

# Hormone Autotrophic Growth and Differentiation Identifies Mutant Lines of Arabidopsis with Altered Cytokinin and Auxin Content or Signaling<sup>1</sup>

Markus Frank, Hans-Michael Rupp, Els Prinsen, Václav Motyka, Harry Van Onckelen, and Thomas Schmülling\*

Universität Tübingen, Center for Plant Molecular Biology (ZMBP), Allgemeine Genetik, Auf der Morgenstelle 28, D-72076 Tübingen, Germany (M.F., H.-M.R., T.S.); University of Antwerp, B-2610 Antwerp, Belgium (E.P., H.V.O.); and Institute of Experimental Botany, Academy of Science of the Czech Republic, Rozvojová 135, CZ-16502 Prague 6, Czech Republic (V.M.)

---

We describe mutant tissue lines of Arabidopsis that are able to grow in vitro as callus on hormone-free medium. The 14 lines presented here show different hormone autotrophic differentiation behaviors that can be classified into three categories: (a) forming roots (*rooty callus*), (b) forming shoots or shoot-like structures (*shooty callus*), or (c) growing without organ formation (*callus*). Three fast-growing lines showed altered steady-state mRNA levels of the *Cdc2* and *CycD3* cell cycle genes. Three of the six *rooty callus* lines contained about 20- to 30-fold higher levels of auxins than wild-type callus. These and two other lines with normal auxin content showed an increased steady-state level of *IAA1* and *IAA2* transcripts in the absence of exogenous auxin. Five of the six *shooty callus* lines had increased steady-state mRNA levels of the *CK1* gene and/or of the homeobox genes *KNAT1* and *STM*, suggesting that the phenotype is linked to altered cytokinin signaling. Also, one cytokinin-overproducing line with only 5% of wild-type cytokinin oxidase activity was identified. These results indicate that screening for hormone-autonomous growth identifies mutants with altered hormone content or signaling.

---

Plant cell division, growth, and differentiation need to be precisely controlled during development to ensure coordinated growth of tissues. Most mitotic activity is restricted to the meristems and the young growing tissues surrounding them. A loss of this control could lead to cell division and growth at ectopic positions and eventually to the development of unorganized tissue growth (i.e. plant tumors).

In many instances, de-differentiation and tumor formation in plant tissue is caused by an imbalance of the hormones auxin and cytokinin. Plant tumors are often able to grow in vitro as callus on medium without auxin or cytokinin. For example, pathogenesis of the crown gall disease

after infection by *Agrobacterium tumefaciens*, a well-studied case of plant tumor formation, is caused by the transfer and expression of auxin- and cytokinin-synthesizing genes to the plant cell (for review, see Morris, 1995). The auxin-to-cytokinin ratio influences qualitatively and quantitatively growth and differentiation of tumors in planta or callus grown in vitro. A high auxin-to-cytokinin ratio leads to root formation in calli, while a low auxin-to-cytokinin ratio favors shoot formation (Skoog and Miller, 1957).

Not only altered hormonal content, but also changes in hormone sensitivity or signal transduction can lead to the formation of tumors with a distinct differentiation behavior. The transgenic overexpression of the *Agrobacterium rhizogenes rolB* gene enhances auxin sensitivity and induces the formation of ectopic roots (Cardarelli et al., 1987; Maurel et al., 1991; Schmülling et al., 1993). Overproduction of the *CK1*-encoded His kinase homolog mimics enhanced cytokinin signaling and induces shoot formation (Kakimoto, 1996). In *Nicotiana tabacum*, plants carrying the *Hl-1* allele form tumors after infection with the auxin-synthesizing genes of *A. tumefaciens*. In wild-type plants tumors form after treatment with auxin and cytokinin (Meyer et al., 1997). This indicates that *Hl-1* enhances the sensitivity of certain tissues to cytokinins or activates growth-factor-independent pathways.

Other examples of genes that deregulate proper control of cell division and growth are the oncogenes *6b* and *Iso* of *A. tumefaciens* T-DNA. Infection with either gene leads to the formation of undifferentiated tumors on a limited number of host plants (Hooykaas et al., 1988; Otten and Schmidt, 1998). Similarly, overexpression of the *KNAT2* and *CycD3* genes causes an auxin- and/or cytokinin-independent tumor formation on Arabidopsis leaves (Dockx et al., 1996; Riou-Khamlichi et al., 1999). Arabidopsis tumors that show hormone-independent growth are also formed as a consequence of somatic mutations after  $\gamma$ -ray irradiation (Persinger and Town, 1991).

Plant tumors also arise spontaneously in certain combinations of genotypes and in high-inbred lines. These so-called genetic tumors have been especially well studied in the genus *Nicotiana*, e.g. in *Nicotiana glauca*  $\times$  *Nicotiana langsdorffii* hybrids (Smith et al., 1976; Ichikawa and Syono,

---

<sup>1</sup> This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 446), the Volkswagenstiftung, and International Association for the Promotion of Cooperation with Scientists from the New Independent States of the former Soviet Union (INTAS) to T.S. M.F. received a stipend from the Studienstiftung des deutschen Volkes. E.P. and H.V.O. are, respectively, a postdoctoral fellow and the research director of the Fund for Scientific Research Flanders.

\* Corresponding author; e-mail ts@uni-tuebingen.de; fax 49-7071-295042.

1991) and in radish (Buzovkina et al., 1993; Lutova et al., 1997).

We have used a genetic approach to identify elements that normally regulate cell division and differentiation and prevent tumor formation. In light of the close links between tumor formation and auxin and cytokinin metabolism and signaling, we hypothesized that this approach would lead to the identification of potential new hormone mutants. Negative control elements of cell division and differentiation that are not directly related to these hormones may also be identified using this approach. We isolated 14 *Arabidopsis* mutant tissue lines that showed a de-differentiation of organized tissues after germination, and subsequently grew in vitro as callus without exogenous hormones. Based on their differentiation behavior, we distinguish three mutant classes: the *shooty callus* class (*s1-s6*), the *rooty callus* class (*r1-r6*), and the *callus* class (*c1-c2*). We describe changes in hormone concentration and altered steady-state transcript levels of genes such as the *IAA*, *CK11*, and homeobox genes, which play central roles in growth and development.

## MATERIALS AND METHODS

### Mutant Screening and Growth Conditions

Ethylmethane sulfonate (EMS) mutagenized seeds of *Arabidopsis* Heynh. ecotype Columbia (Col-1 *g11*) were purchased from Lehle Seeds (Round Rock, TX). Approximately 29,000 surface-sterilized M2 individuals from four parental families were plated in vitro on hormone-free Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), containing 3% (w/v) Suc and solidified with 9 g/L agar (Agar Agar reinst, Merck, Darmstadt, Germany). Plates were incubated at 24°C under light (16 h)/dark (8 h) cycles. Seedlings were screened after 4 weeks for individuals that de-differentiated and continued to grow as callus-like tissue. Calli were further propagated on hormone-free MS medium and transferred weekly to fresh medium.

### Growth Measurement

For growth measurements, tumor explants (approximately 100 mg fresh weight) were placed on individual Petri dishes on hormone-free medium. The growth rate was expressed as  $W = (W_t - W_0)/W_0$ .  $W_0$  and  $W_t$  are the initial sample weight and the weight after 7 d, respectively. Data are the means of three experiments with five samples per data point in each experiment. Wild-type callus was grown on medium containing 1 mg/L naphthylacetic acid and 0.1 mg/L  $N^6$ -( $\Delta^2$ isopentenyl)adenine riboside (iP).

### Hormone Analysis

The cytokinin and indole-3-acetic acid (IAA) content of callus harvested 7 d after subcultivation was measured as described previously (Prinsen et al., 1995a, 1995b). Cytokinins and IAA were extracted overnight from approximately 1 g of frozen tissue in  $CHCl_3$ /methanol/water/acetic acid (Bielecki, 1964) and purified combining solid-

phase extraction and immunoaffinity chromatography using a broad spectrum anti-cytokinin antibody. The stable isotopes [ $^2H_5$ ]trans-zeatin, [ $^2H_5$ ]zeatin riboside, [ $^2H_5$ ]zeatin 9-glucoside, [ $^2H_5$ ]zeatin 7-glucoside, [ $^2H_5$ ]zeatin O-glucoside, [ $^2H_5$ ]zeatin riboside O-glucoside, [ $^2H_5$ ]zeatin riboside 5'-monophosphate, [ $^2H_6$ ]iP, [ $^2H_6$ ]iP riboside, [ $^2H_6$ ]iP 9-glucoside (20 ng of each, Apex Organics, Honiton, Devon, UK) and [*phenyl*- $^{13}C_6$ ]indole-3-acetic acid (50 ng, Cambridge Isotope Lab, Cambridge, MA) were initially added as internal tracers for recovery and analytical purposes. The different cytokinin fractions obtained after purification were analyzed by micro liquid chromatography with column switch configuration coupled to positive ion electrospray tandem mass spectrometry using multiple reactant monitoring (Prinsen et al., 1995b, 1998). After pentafluorobenzyl derivatization of IAA, PFB-IAA was analyzed by negative ion chemical ionization gas chromatography-selected ion monitoring-MS (Epstein and Cohen, 1981). Prior to purification, IAA conjugates were converted to free IAA by alkaline hydrolysis (Bialek and Cohen, 1989).

### Cytokinin Oxidase Activity

Cytokinin oxidase was extracted from 3 to 8 g of callus tissue harvested 7 d after subcultivation. The cytokinin oxidase activity was determined using the method of Chatfield and Armstrong (1986) as modified by Motyka and Kamínek (1994) based on the measurement of the rate of conversion of [ $2,8$ - $^3H$ ]iP to adenine. Separation of the substrate from the product of the enzyme reaction was achieved by thin-layer chromatography on microcrystalline cellulose plates developed with the upper phase of the 4:1:2 (v/v) mixture of ethylacetate/*n*-propanol/water. Zones containing iP and adenine were located under UV light, cut into strips, and their radioactivity was measured using the liquid scintillation technique. Cytokinin oxidase activity determinations were repeated three times for each tissue sample. The SD averaged 8% and did not exceed 17% of the means.

### RNA Analysis

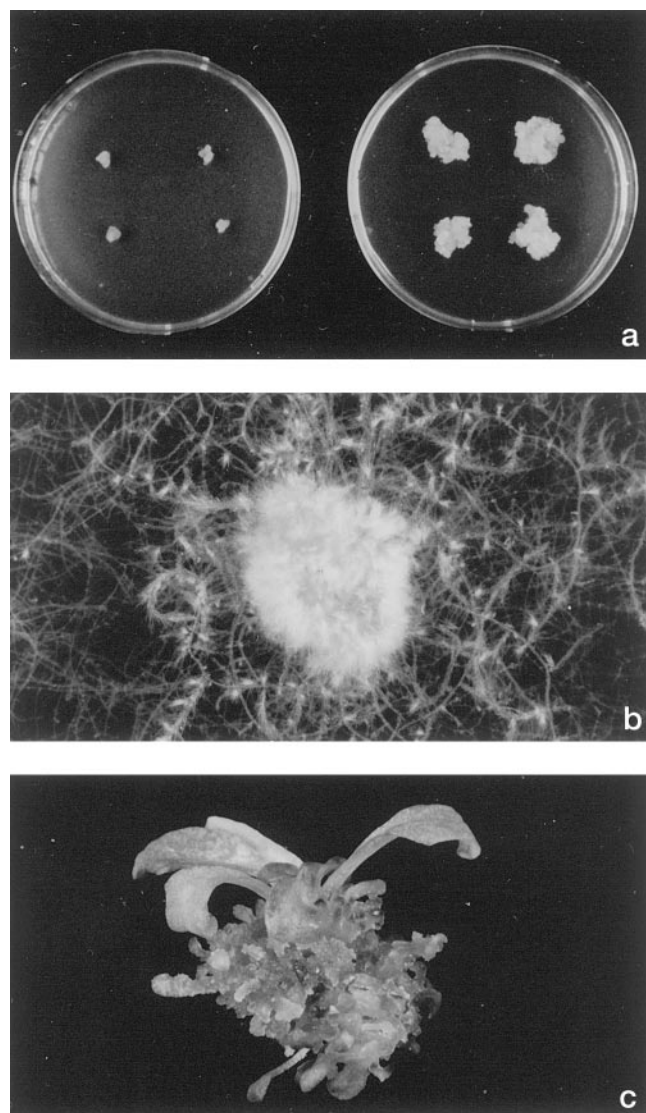
Total RNA was extracted from plant tissues according to Verwoerd et al. (1989). RNA (50  $\mu$ g) was separated in a denaturing 1.5% (w/v) agarose-formaldehyde gel, transferred to nylon membranes (Hybond N, Amersham, Buckinghamshire, UK), and hybridized with radioactive-labeled DNA probes generated with a random primer labeling kit (Prime-It II, Stratagene, Heidelberg). Hybridizations were carried out in hybridization solution (QuickHyb, Stratagene) according to the manufacturer's instructions. The lowest stringency wash was performed in  $0.2\times$  SSC and 0.1% (w/v) SDS at 65°C. As a control for loading, the blot was rehybridized with a 25S rDNA probe.

## RESULTS

### Identification and Classification of Mutant Lines

Among approximately 29,000 mutagenized M2 seedlings, we identified 14 lines that showed tissue de-

differentiation after germination. In all cases tissue dedifferentiation yielded callus or callus-like tissue that could, in contrast to wild-type callus, be propagated on hormone-free medium (Fig. 1a). Based on their main characteristics, we distinguished three mutant classes: (a) six lines that we called *rooty callus* (*r1-r6*) grew as a brownish, soft callus, formed roots, and were uniform in appearance (Fig. 1b); (b) six lines that we called *shooty callus* (*s1-s6*) were green, formed shoots or shoot-like structures (Fig. 1c), and differed in their capability to form shoots and in the size of the shoots; and (c) two lines that we called *callus1* and *callus2* (*c1* and *c2*) formed neither shoots nor roots on hormone-free medium (Fig. 1a). Line *c1* responded readily to exogenous cytokinin by forming shoots, whereas no



**Figure 1.** Phenotype of Arabidopsis mutant lines cultivated in vitro on hormone-free medium. a, Growth of mutant line *c1* (right) compared with growth of control calli on hormone-free MS medium (left). b, Root-forming phenotype of mutant line *r2* grown on hormone-free MS medium. c, Shoot-forming phenotype of mutant line *s3* grown on hormone-free MS medium.

shoots formed in *c2*, even after prolonged incubation with elevated cytokinin concentrations (10 mg/L iP). *c2* formed roots in response to exogenous auxin. Therefore, for further investigation, *c1* was examined in parallel with the *shooty callus* class, *c2* with the *rooty callus* class. The phenotype of all mutant tissue lines has been stable for more than 2 years.

We attempted to regenerate plants from the calli to obtain progeny and to characterize the mutant lines genetically. The *rooty callus* and *callus* lines, as well as the majority of the *shooty callus* lines, could not be regenerated to form plants or were infertile. From line *r1* we obtained two seeds by selfing more than 200 regenerants. One of these seeds germinated in vitro and reproduced the parental phenotype. Dedifferentiation and callus formation occurred in the  $F_2$  progeny of line *s1*, obtained after backcrossing with the wild type, at a ratio of approximately 1:3 (122 of 466 analyzed  $F_2$  seedlings showed the mutant phenotype and 344 were phenotypically wild type), indicating that a single recessive mutation is responsible for the phenotype. The mutated locus has been mapped to chromosome 2 (data not shown). A more detailed analysis of this mutant will be described elsewhere (M. Frank, I. Lorenz-Meyer, A. Guivarch, D. Chriqui, and T. Schmülling, unpublished data).

### Growth and Cell Cycle Regulator Genes

First, we compared the growth rate of the mutant lines growing on hormone-free medium with wild-type callus growing on auxin- and cytokinin-containing medium. The gain in fresh weight was for all mutant lines 2- to 13-fold faster than for wild-type callus. The highest increases in fresh weight were found in the lines *s6* and *r3* (Table I; data not shown).

Next, we investigated steady-state mRNA levels of the cell cycle genes *H4*, *Cdc2a*, and *CycD3* in the mutant lines and compared it with wild-type seedlings and callus. Deregulated expression of the homologous genes in animals is often linked to tumor formation (Hunter, 1997). Results of northern-blot analyses are shown in Figure 2, and the relative signal strength compared with the 25S control hybridization is listed for selected lines in Table I.

The transcript abundance of the histone *H4* gene, a marker for the cell cycle S phase, was in almost all mutant calli comparable to that of wild-type seedlings and calli. The highest *H4*/25S ratios, 2.5 and 1.9, were found in the fastest-growing lines *s6* and *r3*, respectively (Table I).

Expression of *Cdc2a* occurs in dividing cells and in cells with competence to divide (Hemerly et al., 1993). As indicated in Figure 2 and Table I, the fastest-growing mutant lines, *s6*, *r3*, and *c2*, showed a slightly enhanced steady-state mRNA level of *Cdc2a*. The *Cdc2a*/25S ratios were 1.3- to 1.4-fold higher than in the wild type or in the other mutant calli (Table I; data not shown).

*CycD3* regulates the G1/S transition. Its overexpression causes cytokinin-independent tumor formation in plants (Riou-Khamlichi et al., 1999). Figure 2 and Table I show that *CycD3* transcript levels were approximately 1.9- to 3.5-fold increased in lines *s6*, *c2*, and *r3* compared with wild-type seedlings and calli.

**Table I.** Growth and cell-cycle gene expression in wild-type (WT) and mutant *Arabidopsis* lines

The growth coefficient ( $W$ ) for 1 week of growth is expressed as  $(W_t - W_0)/W_0$ .  $W_0$  and  $W_t$  are the initial and final average weights of calli. Data are the means of three independent experiments with five samples per point in each experiment. Intensities of hybridizations shown in Figure 2 were determined by measurement of the optical filter density. Ratios of signal strength were compared with the ratio of WT callus, which was set arbitrarily at 1. ND, Not determined.

Plant Material	$W$	Increase <sup>a</sup>	Ratio				
			<i>H4/25S</i>	<i>Cdc2a/25S</i>	<i>Cdc2a/H4</i>	<i>CycD3/25S</i>	<i>CycD3/H4</i>
Seedling (WT)	ND	—	1.1	0.9	0.8	0.8	0.7
Callus							
WT	0.1	—	1.0	1.0	1.0	1.0	1.0
<i>s2</i>	0.7	7	1.2	0.9	0.9	1.1	0.9
<i>s6</i>	1.7	17	2.5	1.4	0.7	3.5	1.4
<i>c2</i>	0.7	7	1.6	1.4	0.9	2.5	1.6
<i>r2</i>	0.3	3	0.9	1.0	1.0	1.0	1.1
<i>r3</i>	1.5	15	1.9	1.3	0.7	1.9	1.0

<sup>a</sup>Increase in mutant calli compared with wild-type calli.

### Analysis of the Auxin and Cytokinin Content

The mutant lines mimic a hormone effect without the presence of exogenous hormones. We therefore determined the endogenous concentrations of auxin and cytokinins. Table II shows that the IAA content was approximately 10- to 25-fold higher in the lines *r1*, *r2*, and *r6* than in control tissue. In the same lines, the IAA conjugate concentration was increased 5- to 33-fold. In contrast, lines *r3*, *r4*, and *r5*, both *c* lines, and all *s* lines contained similar auxin and auxin conjugate levels as controls (data not shown).

Table III shows that line *c1* contained between 5- and 80-fold higher concentrations of most of the 17 different cytokinin metabolites of the *iP*-, *trans*-zeatin, and dihydrozeatin-type cytokinins compared with wild type. The highest increases were found for zeatin riboside (20-fold) and the *O*-conjugates zeatin *O*-glucoside (81-fold) and dihydrozeatin *O*-glucoside (45-fold). It is noteworthy that the total content of *N*-conjugates was higher than that of the *O*-conjugates (Table III). This indicates that *N*-conjugation is relevant in *Arabidopsis*, while it is weak or does not occur in cytokinin-overproducing tobacco (Motyka et al., 1996; Faiss

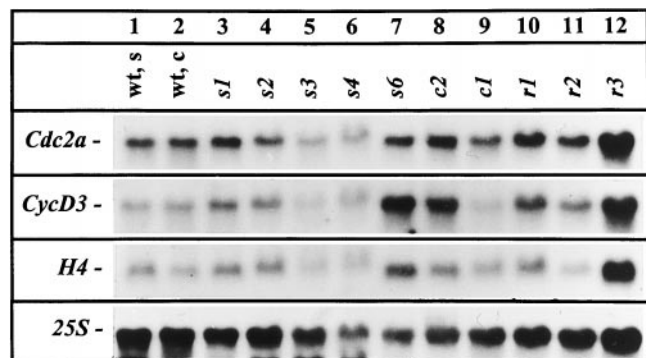
et al., 1997; Rupp et al., 1999). The cytokinin content of all other mutant lines was similar to wild type or did not show consistent differences (data not shown).

### Analysis of Cytokinin Oxidase Activity

The presence of higher cytokinin metabolite concentrations in line *c1* could be due to increased cytokinin synthesis and/or decreased catabolism. Cytokinin oxidase is the key enzyme of cytokinin degradation in plants (Armstrong, 1994). Table IV shows that *c1* has approximately 5% of wild-type cytokinin oxidase activity. None of the other tested lines (*r1*, *c2*, *s1*, *s4*, and *s6*) had significantly altered cytokinin oxidase activities compared with wild-type callus (Table IV; data not shown).

### Steady-State mRNA Levels of *IAA1* and *IAA2*

We used the auxin primary response genes *IAA1* and *IAA2* (Abel et al., 1995) as marker genes to test for altered auxin signaling in the mutants. Figure 3 shows that *r1*, *r2*, *r3*, and *c2* contained approximately 3- to 5-fold elevated steady-state mRNA levels of the *IAA1* and *IAA2* genes. In contrast, the *s* lines and line *c1* contained no detectable or only low levels of these transcripts (Fig. 5 and data not



**Figure 2.** Northern-blot analyses of *H4*, *Cdc2*, and *CycD3* steady-state mRNA levels in wild-type seedlings (wt, s), wild-type calli (wt, c), and mutant calli. Total RNA (50  $\mu$ g) was separated in a denaturing formaldehyde gel and, after blot transfer to a nylon filter, hybridized with <sup>32</sup>P-labeled specific cDNA probes. Hybridization with a 25S rDNA probe served as a control for loading.

**Table II.** Content of free and conjugated IAA in wild-type (WT) seedlings and mutant calli

The mutant calli and WT seedlings were grown in vitro on solidified, hormone-free MS medium. IAA and IAA conjugate concentrations were determined by gas chromatography-selected ion reactant-MS as described in "Materials and Methods." The data are the means  $\pm$  SE of three independent biological replications.

Plant Material	IAA	IAA Conjugates
WT seedling	12.7 $\pm$ 4.6	13,400 $\pm$ 5,300
Callus		
<i>r1</i>	295 $\pm$ 41	430,000 $\pm$ 173,000
<i>r2</i>	205 $\pm$ 44	179,000 $\pm$ 23,000
<i>r6</i>	121 $\pm$ 12	71,000 $\pm$ 19,000

**Table III.** Cytokinin content of mutant line *c1* compared to wild-type (WT) seedlings

Mutant calli and wild-type seedlings (wt, s) were grown in vitro on solidified hormone-free MS medium. Cytokinin concentrations were determined by micro liquid chromatography with column switch configuration coupled to positive ion electrospray tandem mass spectrometry using multiple reactant monitoring as described in "Materials and Methods." The data are the means  $\pm$  SE of two biological independent replications.

Cytokinin Metabolite	WT Seedling	<i>c1</i> Callus	Increase <sup>b</sup>
	<i>pmol g<sup>-1</sup> fresh wt</i>		
iP	7.6 <sup>a</sup>	1.2 $\pm$ 0.1	0.2
iP Riboside	3.7 $\pm$ 3.3	22.7 $\pm$ 5.4	6
iP Riboside 5'-monophosphate	1.5 $\pm$ 0.1	12.5 $\pm$ 0.4	8
iP 9-Glucoside	4.7 $\pm$ 2.2	25.7 $\pm$ 4.3	6
<i>trans</i> -Zeatin	0.8 <sup>a</sup>	0.65 $\pm$ 0.0	1
Zeatin riboside	2.2 $\pm$ 1.7	42.9 $\pm$ 5.4	20
Zeatin riboside 5'-monophosphate	2.4 $\pm$ 1.3	11.4 $\pm$ 2.0	5
Zeatin <i>O</i> -glucoside	6.5 $\pm$ 1.0	525 $\pm$ 105	81
Zeatin riboside <i>O</i> -glucoside	2.3 $\pm$ 0.3	14.8 $\pm$ 0.0	6
Zeatin 7-glucoside	148 $\pm$ 26	751 $\pm$ 25	5
Zeatin 9-glucoside	21 $\pm$ 20	257 $\pm$ 50	12
Dihydrozeatin	1.4 <sup>a</sup>	0.2 $\pm$ 0.1	0.2
Dihydrozeatin riboside	1.1 $\pm$ 0.7	2.8 $\pm$ 0.3	3
Dihydrozeatin riboside 5'-monophosphate	1.0 $\pm$ 0.3	1.4 $\pm$ 0.0	1
Dihydrozeatin <i>O</i> -glucoside	7.1 $\pm$ 2.5	322 $\pm$ 46	45
Dihydrozeatin riboside <i>O</i> -glucoside	2.3 $\pm$ 0.3	11.7 $\pm$ 3.1	5
Dihydrozeatin 7-glucoside	2.8 $\pm$ 1.6	15.1 $\pm$ 7.2	5
Dihydrozeatin 9-glucoside	9.7 $\pm$ 9.1	13.9 $\pm$ 0.0	1

<sup>a</sup> Data from a single sample. <sup>b</sup> Increase in *c1* callus compared with wild-type seedlings.

shown). Lines *r3* and *c2* do not have an elevated auxin content, which indicates altered auxin signal transduction.

### Steady-State mRNA Level CK11

The *shooty callus* mutant lines showed cytokinin-autonomous proliferation and shoot formation similar to Arabidopsis calli overexpressing the *CK11* gene (Kakimoto, 1996). Figure 4 shows that lines *s1*, *s2*, and *s4* contained at least a 5-fold-increased steady-state mRNA level of *CK11* compared with wild-type control tissues. Lines *s3* and *s5*

contained elevated levels of *CK11* mRNA, but the increase was not as large (Fig. 4).

### Steady-State mRNA Levels of *KNAT1* and *STM*

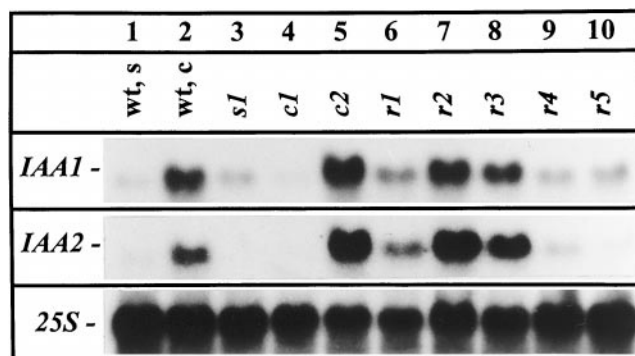
The transgenic overproduction of cytokinins, *KNAT1*, or its maize homolog, *KN1*, are sufficient to induce ectopic shoot formation in tobacco and Arabidopsis, indicating that they might act on a common pathway (Sinha et al., 1993; Hewelt et al., 1994; Chuck et al., 1996). Therefore, deregulated expression of homeobox genes could be linked to the phenotypic traits of the *shooty callus* mutant class.

**Table IV.** Cytokinin oxidase activity in mutant lines

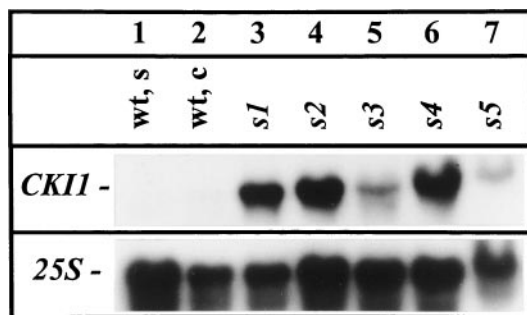
Cytokinin oxidase assays were based on the rate of conversion of [2,8-<sup>3</sup>H]iP to adenine. Substrate and product were separated by thin-layer chromatography. Radioactivity was measured using a liquid scintillation counter. Other details are described in "Materials and Methods." Wild-type (WT) seedlings and mutant calli were grown on solidified, hormone-free MS medium. WT callus was grown on 1 mg/L naphthylacetic acid and 0.1 mg/L iP. The values represent the means  $\pm$  SE of three independent samples.

Plant Material	Cytokinin Oxidase Activity
	<i>pmol mg<sup>-1</sup> protein h<sup>-1</sup></i>
Seedling (WT)	11.4 $\pm$ 1.7
Callus	
WT	4.9 <sup>a</sup>
<i>c1</i>	0.6 $\pm$ 0.3
<i>c2</i>	4.5 $\pm$ 2.5
<i>s1</i>	4.5 $\pm$ 0.5
<i>r1</i>	4.8 $\pm$ 0.7

<sup>a</sup> Data from a single replicate.



**Figure 3.** Northern-blot analyses of *IAA1* and *IAA2* steady-state mRNA levels in wild-type seedlings (wt, s), wild-type calli (wt, c), and mutant calli. Total RNA (50  $\mu$ g) was separated in a denaturing formaldehyde gel and, after blot transfer to a nylon filter, hybridized with <sup>32</sup>P-labeled specific cDNA probes. Hybridization with a 25S rDNA probe served as a control for loading.



**Figure 4.** Northern-blot analysis of *CKII* steady-state mRNA levels in wild-type seedlings (wt, s), wild-type calli (wt, c), and mutant calli. Total RNA (50  $\mu$ g) was separated in a denaturing formaldehyde gel and, after blot transfer to a nylon filter, hybridized with  $^{32}$ P-labeled specific cDNA probes. Hybridization with a 25S rDNA probe served as a control for loading.

Figure 5 shows that the steady mRNA levels of *STM* was 3- to 4-fold increased in all *shooty callus* lines (especially in lines *s1*, *s2*, and *s5*) compared with wild-type seedlings and calli. *KNAT1* mRNA accumulated also in these lines. In contrast, lines *s3*, *s4*, and *s6* contained *KNAT1* mRNA levels comparable to wild-type and seedlings and calli (Fig. 5). Both genes were poorly expressed in the *rooty callus* lines (data not shown).

## DISCUSSION

We have isolated three mutant classes that exhibited loss of control of cell division activity after germination and could grow as a callus on hormone-free medium. These mutant tissue lines resemble phenotypically somatic mutants isolated previously after  $\gamma$ -radiation of Arabidopsis seeds and seedlings (Persinger and Town, 1991; Campell and Town, 1992). The lines described here were analyzed for differences in cell cycle gene expression and, in particular, for differences in auxin and cytokinin content or signaling to detect an eventual correlation between the mutant phenotype and changes of these parameters.

All mutant lines, in particular *s6* and *r3*, had a higher growth rate than wild-type callus grown on auxin- and cytokinin-containing medium (Table I). The steady-state mRNA level of cell cycle genes was not strongly altered in the mutant lines, unlike the deregulation that occurs in animal tumors (Hunter, 1997). In particular, the human *cyclin D1* gene, and perhaps other D-type cyclins, are putative proto-oncogenes possibly activated by deregulated transcription (Motokura and Arnold, 1993). In plants, the correlation between overexpression of genes encoding cell cycle regulators and differentiation is less clear. *Cdc2* and *CycB1* overexpression in Arabidopsis neither altered development nor caused neoplasia (Hemerly et al., 1995; Doerner et al., 1996). In contrast, ectopic and enhanced expression of *CycD3* perturbed plant growth and differentiation (Riou-Khamlichi et al., 1999).

Most mutant lines showed a ratio of the S-phase marker *H4* and *Cdc2a* similar to wild type. The *Cdc2a/H4* ratio can be taken as a measure of the ratio of division-competent cells to cells that are actually dividing. A decrease in the

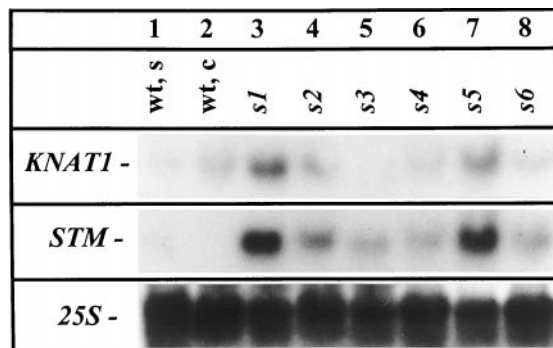
*Cdc2a/H4* ratio indicates a higher percentage of dividing cells. Indeed, the *Cdc2/H4* mRNA ratios were lowest in the fast-growing mutant lines *s6* and *r3* (Table I), indicating that cell division, and not just cell expansion, contributes to the faster growth. The fast-growing mutant lines *s6*, *c2*, and *r3* also have the highest *CycD3* steady-state levels (Table I). This could indicate that *CycD3* is limiting for growth in wild type and the other mutant lines and that deregulated *CycD3* expression might be causally linked to the rapid growth.

The *rooty callus* mutants mimic an auxin phenotype, and the *shooty callus* mutants a cytokinin phenotype in the absence of exogenous hormones. This led us to study hormone content and signal transduction in the mutant lines. In the following sections the results are discussed separately for *rooty* and *shooty callus* lines.

## The *rooty* Mutant Lines

In three of the *rooty callus* lines (*r1*, *r2*, and *r6*), we detected a significantly elevated content of free and conjugated IAA (Table II). Two mutants that overproduce auxin have been previously reported in Arabidopsis. One mutant overproliferating lateral roots was independently isolated several times and called *sur1* (Boerjan et al., 1995), *rty* (King et al., 1995), *hls3* (Lehman et al., 1996), and *alf1* (Celenza et al., 1995). The *RTY/SUR1* gene encodes a protein similar to Tyr aminotransferases possibly implicated in auxin synthesis (Golparaj et al., 1996). *sur1* mutants produce calli that grow on hormone-free medium (Boerjan et al., 1995; King et al., 1995; Delarue et al., 1998). Recently, a second auxin-overproducing mutant, *sur2*, has been identified in Arabidopsis (Delarue et al., 1998). However, *sur2* explants are unable to sustain auxin-autonomous growth. It is possible that the auxin-overproducing *rooty callus* harbor mutated alleles of the *RTY/SUR1* gene.

Lines *r3* and *c2* had normal auxin contents but increased *IAA1* and *IAA2* transcript levels. This indicates that auxin signal transduction is affected in these mutant lines. Possibly, a loss-of-function mutation of a repressor of the



**Figure 5.** Northern-blot analysis *KNAT1* and *STM* steady-state mRNA levels in wild-type seedlings (wt, s), wild-type calli (wt, c), and mutant calli. Total RNA (50  $\mu$ g) was separated in a denaturing formaldehyde gel and, after blot transfer to a nylon filter, hybridized with  $^{32}$ P-labeled specific cDNA probes. Hybridization with a 25S rDNA probe served as a control for loading.

auxin response results in a constitutive auxin response. Similar mutants have yet to be described. Two mutants, *age1* and *age2*, that show enhanced auxin sensitivity of an *IAA* gene promoter or ectopic promoter activity in the absence of exogenous IAA were isolated previously (Oono et al., 1998). In contrast to the lines described here, these mutants did not show altered tissue differentiation.

Interestingly, *r4* and *r5* have neither an altered auxin content nor an altered transcript level of the *IAA1* and *IAA2* genes (Fig. 3). However, *r4* and *r5* have enhanced steady-state mRNA levels of another auxin response gene, *IAA9* (M. Frank and T. Schmülling, unpublished results). This suggests either that *r4* and *r5* are mutated in a different auxin response pathway than the other *shooty callus* mutants, or they are mutated in a gene that regulates functions downstream of *IAA1* and *IAA2*. In comparison, undifferentiated or root-forming Arabidopsis mutant lines obtained by  $\gamma$ -radiation did neither contain increased auxin concentrations, which supports the notion that this phenotype can be induced without increased auxin (Campbell and Town, 1991). In conclusion, these results indicate that the screening procedure has the potential to identify new loci of the auxin response pathway.

### The *shooty* Mutant Lines

None of the *shooty callus* lines had a significantly altered auxin or cytokinin content. Only in line *c1* did we detect strongly elevated cytokinin concentrations (Table III). The strong reduction of cytokinin oxidase activity in this line (Table IV) could affect *c1* hormone homeostasis. The accumulation of cytokinin conjugates might be a detoxification mechanism that operates to avoid the accumulation of toxic concentrations of biologically active cytokinins. The results presented here suggest that a reduction in cytokinin oxidase activity may result in accumulation of conjugates and unorganized growth of tissue. A cytokinin oxidase gene has been cloned from maize (Houba-Hérin et al., 1999; Morris et al., 1999), and several homologous candidate genes are identifiable by comparison with the genomic sequence in Arabidopsis (K. Lemcke, T. Werner, and T. Schmülling, unpublished results). Therefore, loss-of-function studies become feasible for this enzyme. Experiments to test the influence of reduced cytokinin oxidase activity on differentiation processes are in progress.

The steady-state mRNA level of the *CK11* His kinase gene is enhanced in all *shooty callus* mutant lines (Fig. 4). *CK11* is likely to be involved in cytokinin signaling (Kakimoto, 1996). Transgenic overexpressors of the *CK11* gene share phenotypic characteristics such as cytokinin-independent proliferation and shoot formation with the *shooty callus* lines. Mutations in negative regulatory elements of *CK11* could be the reason for the enhanced *CK11* transcript levels and the constitutive cytokinin response phenotype of the *shooty callus* lines. However, the present data do not exclude the possibility that the higher gene expression levels are merely a consequence of the mutant's morphology and not a cause.

Similarly, we observed in the *shooty callus* lines an increase in the steady-state mRNA levels of the shoot-

meristem-specifying class I homeobox gene *STM* (Fig. 5). We hypothesize that lines overexpressing both *CK11* and *STM* are mutated in a pathway that links *CK11* and *STM* functionally. The differences in relative transcript abundance could be due to different types of mutations and/or to overproliferation of different tissues. Likewise, differences in the up-regulation of *KNAT1* in the different mutant lines and in comparison with *STM* accumulation (Fig. 5) could reflect a different contribution of the original *KNAT1* expression domain to the callus tissue. *KNAT1* transcript is localized in the wild type primarily in the peripheral zone of the vegetative shoot apical meristem (Lincoln et al., 1994), while *STM* is expressed in a central region (Endrizzi et al., 1996; Long et al., 1996).

In this context it is noteworthy that the cytokinin-overproducing *amp1* mutant and transgenic cytokinin overproducers also have increased steady-state mRNA levels of *STM* and *KNAT1*. It was therefore hypothesized that cytokinins participate in the regulation of homeobox gene expression (Rupp et al., 1999). The fact that *STM*- and *KNAT1*-overexpressing *shooty callus* lines do not have an altered cytokinin content is compatible with the hypothesis that cytokinins act upstream of *KNAT1* and *STM*.

Enhanced cytokinin signaling by *CK11* in the *shooty callus* lines might be causally related to enhanced shoot meristem activity through deregulation of shoot-meristem-specifying homeobox genes. This raises the possibility that cytokinins, *CK11*, and these homeobox genes act on the same pathway. Further experimental proof is required to support this hypothetical model.

### CONCLUSIONS

Our experimental results indicate that the phenotype of all *shooty callus* lines and of line *c2* is linked to increased auxin content or signaling. In all *shooty callus* lines and in line *c1*, we found increased cytokinin content or indications of enhanced cytokinin signaling. Thus, the analyses of hormone content and hormone sensitivity have so far confirmed our operational concept. This supports the idea that tightly regulated auxin and cytokinin metabolism and signaling are a prerequisite for coordinated growth and development.

This pilot study demonstrates that screening for such mutants and analysis of known hormonal parameters provides a useful approach to identify new factors involved in auxin and cytokinin metabolism and/or signal transduction. The phenotypic differences between different *shooty callus* lines and differences in the expression of marker genes suggests that mutations of different genes have occurred in the various lines. One new genetic locus has been identified in the progeny of line *s1* and we have been able to identify similar mutants from mutagenized single lines that map to different chromosomal locations. We are currently analyzing the early events during the de-differentiation process caused by these mutations (M. Frank, I. Lorenz-Meyer, A. Guivarch, D. Chriqui, and T. Schmülling, unpublished results).

## ACKNOWLEDGMENTS

We thank Franka Schermutzki for help with seed sterilization and plating, and S. Öden for skillful technical assistance. Plasmids harboring part of the *KNAT1* and *STM* cDNAs were a generous gift of Dr. Thomas Laux. The *CycD3* gene probe was kindly provided by Dr. James A. H. Murray, and the *IAA1* and *IAA2* gene probes were kindly provided by Drs. Steffen Abel and Anastasios Theologis. We also acknowledge proofreading by Catherine Scott-Taggart.

Received August 9, 1999; accepted November 12, 1999.

## LITERATURE CITED

- Abel S, Nguyen MD, Theologis A (1995) The *PS-IAA4/5*-like family of early auxin-inducible mRNAs in *Arabidopsis thaliana*. *J Mol Biol* **251**: 533–549
- Armstrong J (1994) Cytokinin oxidase and the regulation of cytokinin degradation. In DWS Mok, MC Mok, eds, *Cytokinins: Chemistry, Activity and Function*. CRC Press, Boca Raton, FL, pp 139–154
- Bialek K, Cohen JD (1989) Quantitation of indoleacetic acid conjugates in bean seeds by direct tissue hydrolysis. *Plant Physiol* **90**: 398–400
- Bialeski WJ (1964) The problem of halting enzyme action when extracting plant tissues. *Anal Biochem* **9**: 431–442
- Boerjan W, Cervera MT, Delarue M, Beeckman T, Dewitte W, Bellini C, Caboche M, Van Onckelen H, Van Montagu M, Inzé D (1995) *superroot*, a recessive mutation in *Arabidopsis*, confers auxin overproduction. *Plant Cell* **7**: 1405–1419
- Buzovkina IS, Kneshe I, Lutova LA (1993) The model of tumorigenesis in vitro in inbred and hybrids of radish. *Genetika* **29**: 1002–1008
- Campell BR, Town CD (1991) Physiology of hormone autonomous tissue lines derived from radiation-induced tumors of *Arabidopsis thaliana*. *Plant Physiol* **97**: 1166–1173
- Campell BR, Town CD (1992) Characterization of overexpressed cDNAs isolated from a hormone-autonomous, radiation-induced tumor tissue line of *Arabidopsis thaliana*. *Plant Physiol* **100**: 2018–2023
- Cardarelli M, Mariotti D, Pomponi M, Spano L, Capone I, Costantino P (1987) *Agrobacterium rhizogenes* T-DNA genes capable of inducing hairy root phenotype. *Mol Gen Genet* **209**: 475–480
- Celenza JL, Grisafi PL, Fink GGR (1995) A pathway for lateral root formation in *Arabidopsis thaliana*. *Genes Dev* **9**: 2131–2142
- Chatfield JM, Armstrong DJ (1986) Regulation of cytokinin oxidase activity in callus tissues of *Phaseolus vulgaris* L. cv Great Northern. *Plant Physiol* **80**: 493–499
- Chuck C, Lincoln C, Hake S (1996) *KNAT1* induces lobed leaves with ectopic meristems when overexpressed in *Arabidopsis*. *Plant Cell* **8**: 1277–1289
- Delarue M, Prinsen E, Van Onckelen H, Caboche M, Bellini C (1998) *Sur2* mutations of *Arabidopsis thaliana* define a new locus involved in the control of auxin homeostasis. *Plant J* **14**: 603–611
- Dockx J, Kock P, Willemsen V, Weisbeek P, Smeekens S, Scheres B (1996) The role of the homeobox gene *KNAT2* in *Arabidopsis* development. Presented at the 7th International Conference on *Arabidopsis* Research, Norwich, UK, poster 196
- Doerner P, Jorgensen JE, You R, Stepphuhn J, Lamb C (1996) Control of root growth and development by cyclin expression. *Nature* **380**: 520–523
- Endrizzi K, Moussian B, Haecker A, Levin JZ, Laux T (1996) The *SHOOT MERISTEMLESS* gene is required for maintenance of undifferentiated cells in *Arabidopsis* shoot and floral meristems and acts at a different regulatory level than the meristem genes *WUSCHEL* and *ZWILLE*. *Plant J* **10**: 967–979
- Epstein E, Cohen JD (1981) Microscale preparation of pentafluorobenzyl esters: electron-capture gas chromatographic detection of indole-3-acetic acid from plants. *J Chromatogr* **209**: 413–420
- Faiss M, Zalubílova J, Strnad M, Schmülling T (1997) Conditional transgenic expression of the *ipt* gene indicates a function for cytokinins in paracrine signaling in whole tobacco plants. *Plant J* **12**: 401–415
- Golparaj M, Tseng TS, Olszewski N (1996) The *ROOTY* gene of *Arabidopsis* encodes a protein with high similarity to aminotransferases (abstract no. 469). *Plant Physiol* **111**: S-114
- Hemerly A, Engler JdA, Bergounioux C, Van Montagu M, Engler G, Inzé D, Ferreira P (1995) Dominant negative mutants of the Cdc2 kinase uncouple cell division from iterative plant development. *EMBO J* **14**: 3925–3936
- Hemerly AS, Ferreira P, de Almeida Engler J, Van Montagu M, Engler G, Inzé D (1993) *cdc2a* expression in *Arabidopsis* is linked with competence for cell division. *Plant Cell* **5**: 1711–1723
- Hewelt A, Prinsen E, Schell J, Van Onckelen H, Schmülling T (1994) Promoter tagging with a promoterless *ipt* gene leads to cytokinin-induced phenotypic variability in transgenic tobacco plants: implications of gene dosage effects. *Plant J* **6**: 879–891
- Hooykaas PJJ, den Dulk-Ras H, Schilperoort RA (1988) The *Agrobacterium tumefaciens* T-DNA gene 6b is an *onc* gene. *Plant Mol Biol* **11**: 791–794
- Houba-Hérin N, Pethe C, d'Alayer J, Laloue M (1999) Cytokinin oxidase from *Zea mays*: purification, cDNA cloning and expression in moss protoplasts. *Plant J* **17**: 615–626
- Hunter T (1997) Oncoprotein networks. *Cell* **88**: 333–346
- Ichikawa T, Syono K (1991) Tobacco genetic tumors. *Plant Cell Physiol* **32**: 1123–1128
- Kakimoto T (1996) CKI1, a histidine kinase homolog implicated in cytokinin signal transduction. *Science* **274**: 982–985
- King JJ, Stimart DP, Fisher RH, Bleecker AB (1995) A mutation altering auxin homeostasis and plant morphology in *Arabidopsis*. *Plant Cell* **7**: 2023–2037
- Lehman A, Black R, Ecker J (1996) *HOOKLESS1*, an ethylene response gene, is required for differential cell elongation in the *Arabidopsis* hypocotyl. *Cell* **85**: 183–194
- Lincoln C, Long J, Yamaguchi J, Serikawa K, Hake S (1994) A *knotted1*-like homeobox gene in *Arabidopsis* is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. *Plant Cell* **6**: 1859–1876
- Long JA, Moan EI, Medford JL, Barton MK (1996) A member of the *KNOTTED* class of homeodomain proteins encoded by the *STM* gene of *Arabidopsis*. *Nature* **379**: 66–69
- Lutova LA, Buzovkina IS, Smirnova OA, Tikhodeyev ON, Shishkova SO, Trifonova IM (1997) Genetic control of in vitro differentiation processes in radish. *In Vitro Cell Dev Biol Plant* **33**: 269–274
- Maurel C, Barbier-Brygoo H, Spena A, Tempe J, Guern J (1991) Single *rol* genes from *Agrobacterium rhizogenes* TL-DNA alter some of the cellular responses to auxin in *Nicotiana tabacum*. *Plant Physiol* **97**: 212–216
- Meyer AD, Aebi R, Meins F (1997) Tobacco plants carrying a *tms* locus of Ti-plasmid origin and the *H1-1* allele are tumor prone. *Differentiation* **61**: 213–221
- Morris RO (1995) Genes specifying auxin and cytokinin biosynthesis in prokaryotes. In PJ Davies, ed, *Plant Hormones: Physiology, Biochemistry and Molecular Biology*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 318–339
- Morris RO, Bilyeu KD, Laskey JG, Cheikh NN (1999) Isolation of a gene encoding a glycosylated cytokinin oxidase from maize. *Biochem Biophys Res Commun* **255**: 328–333
- Motokura T, Arnold A (1993) Cyclin D and oncogenesis. *Curr Opin Gene Dev* **3**: 5–10
- Motyka V, Faiss M, Strnad M, Kamínek M, Schmülling T (1996) Changes in cytokinin content and cytokinin oxidase activity in response to derepression of *ipt* gene transcription in transgenic tobacco calli and plants. *Plant Physiol* **112**: 1035–1043
- Motyka V, Kamínek M (1994) Cytokinin oxidase from auxin- and cytokinin-dependent callus cultures of tobacco (*Nicotiana tabacum* L.). *J Plant Growth Regul* **13**: 1–9



- Murashige T, Skoog F** (1962) A revised medium for plant growth and bioassays with tobacco tissue culture. *Physiol Plant* **15**: 473–497
- Oono Y, Chen-Qianhong G, Overvoorde PJ, Koehler C, Theologis A** (1998) *age* mutants of *Arabidopsis* exhibit altered auxin-regulated gene expression. *Plant Cell* **10**: 1649–1662
- Otten L, Schmidt J** (1998) A T-DNA from the *Agrobacterium tumefaciens* limited-host-range strain AB2/73 contains a single oncogene. *Mol Plant-Microbe Interact* **11**: 335–342
- Persinger SM, Town CD** (1991) Isolation and characterization of hormone-autonomous tumors of *Arabidopsis thaliana*. *J Exp Bot* **42**: 1363–1370
- Prinsen E, Redig P, Strnad M, Galis I, Van Dongen W, Van Onckelen H** (1995a) Quantifying phytohormones in transformed plants. In KMA Gartland, MR Davey, eds, *Methods in Molecular Biology*, Vol 44. Humana Press, Totowa, NJ, pp 245–262
- Prinsen E, Redig P, Van Dongen W, Esmans EL, Van Onckelen H** (1995b) Quantitative analysis of cytokinins by electrospray tandem mass spectrometry. *Rapid Commun Mass Spectrom* **9**: 948–953
- Prinsen E, Van Dongen W, Esmans EL, Van Onckelen H** (1998) Micro and capillary LC-MS/MS: a new dimension in phytohormone research. *J Chromatogr A* **826/1**: 25–37
- Riou-Khamlichi C, Huntley R, Jaqmar A, Murray JAH** (1999) Cytokinin activation of *Arabidopsis* cell division through a D-type cyclin. *Science* **283**: 1541–1544
- Rupp HM, Frank M, Werner T, Strnad M, Schmülling T** (1999) Increased steady state mRNA levels of the *STM* and *KNAT1* homeobox genes in cytokinin overproducing *Arabidopsis thaliana* indicate a role for cytokinins in the shoot apical meristem. *Plant J* **18**: 557–563
- Schmülling T, Fladung M, Grossmann K, Schell J** (1993) Hormonal content and sensitivity of transgenic tobacco and potato plants expressing single *rol* genes of *Agrobacterium rhizogenes* T-DNA. *Plant J* **3**: 371–382
- Sinha NR, Williams RE, Hake S** (1993) Overexpression of the maize homeobox gene, *KNOTTED-1*, causes a switch from determinate to indeterminate cell fates. *Genes Dev* **7**: 787–795
- Skoog F, Miller CO** (1957) Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. *Symp Soc Exp Biol* **11**: 118–131
- Smith HH, Kao KN, Combatti NC** (1976) Interspecific hybridization by protoplast fusion in *Nicotiana*. *J Hered* **67**: 123–128
- Verwoerd TC, Dekker MM, Hoekema A** (1989) A small-scale procedure for the rapid isolation of plant RNA. *Nucleic Acid Res* **17**: 2362