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Physiology of Hormone Autonomous Tissue Lines Derived From Radiation-Induced Tumors of *Arabidopsis thaliana*[1]

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ABSTRACT

γ-Radiation-induced tumors of *Arabidopsis thaliana* L. have been produced as a novel approach to isolation of genes that regulate plant development. Tumors excised from irradiated plants are hormone autonomous in culture and have been maintained on hormone-free medium for up to 4 years. Five tumor tissue lines having different morphologies and growth rates were analyzed for auxin, cytokinin, and 1-aminocyclopropane-1-carboxylic acid (ACC) content, ethylene production, and response to exogenous growth regulators. Normal tissues and two crown gall tissue lines were analyzed for comparison. Rosettes and whole seedlings each contained approximately 30 nanograms·(gram fresh weight)$^{-1}$ free indoleacetic acid (IAA), 150 nanograms·(gram fresh weight)$^{-1}$ ester-conjugated IAA, and 10 to 20 micrograms·(gram fresh weight)$^{-1}$ amide-conjugated IAA. The crown gall lines contained similar amounts of free and ester-conjugated IAA but less amide conjugates. Whereas three of the radiation-induced tumor lines had IAA profiles similar to normal tissues, one line had 10- to 100-fold more free IAA and three- to 10-fold less amide-conjugated IAA. The fifth line had normal free IAA levels but more conjugated IAA than control tissues. Whole seedlings contained approximately 2 nanograms·(gram fresh weight)$^{-1}$ of both zeatin riboside and isopentenyladenosine. The crown gall lines had 100- to 1000-fold higher levels of each cytokinin. In contrast, the three radiation-induced tumor lines analyzed contained cytokinin levels similar to the control tissue. The radiation-induced tumor tissues produced very little ethylene, although each contained relatively high levels of ACC. Normal callus contained similar amounts of ACC but produced several times more ethylene than the radiation-induced tumor lines. Each of the radiation-induced tumor tissues displayed a unique set of responses to exogenously supplied growth regulators. Only one tumor line showed the same response as normal callus to both auxin and cytokinin feeding. In some cases, one or more tumor lines showed increased sensitivity to certain growth substances. In other cases, growth regulator feeding had no significant effect on tumor tissue growth. Morphology of the radiation-induced tumor tissues generally did not correlate with auxin to cytokinin ratio in the expected manner. The results suggest that a different primary genetic event led to the formation of each tumor and that growth and differentiation in the tumor tissue lines are uncoupled from the normal hormonal controls.

Investigations of the hormone physiology of plant tumors have the potential to elucidate the process of tumor formation and, more importantly, the roles of hormones in regulating normal plant growth and development. Tumors arising from infection by pathogens such as *Agrobacterium* have received the most attention in this regard. The *tms*[3] and *tmr* oncogenes of *Agrobacterium tumefaciens* code for enzymes that synthesize auxin and cytokinin, respectively (14). Furthermore, *in vivo* and *in vitro* morphologies of crown gall tumors usually reflect their endogenous auxin to cytokinin ratios (reviewed in refs. 14 and 21), *i.e.* the pattern of differentiation observed in crown gall tissues corresponds to that obtained by manipulating the concentrations of exogenous auxin and cytokinin supplied to normal tobacco tissues (29). Exceptions to this pattern are known to occur, however. Several species show a fully virulent response when transformed by *A. tumefaciens* strains carrying mutated oncogenes (reviewed in ref. 3). In at least one case, this "compensation" is not due to increased host-directed hormone synthesis (4).

The "genetic" tumors of interspecific *Nicotiana* hybrids (reviewed in ref. 2) and other nonpathogenic neoplasms of predisposed species (reviewed in ref. 10) make up a second, poorly characterized class of plant tumors. Early reports indicated that tumor-prone *Nicotiana* hybrids contained higher auxin levels than the parent species, but a recent study in which more reliable methods were used did not find higher IAA levels in tumors of *Nicotiana glauca* × *Nicotiana langsdorffii* hybrids (5). Tissue of hybrid tumors was also found to contain cytokinins at levels similar to those found in stem tissue of the parent species (19, 20). Light quality and other environmental factors have been shown to influence spontaneous tumor formation in susceptible tomato species (10, 15), but to our knowledge the hormonal status of these tumors has not been investigated.

A third mechanism for induction of tumors is treatment of plants with radiation or mutagenic chemicals. For example, Hirono *et al.* (9) described the induction of tumors in *Arabidopsis* by ionizing radiation. Because this type of tumorigenesis presumably involves changes in the organism's genetic makeup, mutagenically produced tumors may provide the most useful system for investigating the control of plant growth and development. Toward that end, γ-radiation-

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[3] Abbreviations: *tms*, tumor morphology shooty; *tmr*, tumor morphology rooty; ACC, 1-aminocyclopropane-1-carboxylic acid; N^{6}BA, N^6-benzyladenine; CCC, chlorocholine chloride; iPA, N^6-Δ^2-isopentenyladenosine; NAA, α-naphthaleneacetic acid; ZR, *trans*-zeatin riboside; MS, Murashige and Skoog medium.

induced tumors of *Arabidopsis thaliana* have been produced in this laboratory (28). When γ-irradiated seeds were sown and raised aseptically, tumors appeared on hypocotyls and shoot tips of the resulting plants. Tumors excised from irradiated plants were placed directly on hormone-free medium, and the tissue lines have been grown exclusively on hormone-free medium for more than 4 years. Hormone autonomy of the radiation-induced tumor tissues in culture may be due to genetic changes causing increased hormone synthesis or increased hormone sensitivity (*e.g.* modification of a hormone receptor). Alternatively, tumors may arise from changes in processes downstream from perception of hormonal stimuli, which control cell division.

The array of phenotypes presented by the radiation-induced tumor lines suggested an altered hormone balance in at least some cases. The 10 lines we have maintained range from dark green to white and from compact to friable to very soft. The tumor lines also vary greatly in growth rate, and their morphology may be shooty, rooty, or undifferentiated (28). This report is the first study of the hormone physiology of radiation-induced tumors. We present data concerning the endogenous hormone contents of a selected group of radiation-induced tumor lines and the effects of exogenously supplied growth regulators on growth of these tumor tissues.

MATERIALS AND METHODS

Plant Material

All plant tissues were derived from the Landsberg *erecta* ecotype of *Arabidopsis thaliana* L. The origin and phenotype of each tumor line used in this study is given in Table I. Isolation of the radiation-induced tumors was reported in detail elsewhere (28). Line 2.10A is a faster growing, morphologically distinct variant of line 2.10, which appeared spontaneously in culture and has since been maintained as a separate line. Crown gall tumors were elicited on plants grown under sterile conditions by puncturing the stems with a needle dipped in a rapidly growing culture of *Agrobacterium tumefaciens* strain B6 or C58. The resulting tumors were excised and cultured on medium containing 50 $\mu g \cdot mL^{-1}$ carbenicillin and 100 $\mu g \cdot mL^{-1}$ cefotaxime until free of bacteria. Production of opines by the crown gall tissue lines was confirmed by the assay of Aerts *et al.* (1) after incubation overnight on medium containing 1 mM arginine. Normal callus tissue was initiated by placing hypocotyl segments excised from young plants onto modified MS medium (see below) containing 10.7 μM NAA and 0.23 μM kinetin. Established callus was maintained on medium containing 3.0 μM NAA and 1.0 μM N^6-BA, suboptimal concentrations chosen to produce a growth rate similar to the more rapidly growing tumor lines. Whole rosettes for hormone analysis were obtained from plants grown at 22°C under continuous cool-white fluorescent illumination (35–60 $\mu mol \cdot m^{-2} \cdot s^{-1}$) in a mixture of peat, perlite, and vermiculite (1:1:1) and irrigated with nutrient solution. Whole seedlings for hormone analysis were raised by incubating sterilized seeds in half-strength liquid MS medium on a rotary shaker (140 rpm) under continuous fluorescent light.

Plant Tissue Culture

Tissue stocks were maintained in 125-mL Erlenmeyer flasks containing 40 mL of MS medium (16), modified to include 30 $g \cdot L^{-1}$ sucrose, 1 $mg \cdot L^{-1}$ nicotinic acid, 10 $mg \cdot L^{-1}$ thiamine-HCl, 1 $mg \cdot L^{-1}$ pyridoxine-HCl, and 4 $mg \cdot L^{-1}$ glycine, and solidified with 9 $g \cdot L^{-1}$ TC agar (Hazleton Biologics). The radiation-induced tumor tissues have grown *in vitro* for more than 4 years with no exposure to exogenous growth regulators. Tumor and callus tissues were raised under continuous cool-white fluorescent illumination (45–70 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 26°C and subcultured at intervals of no more than 30 d. For growth experiments, tissue was grown in 95- × 25-mm shell vials containing 10 mL of MS medium plus growth regulators as indicated. Growth is expressed as $(W - W_0)/W_0$, where W and W_0 are the final and initial fresh weights of the tissue,

Table I. *Origins and Phenotypes of the Tissues Used in This Study*
Slow growing lines have doubling times >5 d.

Tissue Line	Origin	Phenotype
Erecta callus	Hormone-dependent callus derived from hypocotyl tissue	Dark green, friable, shooty and rooty (depending on NAA and N^6-BA concentrations in medium), rapid growth
1.2	Radiation-induced tumor	Dark green, hard, rooty, slow growth
2.10	Radiation-induced tumor	Light green, hard, undifferentiated, slow growth
2.10A	Variant of 2.10	Light green, very soft, undifferentiated, rapid growth
3.4	Radiation-induced tumor	Dark green, friable, undifferentiated, rapid growth
5.5	Radiation-induced tumor	Light green, friable, shooty, rapid growth, rapid necrosis
EB6-1	Crown gall tumor elicited by *A. tumefaciens* strain B6	Light green, friable, shooty, rapid growth
EC58-1	Crown gall tumor elicited by *A. tumefaciens* strain C58	Light green, friable, shooty, rapid growth

respectively. Final fresh weights were determined after 28 d in culture for lines 1.2 and 2.10 and after 21 d for all other lines. Each experiment was performed two to four times. Growth curves shown in "Results" illustrate representative experiments. Tissue for hormone analysis was raised on MS medium either in shell vials or in 100- × 25-mm Petri dishes.

Hormone Analysis

Tissue harvested for auxin and cytokinin analysis (approximately 3 and 10 g per extraction, respectively) was frozen in liquid N_2 and stored at −70°C until extraction. Free, ester-conjugated, and amide-conjugated IAA were extracted and purified by the method of Chen *et al.* (6) using C_{18} and NH_2 solid phase extraction columns and then analyzed by reverse phase C_{18} HPLC with fluorescence detection. Recovery was estimated by adding [^{3}H]IAA to each extract. Samples containing too little IAA for accurate quantitation by this method (less than approximately 50 ng·(g fresh weight)$^{-1}$) were methylated following HPLC and analyzed by GC-selected ion monitoring-MS using [$^{13}C_6$]IAA as the internal standard (7). Accuracy of the HPLC quantitation was verified by subjecting a representative assortment of samples to GC-selected ion monitoring-MS analysis following HPLC. Similar results were obtained using both methods. Cytokinins were extracted and purified by the procedures of MacDonald and Morris (12). After extraction, addition of [^{3}H]ZR-trialcohol tracer, and treatment with acid phosphatase and β-glucosidase, cytokinins were purified by chromatography through DEAE-cellulose, C_{18}, and immunoaffinity columns (antibodies were kindly provided by Dr. Roy Morris). This procedure yielded samples clean enough to allow quantitation and tentative identification of cytokinins by reverse phase C_{18} HPLC with UV absorbance detection (12). Ethylene was measured using a Perkin-Elmer model 8410 gas chromatograph equipped with flame ionization detector and GS-Q megabore column (30 m × 0.534 mm, J & W Scientific). The ethylene precursor, ACC, was extracted by crushing tissue in 80% ethanol in a 2-mL centrifuge tube. After centrifugation, ACC in the supernatant was quantified by the method of Lizada and Yang (11).

RESULTS

Endogenous Hormone Content of Radiation-Induced Tumor Lines

Our first goal for investigating the hormone autonomous tumor lines was to determine whether these tissues synthesize auxins and cytokinins. We measured the levels of free and conjugated IAA in five radiation-induced tumor tissue lines, two crown gall tissue lines, and normal *Arabidopsis* tissues. Two sources of normal plant material were used for IAA extractions: whole rosettes harvested just before bolting and whole seedlings grown in aseptic liquid culture. Both tissues were found to contain approximately 30 ng·(g fresh weight)$^{-1}$ free IAA (Table II). This figure is in the range reported for rapidly growing organs such as etiolated maize coleoptiles (22) and young tobacco leaves (23). Both control tissues contained large amounts of amide-conjugated IAA (10–20 μg·[g fresh weight]$^{-1}$) and relatively little ester-conjugated IAA, implicating conjugation to amino acids as the prevailing pathway of IAA metabolism in *Arabidopsis*. The two crown gall tissue lines contained free and ester-conjugated IAA in amounts similar to normal plants but less than half as much amide-conjugated IAA.

Table II. *The IAA Content of Arabidopsis Plants and Tumor Tissue Lines*

Age is expressed as days after seed imbibition (plants) or subculture (tumor lines). ND, not determined.

Tissue	Age	Experiment	[IAA]		
			Free	Ester	Amide
	d		*ng·g fresh wt*$^{-1}$		
Erecta whole seedlings	4	1	27[a]	210[a]	11,317
	8	2	20[a]	194	19,902
	4	3	15[a]	ND	ND
Erecta rosettes	19	1	30[a]	158[a]	19,802[a]
	19	2	45[a]	<50	17,154
1.2	14	1	54[a]	589	5,902
	14	2	21[a]	355	27,674
	14	3	27[a]	ND	ND
2.10	14	1	15[a]	632	2,864
	14	2	18[a]	270	6,191
	14	3	25[a]	ND	ND
2.10A	7	1	251	4,017	1,722
	14	1	350	3,805	5,263
	21	1	2,499[a]	16,672	1,185
	7	2	3,517	2,856	526
	14	2	920[a]	5,095[a]	6,520[a]
	21	2	2,260	18,231	718
3.4	7	1	18[a]	102	34,806
	14	1	16[a]	853[a]	51,202[a]
	21	1	18[a]	2,782	33,490
	7	2	<50	3,155[b]	29,474
	14	2	53[a]	5,402	44,744
	21	2	<50	4,414[b]	39,078
5.5	7	1	51[a]	<50	21,701
	7	2	43[a]	1,026	11,037
	7	3	<50	99[a,b]	10,008[a]
	7	4	18[a]	ND	ND
EB6-1	14	1	47[a]	31	1,952
	14	2	60[a]	159	3,816
	14	3	24[a]	ND	ND
EC58-1	14	1	30[a]	51	4,765
	14	2	42[a]	54	1,591
	14	3	26[a]	ND	ND

[a] Denotes value obtained by GC-MS. [b] Includes free IAA.

The five radiation-induced tumor lines all synthesized IAA, although total IAA content and the pattern of conjugate accumulation varied considerably (Table II). Tumor lines 1.2, 2.10, and 5.5 all contained free IAA in quantities similar to those found in normal tissue. Lines 1.2 and 2.10 had somewhat more ester-conjugated IAA, whereas the ester content of line 5.5 varied, ranging above and below normal levels. The amount of amide-conjugated IAA in these three lines also varied but, in general, did not differ greatly from normal tissue. Tumor line 3.4 contained the basal level of free IAA but consistently yielded more amide-conjugated IAA than any other tissue line. This line also contained more ester conjugates than normal in five of six experiments. The variant

Table III. *Cytokinin Content of Arabidopsis Plants and Tumor Tissue Lines*

Tissue	Age	Experiment	Cytokinin Content	
			ZR	iPA
	d		*ng·g fresh wt*$^{-1}$	
Erecta seedlings	8	1	0.5	1.8
		2	4.2	2.9
		3	1.3	1.5
2.10A	14	1	1.5	2.6
		2	2.7	0.4
		3	0.2	0.2
3.4	14	1	6.8	28.7
		2	<0.1	0.9
		3	0.2	0.7
5.5	7	1	2.6	24.2
		2	<0.1	0.3
		3	<0.1	0.2
EB6-1	14	1	1,903	204
		2	4,181	539
		3	15,222	2,304
EC58-1	14	1	6,746	4,501
		2	3,717	2,640
		3	2,832	1,668

line 2.10A displayed greatly elevated levels of free IAA. This line contained much more free and ester-conjugated IAA than its parent, line 2.10, and a similar amount of amide conjugates. The total amount of IAA in line 2.10A was usually less than or equal to that found in normal tissue, but the proportion of amide conjugates was lower, suggesting a block in the amide conjugation pathway.

Because the free IAA content of tobacco crown gall tissue is known to fluctuate with age in culture (23), we extracted lines 3.4 and 2.10A at different times after subculturing. The values obtained for the three IAA pools did vary over time in both lines (Table II). However the magnitude of these variations was not much greater than that observed in replicate extractions of material of the same age. Therefore, we chose to extract IAA from the other cultured tissue lines at a fixed time midway between passages, when the tissues were growing rapidly.

The three fast growing radiation-induced tumor lines were analyzed for cytokinin content and compared with the crown gall lines and whole seedlings (we could not amass sufficient tissue to analyze the slower growing lines). After immunoaffinity purification, extracts from normal seedlings contained low levels of presumptive cytokinins having the same HPLC retention times as authentic iPA and ZR (Table III). These values are similar to those reported for other vegetative tissues such as *Nicotiana* stem (19, 20). The figures shown presumably include hydrolyzed *O*-glucosides and 5′-phosphates, because β-glucosidase and acid phosphatase were added to the tissue extracts before immunoaffinity purification (the conjugated forms are not recognized by the antibodies). The two crown gall lines each contained 100- to 1000-fold more iPA and ZR than seedling tissue. The radiation-induced tumor lines generally contained cytokinins in amounts similar to normal tissue, although lines 3.4 and 5.5 each had somewhat higher levels of iPA in one experiment.

Ethylene production was measured because this hormone is known to stimulate the growth of certain tissues in culture (25). The ethylene precursor ACC was also assayed as an indirect measure of auxin activity, because auxins are known to stimulate ethylene production by inducing ACC synthesis (25). Accumulation of ACC has been shown to parallel that of IAA in crown gall tissues of several species (4, 13, 26), and a variety of natural and synthetic auxins induce ACC accumulation in tobacco crown gall cells (25). Because plants can produce biologically active auxins that we did not measure directly, it was desirable to measure ACC levels in the tumor lines. The radiation-induced tumor lines all evolved very small volumes of ethylene (Table IV). Callus tissue synthesized somewhat more, and crown gall line EB6-1 produced >10-fold more, ethylene than the radiation-induced tumor tissues. In contrast to their low rate of ethylene production, all tissues examined contained relatively high levels of the ethylene precursor ACC (Table IV). No large differences in ACC content were observed among the tissue lines. Surprisingly, line 2.10A, which has the highest levels of free IAA, evolved the least ethylene of any tissue line and did not contain more ACC than the other lines examined.

Response to Exogenous Plant Growth Substances

To determine whether altered hormone response might play a role in radiation-induced tumorigenesis, we measured growth of the tumor lines and callus tissue in the presence of exogenously supplied plant growth substances. The optimum concentration of the synthetic auxin, NAA, for growth of normal tissue was found to be 10 μM (Fig. 1a). Higher concentrations were clearly toxic, causing the callus tissue to turn yellow or brown and ultimately die. Tissue placed on suboptimal concentrations of NAA appeared healthy but grew at a slower rate. Growth of crown gall tissue line EC58-1 was not stimulated by NAA; treatment with 10 μM NAA resulted in significant growth inhibition. Similar results were obtained with line EB6-1, except this crown gall tissue proved even more sensitive to auxin feeding, with 10 μM NAA causing death. Growth of line 3.4, which was found to contain the most total IAA but a low level of free IAA, was increased by

Table IV. *Ethylene Biosynthesis Rate and ACC Content of Arabidopsis Callus and Tumor Tissue Lines*

Tissue was analyzed 7 d after subculture for line 5.5 and 14 d after subculture for all other lines. Growth medium for callus tissue contained 3 μM N^6-BA and 10 μM NAA. Results are means ± SE (*n*).

Tissue Line	Ethylene Synthesis	ACC Content
	pmol·(g fresh wt)$^{-1}$*·h*$^{-1}$	*nmol·(g fresh wt)*$^{-1}$
callus	31.5 ± 4.6 (5)	25.6 ± 9.6 (3)
1.2	6.9 ± 3.2 (5)	35.3 ± 7.0 (4)
2.10	5.4 ± 1.1 (7)	15.5 ± 5.0 (6)
2.10A	0.9 ± 0.2 (5)	33.0 ± 5.8 (6)
3.4	1.8 ± 0.5 (8)	66.8 ± 12.5 (7)
5.5	6.4 ± 2.1 (5)	31.2 ± 11.3 (5)
EB6-1	113.4 ± 12.0 (6)	19.4 ± 7.7 (3)

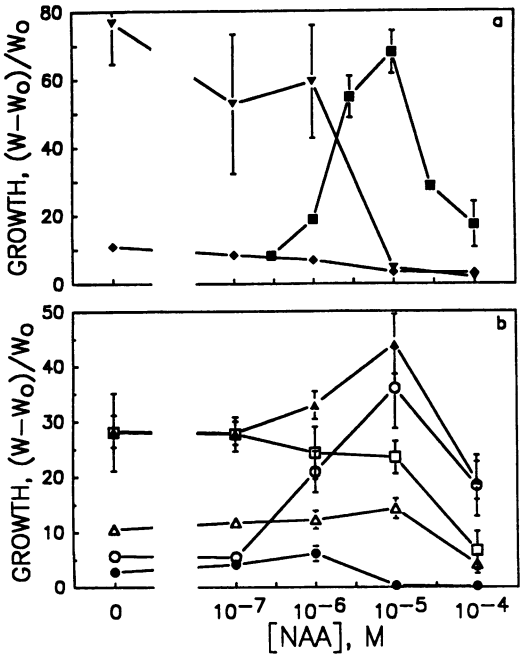

Figure 1. NAA dose response of (a) normal callus and crown gall tissue lines and (b) radiation-induced tumor tissue lines. Growth medium for callus tissue contained 3 μM N^6-BA. Bars, SE (n = 5 for lines 1.2 and 2.10; n = 9 for all other lines). ■, callus; ▼, EB6-1; ◆, EC58-1; ○, 1.2; ●, 2.10; △, 2.10A; ▲, 3.4; □, 5.5.

10 μM NAA (Fig. 1b). Tissue fed 100 μM NAA grew rapidly at first but was always dead after 21 d. The response of line 5.5, which also contained little free IAA, was variable. Feeding 10 μM NAA inhibited growth in two experiments and increased growth in two other experiments. Line 2.10 was 10-fold more sensitive than callus tissue to exogenous auxin. Its growth was stimulated by 0.1 and 1 μM NAA but was strongly inhibited by 10 μM. Line 2.10A, the fast growing variant that had high free IAA levels, was not significantly affected by NAA concentrations up to 10 μM. The growth of line 1.2 was increased up to 700% by 1 to 10 μM NAA. This level of NAA also caused the appearance of roots over the surface of the tissue. This was the only growth substance-induced change in morphology observed with any of the radiation-induced tumor lines. In summary, the two slow growing lines, 1.2 and 2.10, and line 3.4 grew more rapidly when supplied with NAA. Lines 2.10A and 5.5 were not consistently stimulated or inhibited by NAA. Line 2.10 showed increased auxin sensitivity relative to callus.

The effect of the synthetic cytokinin N^6-BA on tumor and callus growth was also investigated (Fig. 2a). The optimum concentration of N^6-BA for normal tissue was 3 to 10 μM, depending on the amount of NAA present. N^6-BA was toxic to callus and to every tumor line at 100 μM. Growth of crown gall lines EC58-1 and EB6-1 was not enhanced by N^6-BA at any concentration tested. Radiation-induced tumor line 3.4 was stimulated by 1 to 10 μM N^6-BA, the same range found optimal for callus (Fig. 2b). Lines 5.5 and 2.10A were also stimulated by N^6-BA, but the optimum concentration varied in different experiments. The most favorable N^6-BA concentrations ranged from 0.1 to 10 and from 0.1 to 1 μM for lines 5.5 and 2.10A, respectively. Neither line 1.2 nor 2.10 was consistently stimulated by N^6-BA feeding. Growth of each line was slightly increased or decreased by N^6-BA in different experiments. In summary, growth of the three rapidly proliferating radiation-induced tumor lines could be increased by N^6-BA treatment, although the optimum concentration differed for each line. The two slower growing lines and the two crown gall lines were not stimulated by N^6-BA.

Gibberellins have been shown to occur in tobacco crown gall tissues, particularly shooty teratomas (17), and GA_3 can substitute for auxin in supporting the growth of unorganized tissue from tobacco teratomas (24). In light of these observations, and the well-documented effects of gibberellins on cell division and expansion in other systems, we measured the effects of GA_3 (not native to *Arabidopsis* but biologically active [30]) on growth of the radiation-induced tumor lines. GA_3 did not increase the growth of any tissue line tested (Fig. 3). Concentrations up to 10 μM GA_3 had no significant effect on callus tissue, whereas 100 μM was slightly inhibitory. Lines 2.10A and 3.4 yielded essentially the same result. GA_3 was toxic to lines 1.2 and 5.5 at 100 μM and inhibited growth at 1 to 10 μM. The effect of the gibberellin biosynthesis inhibitor CCC on tissue growth was also tested (Table V). None of the tumor lines was inhibited by 0.5 mM CCC, a concentration that inhibits stem elongation in Landsberg *erecta* plants (not shown). From this evidence it appears unlikely that changes in gibberellin metabolism are involved in the hormone autonomy of the radiation-induced tumor tissues.

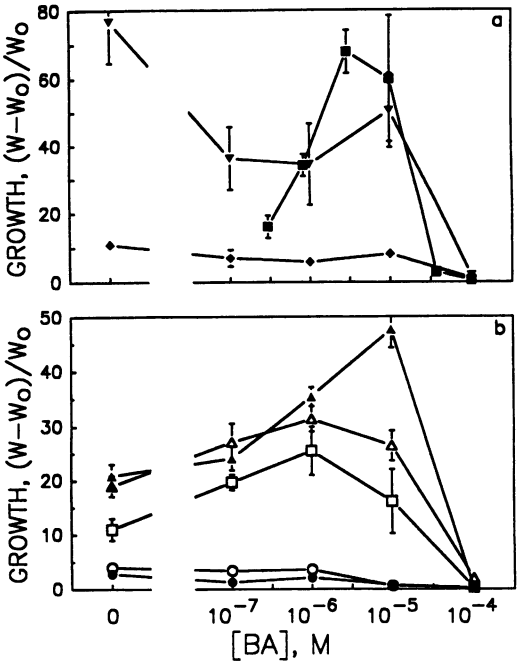

Figure 2. N^6-BA dose response of (a) normal callus and crown gall tissue lines and (b) radiation-induced tumor tissue lines. Growth medium for callus tissue contained 10 μM NAA. Bars, SE (n = 5 for lines 1.2 and 2.10; n = 9 for all other lines). ■, callus; ▼, EB6-1; ◆, EC58-1; ○, 1.2; ●, 2.10; △, 2.10A; ▲, 3.4; □, 5.5.

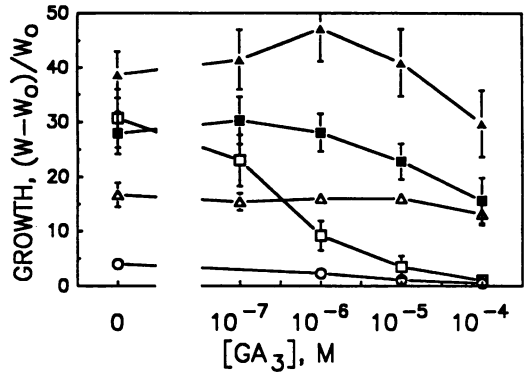

Figure 3. GA_3 dose response of normal callus and radiation-induced tumor tissue lines. Growth medium for callus tissue contained 1 μM N^6-BA and 3 μM NAA. Bars, SE ($n = 5$ for line 1.2; $n = 9$ for all other lines). ■, callus; ○, 1.2; △, 2.10A; ▲, 3.4; □, 5.5.

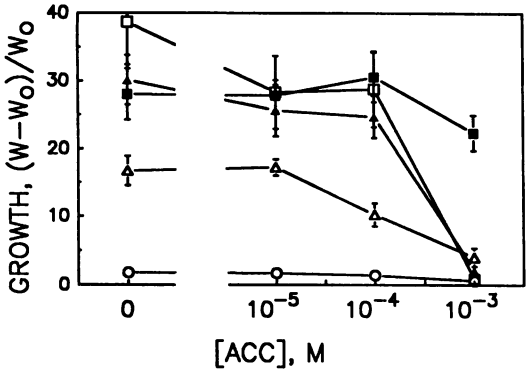

Figure 4. ACC dose response of normal callus and radiation-induced tumor tissue lines. Growth medium for callus tissue contained 1 μM N^6-BA and 3 μM NAA. Bars, SE ($n = 5$). ■, callus; ○, 1.2; △, 2.10A; ▲, 3.4; □, 5.5.

All four radiation-induced tumor lines tested were more sensitive to the ethylene precursor ACC than normal callus (Fig. 4). Growth of callus was not affected by 0.1 mM ACC and only slightly inhibited by 1 mM. In contrast, the tumor lines were all strongly inhibited by 1 mM ACC; tissue from lines 1.2, 3.4, and 5.5 had turned brown and died by the end of the culture period. The tumor lines were also inhibited to varying degrees by 0.1 mM ACC. Somewhat different results were observed when the ethylene-releasing agent 2-chloroethylphosphonic acid (Ethephon) was included in the growth medium (Fig. 5). Normal callus and all tumor lines tested were inhibited by 0.1 mM Ethephon. At 1 mM, Ethephon was toxic to all tissue lines. These results, together with the ethylene and ACC determinations (Table IV), suggest that the tumor lines are less efficient than callus at metabolizing ACC, either through conjugation or conversion to ethylene.

DISCUSSION

The foremost conclusion that can be drawn from this study is that the radiation-induced tumor tissue lines do not require high levels of endogenous auxin and cytokinin for hormone autonomous growth. With the exception of line 2.10A, which had 10- to 100-fold more IAA, none of the lines examined contained substantially more iPA, ZR, or free IAA than normal seedlings. In this respect, the radiation-induced tumors resemble genetic tumors more than crown gall tumors. *Agrobacterium* strains carrying Ti plasmids with mutant *tms* or *tmr* oncogenes are still able to elicit tumors on many host species, but strains lacking both oncogenic functions are either avirulent or very weakly virulent, even though T-DNA transfer occurs (3, 21). Thus, it appears that T-DNA-directed synthesis of either auxin or cytokinin is necessary for growth of crown gall tissue. In contrast, genetic tumors that have been examined to date do not contain elevated levels of IAA or cytokinin (5, 19, 20).

These findings suggest that genetic and radiation-induced tumors may both result from abnormalities in gene expression which render cell division independent of hormonal regulation. Uncoupling of developmental processes from the normal hormonal control mechanisms has been observed in other systems. Tissue growth rate and morphology, IAA synthesis, and sensitivity to exogenous auxin have been shown to vary independently in certain tobacco crown gall tissue lines (26, 27; B. R. Campell and W. L. Pengelly, unpublished results). It was also shown that host compensation in *Nicotiana glutinosa, i.e.* production of tumors having the "high auxin phenotype" (fast growing, friable, undifferentiated) when transformed by a *tms2* mutant *Agrobacterium* strain, does not result from increased IAA accumulation or enhanced auxin sensitivity (4). Compensation for defective *Agrobacterium* oncogenes occurs in many other host species (3), but the hormonal status of the resulting tumors has not been investigated. The hormone autonomous growth of some "habituated" tissue lines also occurs without the accumulation of elevated hormone levels. Two notable examples are the cytokinin autotrophic, epigenetic variants derived from "Havana 425" tobacco (8) and an auxin and cytokinin autotrophic tobacco cell line induced by UV irradiation (18).

A particularly striking aspect of this study is that morphology of the radiation-induced tumor tissues frequently does not correlate with auxin to cytokinin ratio (endogenous or exogenous) in the expected manner. Skoog and Miller (29) showed that, when exogenous hormones are supplied to tobacco pith tissue, high auxin to cytokinin ratios promote root formation and low ratios promote shoot formation. We have

Table V. *Effect of CCC on Growth of Radiation-Induced Tumor Tissue Lines and Normal Callus*

Growth medium for callus contained 1 μM N^6-BA and 3 μM NAA. Values are means ± SE. For line 2.10, $n = 5$; $n = 9$ for all other lines.

Tissue Line	Growth	
	Control	0.5 mM CCC
	$w - w_o/w_o$	
Callus	12.3 ± 0.9	13.5 ± 0.6
1.2	2.0 ± 0.5	2.8 ± 0.7
2.10	2.8 ± 0.7	1.9 ± 1.0
2.10A	35.7 ± 1.8	36.7 ± 1.5
3.4	33.5 ± 2.4	31.7 ± 2.7
5.5	32.6 ± 5.1	36.2 ± 5.2

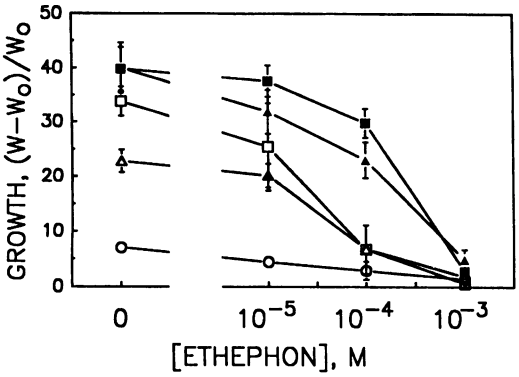

Figure 5. Ethephon dose response of normal callus and radiation-induced tumor tissue lines. Growth medium for callus contained 1 μM N^6-BA and 3 μM NAA. Bars, SE (n = 9 for callus; n = 5 for all other lines). ■, callus; ○, 1.2; △, 2.10A; ▲, 3.4; □, 5.5.

obtained similar results with normal *Arabidopsis* tissue (B. R. Campell and C. D. Town, unpublished results). But, except for the roots formed on NAA-treated tissue of line 1.2, none of the radiation-induced tumor lines showed any morphological change in response to auxin or cytokinin feeding. Both crown gall lines, which have high cytokinin content and relatively low total IAA, form shoots in culture. In contrast, the shooty radiation-induced tumor line, 5.5, contains normal IAA and cytokinin levels. Line 1.2, which intermittently produces roots, does not contain high levels of IAA. Line 2.10A, which has the most free IAA, and line 3.4, which contains the most total IAA, are undifferentiated and have never formed roots. Thus, it appears that *Arabidopsis* tissue ordinarily differentiates according to the auxin to cytokinin paradigm observed in tobacco and other species, but these responses are mostly absent from the radiation-induced tumor lines, suggesting alterations in the mechanisms by which cells of these tissues perceive or respond to hormonal stimuli.

The hypothesis that hormone signal transduction pathways are disrupted in the radiation-induced tumor lines is further supported by the observed changes in hormone sensitivity relative to normal callus tissue. Each of the radiation-induced tissue lines displays a unique set of responses to exogenously supplied growth regulators. In some cases, tumor tissue is more sensitive than callus, *i.e.* the expected growth response is observed at a lower concentration. (Line 2.10 is more sensitive to NAA; lines 5.5 and 2.10A are more sensitive to N^6-BA.) In other cases, tumor tissue is not significantly affected by growth regulator feeding. (Line 2.10A is insensitive to NAA; lines 1.2 and 2.10 are insensitive to N^6-BA.) Both types of change in hormone sensitivity could be due to alterations in a hormone receptor or in part of a signal transduction pathway through which hormones influence cell division and morphogenesis.

The spontaneous variant line, 2.10A, is the only radiation-induced tumor line with higher levels of free IAA than normal tissues. However, the phenotype of the slower growing parent line is not changed by feeding auxin. Growth of line 2.10 is stimulated by NAA, but the growth rate still remains much slower than 2.10A and the tissue maintains its hard phenotype. Exogenously supplied IAA also does not produce the variant (2.10A) phenotype (B. R. Campell and C. D. Town, unpublished results). By analogy with crown gall tissues of tobacco (27), one might expect the elevated level of IAA in line 2.10A to render it more susceptible to auxin-induced growth inhibition, but this line is actually less sensitive to NAA feeding than 2.10. These results suggest that increased accumulation of free IAA may be a consequence of the phenotype change and not the primary causative event.

Based on their hormone physiology, we conclude that no two of the radiation-induced tumor lines studied are alike. All of the tumor lines synthesize IAA and cytokinins, but only one line accumulates higher levels of a hormone (IAA) than control tissues. Our results also appear to rule out increased synthesis of gibberellins or ethylene as a cause of hormone autonomous growth. In many instances, tumor tissues do not respond as normal tissue does to growth regulator feeding, nor do their morphologies correlate with auxin to cytokinin ratios. These results suggest that hormone synthesis may be a result of tumorigenesis in these lines and not the primary cause. A more likely explanation for hormone autonomous growth is that mutations affecting hormone perception or processes downstream from hormone perception have uncoupled the normal regulation of cell division, allowing uncontrolled cell proliferation and tumor formation.

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