Auxin-Dependent Cell Division and Cell Elongation. 1-Naphthaleneacetic Acid and 2,4-Dichlorophenoxyacetic Acid Activate Different Pathways[1]

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During exponential phase, the tobacco (*Nicotiana tabacum*) cell line cv Virginia Bright Italia-0 divides axially to produce linear cell files of distinct polarity. This axial division is controlled by exogenous auxin. We used exponential tobacco cv Virginia Bright Italia-0 cells to dissect early auxin signaling, with cell division and cell elongation as physiological markers. Experiments with 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) demonstrated that these 2 auxin species affect cell division and cell elongation differentially; NAA stimulates cell elongation at concentrations that are much lower than those required to stimulate cell division. In contrast, 2,4-D promotes cell division but not cell elongation. Pertussis toxin, a blocker of heterotrimeric G-proteins, inhibits the stimulation of cell division by 2,4-D but does not affect cell elongation. Aluminum tetrafluoride, an activator of the G-proteins, can induce cell division at NAA concentrations that are not permissive for division and even in the absence of any exogenous auxin. The data are discussed in a model where the two different auxins activate two different pathways for the control of cell division and cell elongation.

Plant growth and morphogenesis are under the control of both environmental stimuli and endogenous developmental programs. In both cases, plant hormones coordinate adaptive changes in cellular division and differentiation. Auxins control several fundamental aspects of the plant development, such as cell expansion, cell division, pattern formation, root development, and apical dominance, and also environmental responses such as photo- and gravitropism (for review, see Hobbie, 1998; Berleth and Sachs, 2001). Despite intensive studies on the physiological responses to auxin, the primary steps of auxin signaling are still far from being understood.

Among the candidates for an auxin receptor, the auxin binding protein 1 (ABP1; Batt et al., 1976; Ray et al., 1977a, 1977b) has been studied most extensively during the more than 25 years since its discovery (Napier, 1997; for review, see Jones, 1994; Napier, 1995; Napier and Venis, 1995). Several experiments based on activation or inhibition of auxin responses via anti-ABP1 antibodies assign an important role in auxin perception to ABP1 (Barbier-Brygoo et al., 1989, 1991; Venis et al., 1992; Rück et al., 1993; Thiel et al., 1993; Leblanc et al., 1999a, 1999b; Steffens et al., 2001). Both ABP1 overexpression and loss-of-function mutants (*abp1* insertional null mutations) revealed the direct involvement of ABP1 in auxin-induced cell expansion (Jones et al., 1998; Chen et al., 2001a, 2001b). However, it is very likely that the activity of ABP1 is complemented by alternative auxin receptors (Hertel, 1995; Venis, 1995; Dharmasiri et al., 2003; Yamagami et al., 2004; for review, see Lüthen et al., 1999).

Despite the important role of ABP1, tobacco (*Nicotiana tabacum*) plants overexpressing Arabidopsis (*Arabidopsis thaliana*) ABP1 at first sight looked fairly normal (Jones et al., 1998). However, a closer look uncovered that these plants were composed of a lower number of gigantic cells. This means that the increased cell size caused by the overexpression of ABP1 had been compensated for by a reduced frequency of cell divisions. These findings implicate that auxin must control cell division and cell elongation through different pathways. This conclusion is supported by auxin response mutants such as *alf4* or *axr6*, where cell division and cell elongation are uncoupled (Celenza et al., 1995; Hobbie et al., 2000). In fact, this idea had been conceived already more than 60 years before (Thimann, 1936; Wareing and Phillips, 1981) when the differential auxin sensitivity of root and shoot growth was discussed with relation to the different modes of growth (with division being confined to the root, whereas the shoot responds exclusively by cell elongation). Recent work leads to a model where auxin triggers cell elongation after high-affinity binding to ABP1, whereas it promotes cell division through a different unknown low-affinity receptor (Chen, 2001). Consistent with this model, tobacco cells, where ABP1 is eliminated through an antisense approach, are dramatically impaired in 1-naphthaleneacetic acid (NAA)-dependent cell elongation, but are still able to proliferate (Chen et al., 2001a, 2001b).

When auxin controls cell division and cell elongation through different receptors, there must be different pathways. In fact, the response of cell division to auxin has been shown recently to require the activity of a putative heterotrimeric G-protein, whereas the response of cell elongation was not dependent on this G-protein (Ullah et al., 2001, 2003). When different receptors are involved, they are expected to differ in their affinity for different ligands (Zhao et al., 2002). Different auxins should therefore affect cell division and cell elongation with different dose response relations.

To test this prediction, we used a system where the responses of cell division and elongation to exogenous auxins can be conveniently assayed. We decided to utilize the tobacco cell line cv Virginia Bright Italia (VBI)-0, where cell division and cell elongation are triggered by exogenous auxins with a clear temporal separation of cell division from cell expansion (Opatrný and Opatrná, 1976). VBI-0 cells derive from stem pith parenchyma and, like their ancestor cells, they divide axially to generate pluricellular cell files. The files exhibit a clear polarity, evident at the level of the two terminal cells: One of them is rounder with the nucleus shifted close to the cross wall and conventionally defined as basal cell, whereas the other (the apical cell) is more elongated with the nucleus displaced to the periphery of the cell (Opatrný and Opatrná, 1976; Petrášek et al., 1998; Campanoni et al., 2003). Using this experimental system, we tested the influence of the two synthetic auxins NAA and 2,4-dichlorophenoxyacetic acid (2,4-D) on cell division and cell elongation. We observe that 2,4-D efficiently triggers cell division but not cell elongation, whereas NAA triggers cell elongation at much lower concentrations as compared to cell division. By inhibiting or activating G-proteins, we find that the division response to 2,4-D is affected, but not the elongation response to NAA. We discuss our findings in a model, where two different receptors bind the two auxins with different affinity and launch two different signaling pathways with different physiological function.

RESULTS

Cell Division and Elongation Can Be Controlled through Exogenous Auxins

In the standard protocol, the tobacco cell line VBI-0 is cultivated in the presence of the exogenous auxins NAA and 2,4-D. After about 1 week of intensive initial divisions producing pluricellular files, the cells switch to elongation, and eventually the files disintegrate into individual cells. We wanted to know whether this developmental response can be manipulated by modulating the concentration of exogenous auxins. Therefore, stationary cells were subcultivated into fresh medium in the presence of different concentrations of the 2 exogenous auxins, but maintaining the orig-

inal molar ratio between NAA and 2,4-D. Six days after inoculation, we scored the number of cells per cell file as measure for division activity, and we recorded confocal pictures from the different samples to detect potential changes in morphology. The dose response curve for cell division over the concentration of exogenous auxins (Fig. 1A) shows the bell-shaped curve typical for many auxin responses. Thus, the subcultivation in standard conditions (defined as 10 μM auxins, composed of 5.4 μM NAA and 4.5 μM 2,4-D) is optimal for cell division in VBI-0. Any deviation from this optimum either by lowering or raising the concentration is accompanied by a sharp decrease in cell division. For very high auxin concentrations (about 200 μM auxins), cell division is even inhibited to below the basal level observed in the absence of
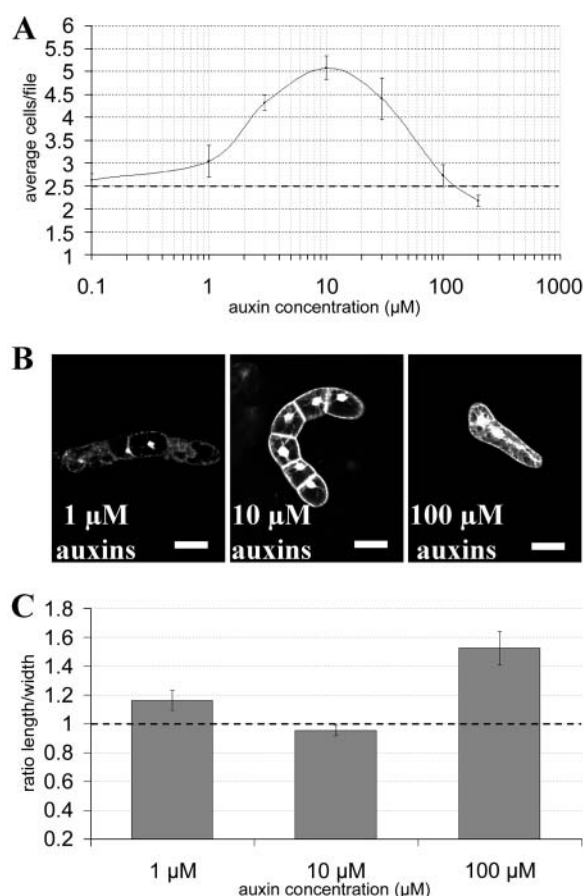

Figure 1. Response of the tobacco cell line VBI-0 to different concentrations of exogenous auxins. Cells were inoculated into fresh medium supplied with different concentrations of NAA and 2,4-D in a fixed ratio (1:1 w/w, respectively) and scored after 6 d of cultivation. A, Average number of cells per file. Each experiment was repeated at least 4 times, counting more than 10^3 cell files/sample every time. The dashed line indicates the basal level of cell division observed in auxin-free medium. B, Morphological effects. The images are confocal sections of cells stained with rhodamin-G6-chloride, a fluorescent membrane dye. Size bars correspond to 50 μm. C, Average ratio of cell length to width as measure of cell elongation. More than 300 individual cell files from 3 independent experiments were viewed by confocal microscopy and images recorded for the central section of the cell.

auxins (dashed line). This is not due to a reduced viability of the cells; in fact, in all samples the cell viability was higher than 90% (data not shown).

To check effects on morphology, cells cultivated for 6 d at 1 μM, 10 μM, and 100 μM auxins were stained with a fluorescent membrane dye and confocal pictures were recorded. Figure 1B shows representative examples. In standard conditions (10 μM auxins), the files show a typical phenotype, whereby cells in the file center exhibit a central nucleus surrounded by radial cytoplasmic strands, whereas the two terminal cells show a clear polarity. The basal cell is more rounded, with the nucleus close to the cross wall, whereas the apical cell is more polar, with the nucleus being displaced laterally. Cell files cultivated at 1 μM of exogenous auxins still maintain this polarity. However, the cells are slightly more elongated, and the few files that undergo several cell divisions are subject to precocious disintegration. For cultivation in 100 μM of exogenous auxins, pluricellular cell files are extremely rare, and the bicellular files that are most common express a hypertrophic polarity with a very small basal cell and an extremely elongated polar cell. These cells contain extraordinarily bundled cytoplasmic strands.

When cell length and width were measured from central confocal sections and the ratio length to width plotted versus auxin concentration (Fig. 1C), cells cultured in standard conditions were found to be roughly isodiametric, whereas cells cultivated at deviating concentrations (1 μM and especially 100 μM) were significantly more elongated.

2,4-D Preferentially Triggers Cell Division, Whereas NAA Triggers Cell Elongation

To investigate whether the two exogenous auxins contribute differentially to cell division and cell elongation, we used the same total concentration but omitted one of these auxins (maintaining the total concentration by increasing the concentration of the other accordingly). Again, the number of cells per file, the morphology of the cells, and the ratio cell length to cell width were recorded under these conditions (Fig. 2). Viability was routinely tested in all the samples, but no relevant differences were noticed (data not shown).

Cell division is reduced in cells treated exclusively with NAA, whereas 2,4-D alone is sufficient to support cell division to a level that even exceeds that of the control (Fig. 2A). NAA, in contrast, enhanced cell elongation by almost 50% as compared to the control, without any other significant effect on the morphology of cell files. In contrast, cell elongation was reduced when 2,4-D was administered alone (Fig. 2C), which was accompanied by a loss of file axiality (Fig. 2B). Indolyl-3-acetic acid (IAA), in concentrations of 10 μM or 50 μM, affected cell division and cell elongation in a manner similar to NAA (P. Campanoni and P. Nick, unpublished data).

We determined the dose dependency of cell division (Fig. 3A) and cell elongation (Fig. 3B) for the 2
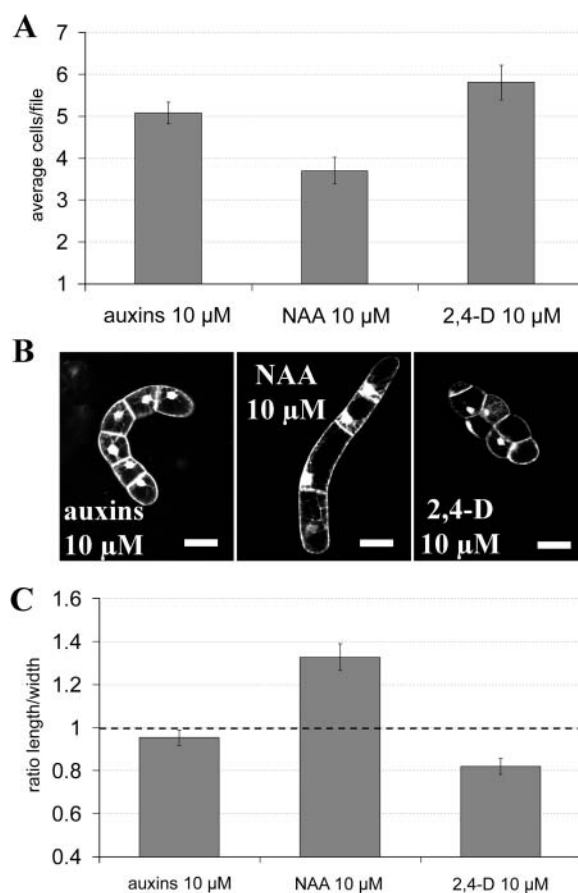

Figure 2. Differential effects of NAA and 2,4-D on division and elongation in VBI-0. Cells were cultivated either with both NAA and 2,4-D at 1:1 w/w ratio (auxins 10 μM) or with either 10 μM NAA or 10 μM 2,4-D alone. A, Average number of cells per file. Each experiment was repeated 8 times, counting more than 10^3 cell files/sample every time. B, Morphological effects. C, Average ratio of cell length to width. For details refer to the legend to Figure 1.

exogenous auxins administered either separately or in a 1:1 ratio, respectively.

2,4-D in the absence of NAA efficiently stimulates cell division with a pronounced optimum at 10 μM (Fig. 3A). This is accompanied by a reduced cell elongation; the observed dose response curve (Fig. 3B) is the mirror image of that for cell division. In contrast, NAA in the absence of 2,4-D, must be administered at 100 μM for an optimal stimulation of cell division (Fig. 3A). The threshold of stimulation is observed between 5 and 10 μM of NAA. However, around 10 μM of NAA, cell elongation is elevated by about 30% (Fig. 3B), decreasing to the basal level when the concentrations of NAA are raised further.

These data show that the optimum for the stimulation of cell division is situated at 10 μM when 2,4-D is used as exclusive auxin, whereas it is shifted by 1 order of magnitude when NAA is used instead. It is not clear, however, whether the intracellular concentrations of both auxins are comparable, due to differences in uptake and efflux mechanisms. However, this
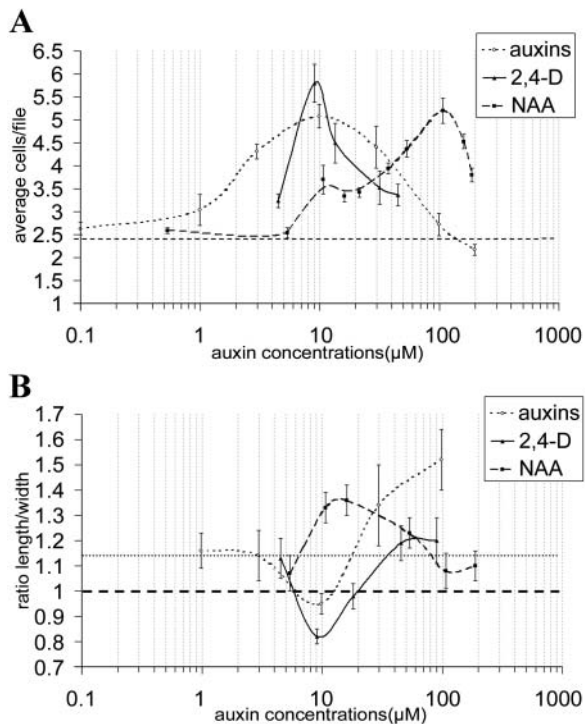
Figure 3. Dose response of cell division (A) and cell elongation (B) over different concentrations of either 2,4-D or NAA. Each data point represents the mean from 3 independent experiments comprising more than 10^3 cell files/sample. The dashed line indicates the basal level of basal cell division in the absence of exogenous auxins. For comparison, the dose response curve for combined addition of NAA and 2,4-D in a 1:1 w/w ratio is shown as well.

problem can be circumvented by comparing for each auxin the stimulation of cell division with that of cell elongation. This comparison shows that NAA stimulates cell elongation at concentrations that are roughly 10-fold lower than those that stimulate cell division. In contrast, 2,4-D does not stimulate cell elongation, even at high concentrations.

Thus, the auxins appear to act complementarily: 2,4-D is a potent trigger of cell division but not of cell elongation, whereas NAA triggers cell elongation much more efficiently than cell division.

The Stimulation of Cell Division by 2,4-D Is Sensitive to Modulators of G-Protein Activity

Recent work has revealed the involvement of G-proteins in auxin signaling to the cell division machinery (Ullah et al., 2001; for review, see Chen, 2001). Therefore, we tested whether pharmacological manipulation of G-proteins would alter the response of VBI-0 to exogenous auxins. For this purpose, we used pertussis toxin (PTX), widely used as a blocker of heterotrimeric G-proteins acting on the α-subunit that has been shown to be effective on G-protein-mediated signal transduction in plants (Warpeha et al., 1991; Wu and Assmann, 1994; Ritchie and Gilroy, 2000). Alternatively, we used aluminum tetrafluoride (AlF_4^-),

which constitutively activates G-proteins mimicking the γ-phosphate of GTP in the active site of the GDP-bound α-subunit of trimeric G-proteins (Bigay et al., 1985). Especially AlF_4^- is not very stable in solution, and therefore the drugs were added at daily intervals. The concentrations and application intervals for both drugs were chosen such that they did not affect cell viability and they did not reach superoptimal concentrations (data not shown). Moreover, we tested whether the results would change when the cells were transferred into fresh medium containing the freshly added drugs (to exclude potential accumulation of the drugs). However, we could not observe any significant difference between these two modi of drug application (data not shown). Five days after inoculation, cell division (Fig. 4A), cell morphology (Fig. 4B), and the ratio cell length to cell width (Fig. 4C) were recorded.

Under standard conditions, i.e. in the presence of both 2,4-D and NAA, PTX caused an inhibition of cell division accompanied by an increase of elongation. When 2,4-D was administered as the only auxin (increasing the concentration to maintain the same total concentration of exogenous auxin as under standard conditions), it was sufficient to maintain a high level of cell division (even exceeding that observed under standard conditions). This response to 2,4-D was blocked by PTX, which was again correlated with an increase of cell elongation over the sample treated with 2,4-D in the absence of the inhibitor.

When both 2,4-D and NAA were present, AlF_4^- caused a strong stimulation of cell division and a strong decrease of cell length, i.e. it acted antagonistically to PTX. When NAA (without AlF_4^-) was administered as the only auxin (again increasing its concentration to reach the same total concentration of exogenous auxin as under standard conditions), it failed to maintain cell division. However, cell length was found to be elevated as compared to the standard treatment. Interestingly, the combination of NAA and AlF_4^- was able to induce cell division to the high level that was observed with 2,4-D, although no 2,4-D was present in the medium. In other words, AlF_4^- could mimic the effect of 2,4-D on cell division. However, in addition, it caused a strong increase in cell elongation, which almost reached the level observed when NAA was given alone. In other words, the combination of AlF_4^- with NAA stimulated both cell division and cell elongation. We tested whether the stimulation of cell division by AlF4$^-$ would require the presence of NAA (Table I). We observed that AlF4$^-$ stimulates division also in the absence of any exogenous auxin, i.e. it does not depend on the presence of NAA.

The morphology of PTX-treated cell files (Fig. 4B) did not show particular differences from the control. Polarity and axiality were clearly manifest; only cell elongation was visibly enhanced. In contrast, AlF_4^--treated files lost their axiality almost completely. This was especially pronounced in the early stages, when the files were composed of a low number of cells (Fig. 4B, right-hand image, inset). During later stages,
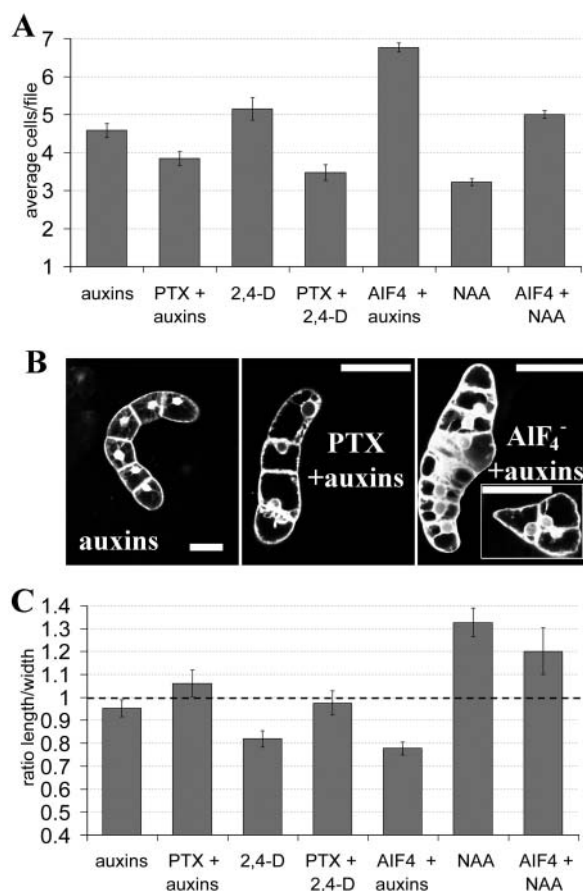
Figure 4. Response of auxin-dependent cell division and cell elongation to PTX and AlF_4^-. Cells were cultivated for 5 d either in standard conditions (1:1 w/w ratio of NAA and 2,4-D for a total auxins concentration of 10 μM) or alternatively in 10 μM 2,4-D or NAA alone. Every 24 h, either 10 ng/mL PTX or 100 μM AlF_4^- was added. The average number of cells per file obtained from 3 independent experimental series comprising more than 10^3 cell files/sample (A) and the average ratio cell length to width from 3 independent experiments with more than 300 individual cell files/sample (C) were measured. Representative images showing the morphological effects of the treatments are shown in B. For details refer to the legend of Figure 1.

axiality appeared to be restored, especially in the terminal regions of the file. However, axiality did not recover in the central cell of the file. This resulted in relatively normal cell files, where the central cell bulged out laterally. In rare cases, the bulging was

observed to lead to a bifurcation of the file (data not shown). It should be emphasized that this morphology differs strikingly from that observed for 2,4-D in the absence of NAA (Fig. 2B, right-hand image), where the axis of division was found to be disoriented all over the original cell file.

PTX and AlF_4^- could act on cell division independently of auxin. If so, the effect of auxin and those drugs on cell division should be additive. To test this, we quantified the effect of AlF_4^- and PTX on the average cell number per file in relation to the corresponding drug-free controls (Table I). We observed that the effect of the drugs (stimulation in case of AlF_4^-, inhibition in case of PTX) was large when the value for the corresponding control was large; it was small when the control value was small. In other words, the different auxins and the pharmacological agents did not interact additively, but multiplicatively, with respect to cell division.

ABP1 Is Expressed in the Presence of NAA or 2,4-D

To test whether both isoforms of the ABP1 are expressed in VBI-0 under the conditions relevant to this study, we checked the abundance of their transcripts by reverse transcription (RT)-PCR (Fig. 5). The control RT-PCR on the constitutively expressed transcription factor *mybB* shows that the starting amount of RNA is equal for each sample (Fig. 5C). As shown in Figure 5A, the *Ntabp1* transcript is abundantly expressed in the presence of both auxins (under standard conditions). The transcript level is even elevated, when 2,4-D or NAA are administered separately at either 10 or 50 μM. The *Ntabp2* transcript is expressed at much lower level, and there is a tendency for weaker expression, when NAA is absent (Fig. 5B).

Summarizing our results, we observe that: (1) Stimulated cell division is mostly accompanied by reduced cell elongation. We observed one exception: When NAA is administered as the only auxin, cell division is reduced whereas cell elongation is increased (following the rule); when NAA is combined with AlF_4^-, cell division is stimulated but cell length remains increased (breaking the rule). (2) 2,4-D efficiently triggers cell division, but does not induce cell elongation. In contrast, NAA induces cell elongation at low concentration and can support cell division only at very

Table I. *Pharmacological manipulation of G-proteins affects cell division depending on the amplitude of induction through auxin*

Averages over cell number per file are reported for corresponding pairs of drug-free controls and drug-treated samples.

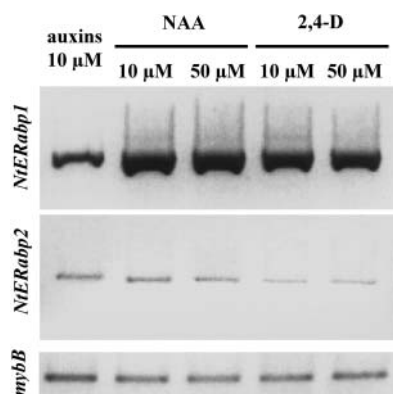| Experimental Condition	Drug-Free Control	After Drug Treatment	Difference
AlF_4^- in auxin-free medium	2.05 ± 0.04	3.14 ± 0.18	1.09 ± 0.22
AlF_4^- in auxins 10 μM	4.5 ± 0.19	6.68 ± 0.12	2.18 ± 0.31
AlF_4^- in NAA 10 μM	3.2 ± 0.09	4.97 ± 0.1	1.77 ± 0.31
PTX in auxins 10 μM	4.5 ± 0.19	3.76 ± 0.19	−0.74 ± 0.38
PTX in 2,4-D 10 μM	5.2 ± 0.09	3.53 ± 0.21	−1.67 ± 0.3

Figure 5. Expression of tobacco ABP1 genes in the VBI-0 cell line under different hormonal treatments. Total RNA from VBI-0 cells cultured in standard conditions (auxin 10 μM) or in medium supplied with 10 or 50 μM NAA or 2,4-D alone were used as template in a OneStep RT-PCR to detect the presence of transcript specific for the tobacco ABP1 genes *NtERabp1*, *NtERabp2*, and the transcription factor *mybB* as internal standard for loading of lanes. The amplificates were separated in 2% (w/v) agarose gel.

high concentrations. (3) PTX, a bona fide blocker of trimeric G-proteins, can block the response of cell division to 2,4-D. (4) AlF$_4^-$, a bona fide activator of G-proteins, can induce cell division. It does not require NAA to do so. Thus, AlF$_4^-$ mimics 2,4-D with respect to the induction of cell division. (5) The interaction of AlF$_4^-$ and PTX with the different auxins on cell division is multiplicative, not additive. (6) The two isoforms of the auxin-binding proteins 1, *Ntabp1*, and *Ntabp2* are expressed in VBI-0 in the presence of NAA and/or 2,4-D.

DISCUSSION

An increasing body of evidence suggests that auxin can activate different signal transduction pathways (Celenza et al., 1995; Hobbie et al., 2000; Yamagami et al., 2004). Jones and coworkers demonstrated that auxin-induced cell division and cell elongation can be uncoupled as distinct processes, suggesting the existence of differential signal transduction pathways leading to the different responses (Jones et al., 1998; Chen, 2001; Chen et al., 2001a, 2001b). In this study, we investigated cell division and cell elongation in exponential cells of the tobacco cell line VBI-0 as physiological markers to assign these different signal pathways to different exogenous auxins.

From the experiments shown in Figures 1 and 2, it becomes evident that auxin-induced cell division and cell elongation are antagonistic processes. Thus, stimulation of cell division is correlated with an inhibition of cell elongation and vice versa. Formally, this antagonism might originate from a trivial cause: If one assumed that cell expansion was independent of auxin, any inhibition of cell division should result in longer cells; when a cake is cut into fewer pieces, the

pieces will be larger in size. However, we observed that it is possible to cause both an increase of cell division and cell elongation by combining AlF$_4^-$ with NAA as the only exogenous auxin (Fig. 4, A and C). In this case, increased cell elongation could be uncoupled from decreased division frequency. This suggests that auxin-induced changes in cell elongation are not just the trivial consequences of auxin-dependent changes of cell division but are regulated by an independent pathway.

A central finding of our study is the qualitative difference of NAA and 2,4-D with respect to the relative sensitivity of cell division and cell elongation. Whereas 2,4-D stimulates cell division and inhibits cell elongation (accompanied by a loss in file axiality), NAA leads to an increase of cell elongation at a reduced frequency of cell divisions (Fig. 2). Only at very high concentrations (Fig. 3A) can NAA induce a substantial response of cell division, which is consistent with findings published for the tobacco cell line BY-2 (Chen et al., 2001b).

The differential effect of 2,4-D and NAA on VBI-0 cell division might be explained, in theory, as a consequence of the different transport properties of the 2 synthetic auxins through the cells. For identical extracellular concentration of NAA and 2,4-D, 2,4-D accumulates more rapidly inside the cells (Delbarre et al., 1996). An external NAA concentration of 10 μM might not be sufficient to establish enough intracellular NAA. In other words, the dose response curves for cell division might not be so different if the effective intracellular concentrations were compared for NAA and 2,4-D (reported in Fig. 3A). We therefore rather compared cell division with cell elongation for each hormone separately. The dose response curve for NAA-dependent cell division is shifted to higher concentrations as compared to the curve obtained for NAA-dependent cell elongation (Fig. 3, A and B, respectively). This demonstrates that the internal concentration of NAA established by 10 μM NAA in the medium is effective to trigger cell elongation, but not cell division. This suggests that NAA has a reduced affinity for the receptor triggering auxin-dependent division, whereas it exhibits a high affinity for the receptor triggering auxin-dependent elongation. On the other hand, 2,4-D can trigger cell division independently of NAA, but fails to stimulate cell elongation, even for very high concentrations.

Thus, cell division and cell elongation are distinct processes differentially affected by different auxin species. This means that the auxin receptor driving cell division shows a different ligand pattern than the receptor controlling cell elongation. If two receptors differ in their ligand pattern, they must be different receptors (Zhao et al., 2002).

Our observations are consistent with a model (Chen, 2001) postulating two distinct auxin receptors that differ in their affinity for NAA (or IAA, in the natural system). The high-affinity receptor would trigger cell elongation, whereas a second low-affinity receptor

would stimulate cell division. Studies on the crystal structure of the ABP1 crystal structure suggest that ABP1 binds NAA (as well as IAA) with high affinity (Woo et al., 2002). The phenotype of ABP1 overexpressors or antisense plants (Jones et al., 1998; Chen et al., 2001a, 2001b) supports ABP1 as the best candidate for a high-affinity receptor triggering auxin-dependent cell elongation. We can demonstrate that the 2 genes encoding this protein, *NTABP1* and *NTABP2*, are effectively expressed in VBI-0 under our experimental conditions (Fig. 5). In the framework of this model, our experiments reported in Figures 2 and 3 mean that the second unknown receptor with low affinity to NAA and responsible for auxin-induced cell division displays high affinity for synthetic auxin 2,4-D (Fig. 6).

When cell division is induced by IAA or NAA, higher doses are required as compared to those that can stimulate cell elongation. The signaling to cell division is then triggered by a low-affinity receptor (which is different from ABP1). The existence of two distinct receptors, which display different affinity to auxin, could also explain the differential auxin sensitivity in root and shoot growth, classically discussed in the context of division playing a major role in root, but not in shoot, growth (Thimann, 1936; Wareing and Phillips, 1981).

The signaling triggered by the low-affinity receptor involves G-proteins (Ullah et al., 2001; for review, see Chen, 2001). The two-pathway model and the involvement of G-protein action in one pathway have also been proposed for auxin-dependent stomatal opening (Cousson, 2003). When the second unknown receptor binds 2,4-D with high affinity, we should be able to activate the 2,4-D pathway (i.e. cell division) in the absence of 2,4-D, simply by activating the transducing

Figure 6. Model of early auxin signaling to cell division and cell elongation in the tobacco cell line VBI-0. A receptor with high affinity for NAA (presumably ABP1) triggers cell elongation, a second unidentified receptor with high affinity for 2,4-D (Rx) activates cell division through a trimeric G-protein. Both pathways are connected by a negative crosstalk (dashed lines 1 and 2). DP stays for a generic docking protein activated by G-proteins.

G-protein cascade. Our finding that AlF_4^-, a bona fide activator of G-proteins (Bigay et al., 1985), can trigger cell division very efficiently in the absence of 2,4-D (Fig. 4A) and even in the absence of NAA (Table I) is predicted by this model (Fig. 6). A second prediction of this model would be the block of cell division by blockers of G-proteins despite the presence of 2,4-D. In fact, PTX, widely used as inhibitor of trimeric G-proteins (Warpeha et al., 1991; Wu and Assmann, 1994; Ritchie and Gilroy, 2000) leads to reduction in division rate and increase in cell elongation despite the presence of 2,4-D in concentrations that should support conspicuous activation of division (Fig. 4A).

It is possible that AlF_4^- and/or PTX alter auxin-dependent cell division, because they affect cell division per se, i.e. independently of auxin. If so, they should interact additively with the different auxin species in the stimulation (AlF_4^-) or inhibition (PTX) of cell division. However, we observe (Table I) that the interaction is not additive, but that the influence of these drugs depends on the amplitude of the auxin effect. In other words, the interaction is multiplicative, not additive, which is to be expected when the G-protein(s), which are a target of these drugs, would act in the signaling chain.

These findings support a model (Fig. 6) where G-proteins transduce the auxin-triggered signal from an unknown receptor with high affinity for 2,4-D toward the machinery responsible for the stimulation of cell division. This unknown receptor (Rx) displays only a low affinity for NAA, which means that NAA can induce cell division only at very high concentration (Fig. 6, dotted arrow). NAA preferentially binds ABP1, leading to an induction of cell elongation. We speculate that ABP1-activated signaling controls axiality as well, because when the ABP1-activated signaling is left silent by using 2,4-D as exclusive auxin, this leads to a loss of axiality (Fig. 2B). This finding is consistent with the fact that ABP1 null mutants in Arabidopsis display disoriented cell division in suspensors and proper embryos (Chen et al., 2001b).

From the antagonism between cell division and cell elongation, one might conclude a negative crosstalk between the 2 pathways initiated by ABP1 or Rx, respectively (Fig. 6, dashed lines 1 and 2). As pointed out above, the observation that AlF_4^- can stimulate both cell division and cell elongation speaks against a trivial relationship where cells are simply larger because they have undergone a lower number of divisions. Whether this negative crosstalk is caused by active inhibition or by competition for a limited supply of a component shared by both pathways remains to be elucidated. Formally, the inhibition of ABP1 signaling through Rx (Fig. 5, dashed line 1) is predicted to be caused by a factor downstream of the G-protein. If it were caused by a factor upstream of the G-protein, a block of the G-protein by PTX should on the one hand block division, whereas on the other hand the inhibitory effect on ABP1 signaling should persist and cause a block of cell elongation. This would mean a
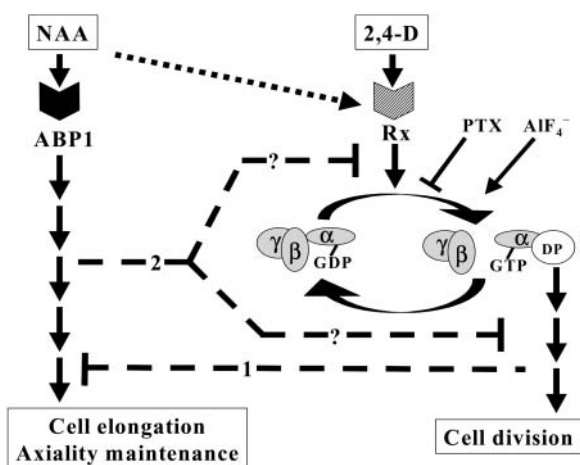
reduced number of shorter cells. We observe, however, that reduced cell division in consequence of treatment with PTX is always accompanied by increased, not by decreased, cell elongation (Fig. 4, A and C). At the present stage it is not possible to decide whether ABP1 signaling (Fig. 6, dashed line 2) inhibits Rx signaling upstream or downstream of the G proteins. A further open question is the concomitant stimulation of both cell division and cell elongation by AlF_4^- in combination with NAA as only exogenous auxin. This effect could be caused by the activation of other targets; AlF_4^- has been reported to activate animal small G-proteins as well (Ahmadian et al., 1997; Vincent et al., 1998). To overcome these limits of a pharmacological approach, it would be necessary to generate transgenic cell lines, where a constitutively active G-protein is expressed under control of an inducible promoter (Okamoto et al., 2001).

In earlier works on VBI-0 cell line, data were presented on changes in endogenous auxin (IAA) and cytokinin concentrations in subcultures grown at various levels of synthetic auxins (Zažimalová et al., 1995, 1996). Those results, together with our data, clearly demonstrate that VBI-0 cells sense variations in quality and quantity of exogenous auxin and control the intracellular content of free IAA and cytokinins in response to it. Measurements of the endogenous content of auxin and cytokinin in cells grown in our experimental conditions will be required to integrate into our model the contribution of IAA and cytokinins in controlling cell division and growth in VBI-0 tobacco cell line.

OUTLOOK

From a physiological and pharmacological analysis of cell division and cell elongation in the tobacco cell line VBI-0, we arrive at a model where different auxin species differentially affect cell division and cell elongation through different signal transduction pathways activated by different receptors. ABP1 preferentially binds NAA and leads to cell elongation. A second unknown receptor, Rx, preferentially binds 2,4-D and activates cell division through G-protein-mediated signal transduction. Both pathways show a negative crosstalk. The regulation of the two receptors during plant development might help to understand how such a simple molecule as auxin can be such a versatile and efficient regulator of plant development.

Starting from the pioneering work of Hertel et al. (1972), where an auxin-binding activity was detected in crude membrane preparations, several auxin-binding proteins have been isolated in affinity labeling and affinity purification assays, although only few are considered as good candidates for receptors (for review, see Napier et al., 2002). ABP1 could be isolated due to its high affinity for IAA and later shown to be an auxin receptor by the use of biotests for cell expansion. Several approaches have been launched

to isolate 2,4-D binding proteins (Ohmiya et al., 1993, 1998; Sugaya and Sakai, 1996; Sugaya et al., 2000), but so far none of them led to the isolation of an auxin receptor. Using cell division in VBI-0 as a bioassay for the activity of a 2,4-D receptor, one could both test the function of previously isolated candidates and screen for 2,4-D independent mutant lines with strong division activity.

MATERIALS AND METHODS

Cell Line

The tobacco (*Nicotiana tabacum*) cell line VBI-0 derives from stem pith explants of cv Virginia Bright Italia (Opatrný and Opatrná, 1976) and was cultivated in slightly modified Heller's liquid medium (Heller, 1953) supplemented with the synthetic auxins 2,4-D (4.5 μM; Fluka Chemie AG, Neu-Ulm, Switzerland) and NAA (5.4 μM; Sigma Chemical, St. Louis). Cells were subcultured every 3 weeks (Petrášek et al., 1998), inoculating 4 mL of stationary cells (5 $\times$ 10^6 cells mL^{-1}) into 30 mL of fresh medium in 100-mL Erlenmeyer flasks, and incubated at 25°C in darkness on an orbital shaker (KS250 basic, IKA Labortechnik, Staufen, Germany) at 120 rpm (1.8-cm diameter).

Morphometry

From each sample, 0.25-mL aliquots were stained with rhodamin-G6-chloride 1:100 v/v (in culture medium) for 10 min on a topover shaker and destained twice in 1-mL culture medium. Cells were then immediately viewed under a confocal laser scanning microscope (DM RBE; Leica, Bensheim, Germany) using a one-channel scan with an argon-krypton laser at 568 nm excitation, a 575-nm beam splitter, and a 590-nm emission filter. Image stacks were recorded at 200-fold magnification along the z axis, and cell length and width were determined from the central section of the cells using the Scion Image Software (Frederick, MD). The ratio of cell length to cell width was used to detect changes in the proportionality of cell expansion. An increase in this ratio therefore indicates that expansion is positively redistributed toward longitudinal growth. For each data point, 300 individual cells from 3 independent experimental series were scored.

Determination of Cell Viability and Division

Cell viability was assayed by the Trypan Blue dye exclusion test (Phillips, 1973). From each sample, 0.5-mL aliquots were stained with a 0.4% (w/v) solution of Trypan Blue (Sigma-Aldrich, Irvine, UK) at a ratio 1:100 v/v. After incubation for 3 min, the frequency of the unstained viable cells was scored as well as the number of cells per individual file using a Fuchs-Rosenthal hematocytometer under a bright-field microscope. For each individual sample, 10^3 cells were scored.

Auxin Treatments and Inhibitor Experiments

For the auxin experiments, stationary cells (cultured for 21 d under standard conditions) were inoculated into 30 mL of the usual culture medium but without exogenous auxins. Hormones were added directly to the respective final concentration into the auxin-starved cultures starting from filter-sterilized stocks of 5 mg mL^{-1} NAA and 5 mg mL^{-1} 2,4-D dissolved in 96% ethanol. An equal volume of sterile 96% (v/v) ethanol was added to the control samples cultured in standard conditions. For dose response curves, the concentrations of NAA were varied between 0.1 and 35 times the relative concentration present in standard conditions (5.4 μM), between 1 and 10 times (4.5 μM) in the case of 2,4-D. Depending on the experiment, the two auxins were administered either in combination or separately. For the pharmacological manipulation of G-protein activity, two drugs were used. AlF_4^- was synthesized as a 20 mM stock solution from a reaction between natrium fluoride (Merck, Darmstadt, Germany) and aluminum chloride (Fluka, Buchs, Switzerland) in a molar ratio of 4:1 in 50 mM Tris-HCl buffer at pH 8. PTX

(Sigma-Aldrich, Neu-Ulm, Germany) was supplied as a sterile suspension in glycerol. To ensure a saturating cellular concentration of the drugs despite the increase in cell number due to cell division and despite the instability of these drugs, they were added directly to the culture prior to inoculation and every 24 h during the subsequent 5 d when the cells were analyzed. The drugs were added each time to a final concentration of 100 μM for AlF_4^- or to 10 ng mL^{-1} for PTX. We tested different concentrations and application intervals for both drugs with respect to cell viability and potential superoptimal concentrations (data not shown). Moreover, we checked whether the results would change when the cells were transferred into fresh medium containing the freshly added drugs (to exclude potential accumulation of the drugs). However, we could not observe any significant difference between these two modi of drug application (data not shown). An equal aliquot of the corresponding sterile solvent was added to the control samples every time.

Extraction of RNA and Detection of Transcripts for the Auxin-Binding Proteins

VBI-0 cells were cultivated for 6 d and then harvested in liquid nitrogen for the extraction of the total RNA using RNeasy Mini kit (Qiagen, Hilden, Germany) following the protocol of the producer. After DNAse I treatment (Invitrogen, Karlsruhe, Germany), 300 pg of total RNA were used as template in a OneStep RT-PCR reaction (Qiagen) together with the primer pairs NtERabp1 5′-TCGCCATGTTCTCGTAG TGGTAGCT-3′/5′-GCTCATCTTTC-CACGAAGTTGTCTG-3′, NtERabp2 5′-CCCGCCACATCATCATACTAGT-TGC-3′/5′-GCTCATCTTTCCACGAAGTTGTCTG-3′, which span several introns on the *NtERabp* gene sequences (Leblanc et al., 1997), or mybB 5′-ATTTGCTTCTTGTTCATCTTTCT-3′/5′- CCCTTTGAACCTTTTCACC-3′, designed to specifically amplify a part of the *NtmybB* gene (accession no. AB056124). Oligonucleotide primers were purchased from Dr. Gabor Igloi (Institut für Biologie III, Freiburg, Germany). Amplificates were obtained from 35 cycles using 58°C for annealing and 1 min of elongation time. Control reactions at longer elongation times were performed to exclude genomic contaminations (data not shown). A total of 7 μL of each reaction were loaded on a preparative 2% (w/v) agarose gel. The identity of the amplificates was directly tested by elution of the PCR-products and sequencing (SeqLab, Göttingen, Germany and Igloi Institut für Biologie).

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

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