursors either toward the existing cell wall space leading to cell expansion or to the cell plate via the phragmoplast leading to division. Knowledge of the specific mechanism is not needed initially to test this hypothesis but one possibility has been proposed. An

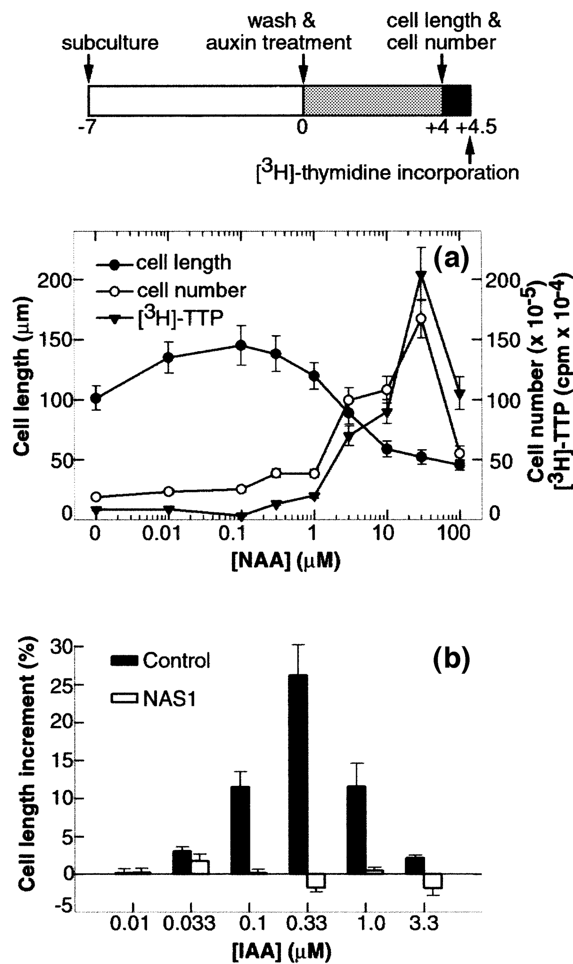

Figure 11. ABP1 antisense suppressed cells lack auxin-inducible cell elongation. Scheme showing experimental time course. Seven-day-old cells were washed in hormone-free media and transferred to media containing the indicated concentrations of auxins. After 4 days, cell length and cell number were determined and [^{3}H]-thymidine added. Twelve hours later, [^{3}H]-thymidine incorporation was determined.
(a) Regulation of cell expansion and cell division by auxin in BY-2 cells. The values represent the mean of cell length (●), cell number (○) and [^{3}H]-thymidine incorporation (▼) ± SEM of three independent experiments.
(b) Auxin-inducible cell expansion occurs at low concentrations of auxin in control cells but is lacking in cells with reduced ABP1 levels (NAS1). Cell length increment is shown as a percentage over growth in the absence of auxin. Closed bars, control cells; open bars, NAS1 cells. The graph shows the mean ± SEM of three replicates. At least 50 cells were measured each replicate.

auxin-ABP1 complex may dock at a specific plasma membrane site to stimulate ion flux and indirectly cause wall loosening and expansion (Barbier-Brygoo *et al.*, 1991; Klämbt, 1990). Alternatively, auxin and ABP1 may directly control secretion of a growth-limiting factor within the endomembrane system. Our hypothesis, which is based on the fact that cytokinesis and karyokinesis in plant cells are not obligatorily coupled, predicts that cells over-expressing *ABP1* would either be binucleate or contain nuclei in the G2 phase because the cell plate would be sacrificed for an extracellular wall. Indeed, we found that cells over-expressing *ABP1* clearly have more nuclei in the G2 phase, but this effect is transient and developmentally regulated.

Over-expression of *ABP1* confers (Jones *et al.*, 1998) and enhances (Bauly *et al.*, 2000) auxin sensitivity to cells in leaves. Leaves expand first at the tip and this expansion moves toward the base, consistent with auxin-induced cell expansion. Keller and van Volkenburgh (1997) have shown that cells at the tip of a young leaf have the capacity to respond to auxin and that this capacity moves basipetally as the leaf expands such that auxin-inducible growth in mature leaves is found eventually at all positions rather than just at the tip. In leaves over-expressing *ABP1*, young leaves behave as if they are mature with respect to the position of auxin-inducible growth, suggesting that ABP1 mediates the observed auxin-inducible growth (Jones *et al.*, 1998). We tested this by measuring ABP1 in young and old leaves of the control plants and found a good correlation between the abundance of ABP1, auxin-induced growth, and epidermal cell size.

The causal relationship between cell volume and DNA content is controversial (Cavallini *et al.*, 1997; Daykin *et al.*, 1997; Folkers *et al.*, 1997; Gendreau *et al.*, 1998; Gilissen *et al.*, 1996; Melaragno *et al.*, 1993; Traas *et al.*, 1998). It is unclear whether the level of endoreduplication sets the size of a given cell under a given condition or whether cell volume sets the ploidy. The work shown here shows that ABP1-induced increases in cell volume precede the G2 phase advance and therefore, at least for leaf cells, the increased cell volume may signal a corresponding increase in nuclear content. Endoreduplication-dependent increases in cell volume may be a requisite for specialized cells such as suspensor and endosperm cells that assume heavy metabolic loads but may not be the case in aging leaf cells. Thus, the discrepancy on cause and effect between nuclear cycling and cell volume control may be due to differences between cell types and/or metabolic loads.

Auxin may be another factor limiting cell growth and division in the expanding leaf. A relatively low level of auxin is found at the tip at a time when cell expansion rate and ABP1 level are maximum. In contrast, at positions and times when levels of auxin are high, cell division is maximum. These correlations prompt a testable hypothesis that there are two separable auxin responses: a high-affinity auxin response leading to cell expansion and a low-affinity auxin response leading to cell division. Furthermore, the hypothesis states that the high affinity response is mediated by ABP1. As a first test of this hypothesis, tobacco cells in culture were assayed for auxin-induced cell expansion and division because these cells can be synchronized (Nagata *et al.*, 1992). In this system, as the current evidence suggests for intact leaves,

cell expansion and division are separable by auxin dosage. We showed that the expansion rate was maximum at low concentrations of auxin while the division rate was maximum at higher concentrations. In cells lacking detectable levels of ABP1, the high affinity response was absent, supporting the idea that ABP1 mediates cell expansion.

What are the possible mechanisms of action for ABP1? As discussed above, ABP1 may behave in a novel manner within a receptor complex at the plasma membrane or as an auxin-dependent regulator of cell wall precursor trafficking. The data so far are consistent with either of these but receptor function is not conclusive since it has not yet been determined that auxin binding, *per se*, is required for ABP1 action. Other possibilities remain that also require auxin binding to ABP1 for functionality but not as a receptor. For example, ABP1 could be a localized auxin sequestering protein despite the fact that the abundance of ABP1 is at most a fraction of the total auxin pool and would have negligible effect as a whole. However, it is possible that ABP1 could alter auxin concentration within a cellular microenvironment, for example in regions of the ER. Finally, the previously described effects of ABP1 under- and over-expression, alteration by exogenous anti-ABP1 sera or peptide do not exclude a role for ABP1 in controlling auxin transport. Thus, the increased levels of ABP1 observed concomitant with or prior to the increase in auxin levels may be cause and effect (Figures 9 and 10).

The results with the cell lines suggest that there are minimally two types of cell expansion that are separably controlled. One type regenerates the volume lost upon division and this type is often described in the literature as meristematic growth. The other type, involving ABP1, leads to an irreversible volume increase beyond the starting cell volume and may be the primary type of expansion utilized in morphogenesis and tropisms.

The phenotypes of some *Arabidopsis* mutants also support a dual auxin pathway because auxin effects on cell division are separable from effects on elongation. For example, an aberrant lateral root formation mutant, *alf4*, lacks auxin-stimulated cell division but is normal with regard to root cell elongation (Celenza *et al*., 1995). Roots of the auxin resistant mutant, *axr6*, have significantly fewer root primordial but elongate faster than wild type (Hobbie *et al*., 2000). On the other hand, many other auxin mutants are impaired in both division and elongation, suggesting that these two pathways either converge or cross regulate.

Leaf morphogenesis begins in the recruitment of cells into the leaf primordium and is regulated throughout expansion via cell-to-cell interactions and the action of a number of transcription factors (Scanlon, 2000). These factors and others yet to be identified establish and/or fix cellular identities and the boundaries of pattern. Our proposal that dual auxin signaling pathways control division and expansion in the leaf does not, at this time, address the issue of pattern formation. Rather, we conclude that these dual pathways are the mechanism in which patterned tissue is filled and partitioned. However, the elements of morphogenesis that establish pattern and those that drive expansion and division are necessarily inextricably linked.

Experimental procedures

Plant growth and treatment

Induction of *Arabidopsis ABP1* by AhTet in intact plants was performed essentially as described by Jones *et al*. (1998). Control plants (designated R7, expressing only the tetracycline repressor) and transformed tobacco plants (MJ10B, expressing both the tetracycline repressor and the tet-inducible *Arabidopsis ABP1*) were grown individually in 10×10-cm pots under normal greenhouse conditions. Plants at the five-leaf stage were watered daily with 0.1% Peter's solution (Peter's Professional, Marysville, OH, USA) plus or minus 4 $\mu g\ ml^{-1}$ of the non-toxic tetracycline analog, anhydrotetracycline (AhTet), via the roots for 4, 8, 11, 18, 26 or 30 days.

Isolation of nuclei and flow cytometric analysis

Selected tissues were excised, immediately chilled on ice, and chopped with a single-edged razor blade in a glass Petri dish containing a chopping buffer (pH 7.0, 4°C) of the following composition: 45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM 3-(N-morpholino) propane sulfonate (MOPS), 0.1% Triton X-100 (Galbraith *et al*., 1983). The nuclear suspensions were passed through 15-μm nylon mesh. Propidium iodide (PI) and RNase were added to a final concentration of 100 $\mu g\ ml^{-1}$ and 10 $\mu g\ ml^{-1}$, respectively. The samples were kept on ice prior to flow cytometric analysis. At least 5000 stained nuclei for each sample were analyzed by flow cytometry (FACScan, manufactured by Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA) with a 488-nm light source from a 15 mW argon ion laser. The flow rate was adjusted to around 50 nuclei per second. Chicken red blood cells were used as an internal standard and for instrument alignment. The FACScan is interfaced to a Cicero data acquisition system (Cytomation, Inc., Fort Collins, CO, USA).

RT–PCR

R7 and MJ10B plants at the five-leaf stage were treated as described for 8 days. Strips of interveinal leaf tissue taken from the middle region of selected leaves (5th, 6th, 7th and 8th) were used to isolated total RNA using the TRIzol reagent (GIBCO BRL, Rockville, MD, USA). cDNA was synthesized using 5 μg of total RNA by Oligo(dT)$_{20}$-primed reverse transcription, using THERMOSCRIPT RT (GIBCO BRL). The first strand cDNA was used as a template for PCR using Takara ExTaq polymerase (PANVERA, Madison, WI, USA). *Arabidopsis ABP1* primers (5′-TGATCGTACTTTCTGTTGGTTCC-3′ and 5′-CCAATAGTAA-GGGA-ACTTCAGCC-3′, corresponding product size 554 bp) and tobacco *ACTIN* primers (5′-CCTCTTAACCCGAAGGCTAA-3′ and 5′-GA-AGGTTGGAAAAGGACTTC-3′, corresponding product size 469 bp, accession number X63603) were added together in each PCR reaction.

Curvature assays

Strips of interveinal leaf tissue taken from the tip, middle or base region of selected leaf (young or mature leaves) were floated on solutions (10 mM sucrose, 10 mM KCl, 0.5 mM Mes, pH 6.0) containing either 10 μM 1-NAA alone, or 10 μM 1-NAA plus 4 μg ml^{-1} AhTet (Jones *et al.*, 1998). Epinastic growth was measured as described by Keller and van Volkenburgh (1997).

Measurement of growth kinetics of tobacco leaf cells

Leaves at the earliest stage possible (less than 15% expanded) were marked with a grid of dots using India ink. Leaves were photographed at 24-h intervals in the afternoons for 10 days and the areas delineated by dots were measured digitally. Growth at each position was expressed as the percent increase in area over each 24-h interval and compared with the area of the leaf as its percent of the maximum. Values were binned, color coded and overlaid onto images of the leaves.

Auxin analyzes

Nicotiana tabacum L. cv. SR1 plants were grown in a greenhouse in Umeå at a day/night temperature of 22°C/17°C and a RH of approximately 70%. The photoperiod was 18 h consisting of natural daylight extended with light from metal halogen lamps (Osram 1043, München, Germany, HQI-TS 400 W DH^{-1}) giving a quantum flux density of 150 μmol m^{-2} s^{-1}. IAA distribution along the longitudinal leaf axis was analyzed in leafs of different developmental stage from four to six individual plants by sampling of 5–50 mg of mesophyll tissue (depending on leaf size) from the tip to the base. The IAA analysis was performed as described (Edlund *et al.*, 1995).

Protein isolation and immunoblot analysis

Microsomal fractions of tobacco leaf extract were obtained in Chapel Hill as described by Shimomura *et al.* (1999). Tobacco leaves were homogenized on ice with five volumes of homogenized medium composed of 0.25 M sucrose, 50 mM Tris–HCl buffer (pH 8.0), 1 mM EDTA, 0.1 mM $MgCl_2$ and 1 mM PMSF. The homogenate was centrifuged at 1,000 × g for 15 min to remove cell debris, and recentrifuged at 40,000 × g for 60 min. The resulting pellets were solubilized in a buffer containing 10 mM Tris (pH 7.0), 50 mM NaCl and 1.0% SDS and shipped to Tsukuba where the immunoblot analysis was performed as described by Shimomura *et al.* (1988). The protein concentrations were assayed by the method of Lowry *et al.* (1951) with modification in the presence of SDS using BSA as standard. Two hundred μg of membrane protein was mixed with SDS sample buffer, heated at 80°C for 10 min, incubated at 37°C for 30 min to 60 min, centrifuged at 15,000 r.p.m. for 10 min to remove insoluble materials prior to loading each lane of a 2-mm-thick 12.5% polyacrylamide gel. Proteins were transferred to nitrocellulose membrane (Schleicher & Schuell BA-S 83, London, UK) using blotting buffer (48 mM Tris, 39 mM glycine, 20% methanol). Polyclonal antibodies specific to tobacco ABP1 were obtained by affinity chromatography (Watanabe and Shimomura, 1998). The second antibody was a peroxidase-conjugated antirabbit IgG goat antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The color development was performed using a Konica Immunostaining Kit HRP-1000 (Konica, Tokyo, Japan).

BY-2 cell culture, auxin treatment, and [^{3}H]-thymidine incorporation

A tobacco BY-2 cell suspension was maintained by weekly 1 : 50 dilution as described by Nagata *et al.* (1992). Stationary phase cells were washed three times with BY-2 cell medium without 2,4-D, resuspended in medium containing different concentrations of 1-NAA (0–100 μM) and cultured under the same conditions as that of maintenance. Four days after adding 1-NAA, 1 μCi of [^{3}H]-thymidine was added to 1-ml aliquots of cell suspension (three replicates). The cell suspensions were continuously cultured for another 12 h, washed three times with dH_2O, centrifuged 1000 × g for 10 min, resuspended in 1 ml of 10% ice-cold trichloroacetic acid (TCA) and left on ice for 30 min. The pellets were washed twice with 5% ice-cold TCA, once with 100% ethanol, and solubilized in 0.1 M NaOH and 0.2% SDS at 37°C for 30 min. Radioactivity was measured by liquid scintillation and [^{3}H]-thymidine incorporation was expressed as CPM per 50 ml of cell suspension. Data of cell length were collected from at least 50 cells using phase contrast microscopy and digital capture. In the IAA treatment, control cells and NAS1 cells antisense suppressed for *ABP1* (Chen *et al.*, 2001), were treated with IAA at 0, 0.01, 0.033, 0.1, 0.33, 1 and 3.3 μM, respectively.

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