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The Role of Phytohormones in Response to Transformation of Pea Lines, Differing in Symbiotic Characteristics, and their Hybrids

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Abstract

This study investigated various morphogenetic processes following wounding, exogenous phytohormone application and oncotransformation in pea lines differing in symbiotic characteristics. The pea lines varied in callus and root formation abilities on medium without phytohormones. Exogenous phytohormones (auxins and cytokinins) decreased the diversity in callus formation but revealed genotypic diversity in shoot formation. Callus, and especially shoot formation capacities decreased greatly after subculture; the effect of phenotype became more visible. Transformation with *Agrobacterium* wild-type strains appeared to be more effective inducing condition revealing contrasting differences between pea genotypes in tumor- and root formation. Pea lines with atypical morphogenetic responses to transformation were revealed. The existing differences between lines for root and tumor formation allowed genetic analysis of these traits. F_1 hybrids were uniform with segregation in the F_2. The capacity for root formation was inherited as a dominant trait, but tumor formation was a recessive trait. Using

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exogenous phytohormones, phenocopies of the typical morphogenetic responses to transformation were obtained. The results suggest an interdependence of these morphogenetic processes with the endogenous phytohormone balance as determined by plant genotype.

Keywords: Pea, *Agrobacterium* transformation, phytohormones, tumor and root formation.

1. Introduction

Phytohormones were shown to trigger different morphogenetic programs, such as callus, root, shoot or embryo formation. The complicated morphogenetic programs composed of dedifferentiation and secondary differentiation processes depend on both internal factors (plant genotype, tissue and age) and environmental factors (Malmberg, 1979; Ezhova et al., 1985). Suitable inducing conditions and strictly controlled experimental conditions are needed to reveal intraspecific variability in characteristics of the morphogenetic processes. Several treatments were used as inducing conditions: 1) wounding (such as organ explantation *in vitro*) (Lutova and Zabelina, 1988; and others), 2) exogenous phytohormones application (Gamborg et al., 1974; Kartha et al., 1974; Malmberg, 1979; Hussey and Gunn, 1984; Rublio et al., 1984; Ezhova et al., 1985) and 3) transformation with oncogenic *Agrobacterium tumefaciens* and *A. rhizogenes* strains (Hobbs et al., 1989; Robbs et al., 1991; Lutova and Sharova, 1993; Lutova et al., 1994) which introduce bacterial genes into the plant genome and either synthesize or activate phytohormones.

Callus formation was studied to explore the dedifferentiation process. Differentiation was examined as root- and shoot formation. These traits characterize plant totipotency. The latter is said to be an attribute of plant tissue which endows them with adaptation capacities (Potrykus, 1992). Morphogenic characteristics depend on the phytohormonal status, which can be determined by the plant genotype (Hussey and Gunn, 1984; Lutova et al., 1994). For instance, intraspecific variability of pea (*Pisum sativum* L.) regeneration processes *in vitro* (callus, root and shoot formation) was demonstrated (Hussey and Gunn, 1984; Rublio et al., 1984; Ezhova et al., 1985; Lutova and Zabelina, 1988; Lutova et al., 1994). Also the intraspecific variability of pea morphogenetic responses to transformation with *Agrobacterium* strains was demonstrated, and genetic forms with atypical responses to transformation were revealed (Hobbs et al., 1989; Puoti-Kaerlas et al., 1990; Robbs et al., 1991; Lutova and Sharova, 1993).

Table 1. Plant material.

Genetic form	Origin	Height	Vegetation period	Red color (presence + absence –)	Nodule formation and nitrogenase activity(n.a.)***
(1)	(2)	(3)	(4)	(5)	(6)
Line 3	Local cv.* of Arhangelskaya region (K3302**)	High	Medium	+	Numerous nodules low n.a.
Line 4	Local cv. of Arhangelskaya region (K3329)	High	Medium and long	+	Numerous medium and large nodules, high n.a.
Line 9	Crupp Pelushke cv. (K5970), Elite group, Germany	High	Long	+	Numerous medium and large nodules, high n.a.
Line 32	Local cv. of Novgorodskaya reg., Pelushke (K6335)	High	Medium and long	+	Numerous very large nodules, very high n.a.
Line 15	Vitalis cv. Great Britain (K6264)	Low	Medium	–	Numerous medium and large nodules, medium n.a.
Line 26	Misog-2 cv. Great Britain (K7389)	Medium	Medium	–	Numerous medium and large nodules, low n.a.
Line 29	Local cv. Afghanistan, Pelushke (K6561)	Medium	Medium	+	Almost does not form nodules with European strains, low n.a.
Sparkle cv.	(T.A. LaRue collection), Boyce Thompson Inst. for Plant Res. Ithaca, USA	Low	Short	–	Numerous small nodules, predominantly on lateral roots

Table 1. Continued.

Genetic form	Origin	Height	Vegetation period	Red color (presence + absence –)	Nodule formation and nitrogenase activity(n.a.)***
(1)	(2)	(3)	(4)	(5)	(6)
Sp. sym5	cv. *Sparkle*, line E2(EMS-treatment) mutation in the 1st chromosome	Short internodes	Short		No or few nodules ethylene- and t^0-dependent resistance to nodulation
Sp. sym8	cv. *Sparkle*, line R-25(g-rays) mutation in the 6th chromosome	Short internodes	Short		No nodules
Line Sprint-2	From Inst. of Cytology and Genetics, Russia (Berdnikov et al., 1989)	Low	Very short	–	Medium nodules predominantly on the main root
S-2 Nod$^-$2	l. Sprint-2 (EMS-treatment) mutation in the sym8 gene (6th chromosome)	Low			No nodules

*Cultivar (cv.) from which the line was breed; **Number according to the All-Russian Plant Research Institute catalogue; ***Nitrogenase activity was detected according to modified acetylene reduction assay (ARA) (Tikhonovich et al., 1987).

This work is devoted to studying the intraspecific variability of pea lines and their F_1 and F_2 hybrid responses to wounding, transformation and exogenous phytohormones. Furthermore, we intend to correlate the morphogenetic responses of different genetic forms to wounding with the responses of these forms to transformation, since the responses to both treatments are expressed as callus, shoot and/or root formation and depend on the phytohormones.

2. Materials and Methods

Plant material (see Table 1)

We used pea lines from the genetic collection received from All-Russian Research Institute for Agricultural Microbiology (ARRIAM). The lines differ in nutrition value, vegetation period, seed productivity, disease resistance, morphological traits (habit, stem and corolla color, pod shape, seed shape and color) and also in symbiotic nitrogen fixation and nodulation capacities. Almost all the lines (except Nod^- mutants) and cv. *Sparkle* form numerous nodules. Only line 29 formed almost no nodules after inoculation with European *Rhizobium* strains. Nitrogenase activity varied from low in lines 3,26 and 29 to very high in line 32. In transformation experiments we also used mutant lines unable to form nodules after inoculation with nitrogen fixing *Rhizobium*: mutants sym5 and sym8 were obtained from *Sparkle* cultivar (from Professor Th.A. LaRue, Ithaca, USA) while line Sprint-2 Nod^--2 was produced from line Sprint-2 (kindly supplied by A.Yu. Borisov All-Russian Institute of Agricultural Microbiology, St. Petersburg). These mutant lines differ from their original parents only in their inability to form nodules. For genetic analysis F_1 and F_2 hybrids from lines with different responses to transformation by *Agrobacterium rhizogenes* strain 8196 were used.

Bacterial strains

Agrobacterium wild-type strains used in transformation experiments were: *A. tumefaciens* strains A281 (supervirulent) and A277; *A. rhizogenes* strains 8196 and 15834. (The strains were obtained from Prof. M. Ondrej, Institute of Molecular Biology, Academy of Sciences of the Czech Republic, Ceske Budejovice, but these strains are used commonly.)

Plant germination

Ten-days-old axenic seedlings used as a source of explants were obtained from seeds sterilized in sulphuric acid. The seeds were germinated on MSO medium (Murashige and Skoog, 1962) containing Difco agar 8 g/l and no added phytohormones. The seedlings were grown in a growth chamber at +25°C and 16/8 hours day/night photoperiod in closed jars.

Regeneration capacity assessment

Internodal explants were used to study regeneration capacity. There was no variation in transformation capacities between different internodes from the same plant (on the same medium), so we used all the internodes of every plant (about 1 cm long) excising them so that meristems were excluded. The basal MSO medium was supplemented by the cytokinin BAP (6-benzylaminopurine, Sigma Co., St. Louis) and/or the auxin NAA (a-naphtaleneacetic acid, Sigma Co.) in the following concentrations. MS + 1 mg/l BAP + 1 mg/l NAA and control hormone- free MSO media were used to assess the effect of hormones on efficiency of callus and root formation. No shoot formation was observed on these media. MS + 1 mg/l BAP + 2 mg/l NAA medium was used to assess callus and shoot formation capacities. The results were assessed 30 days after explantation. Large calli formed on MS + 1 mg/l BAP + 2 mg/l NAA medium were transferred to the same fresh medium or to MS + 5 mg/l BAP and 2 mg/l NAA. The regeneration processes in the 1st passage (on the transferred calli) were assessed 30 days after the transfer procedure.

Emerging shoots were cut off and transferred for rooting to the medium based on MS with half strength sucrose (1%) and NAA (0.1 mg/l). Rooting of the primary regenerants was estimated 30 days later. In order to estimate the regeneration capacity the following traits were assessed:

a) callus formation (frequency in per cent and size in points);
b) root formation (frequency in per cent);
c) shoot formation (frequency in per cent);
d) rooting of the primary regenerants (frequency in per cent).

The 95% confidence intervals were calculated by the f-method of Fisher (Terentyev and Rostova, 1977).

Induction of organogenic calli

To obtain the organogenic calli from immature pea embryos we used the modified procedure by Schroeder et al. (1993). Yellowish-green pods of pea line 3 were gathered in August while the mature pods were gathered in September. Yellowish-green (not yellow) pods of pea line 3 were sterilized for 5 minutes in 96% ethanol. Then seeds were removed and peeled (testae were excised). Every embryo was separated from the cotyledons and pressed with forceps. The embryonic tissue was placed on MS medium containing 5 mg/l BAP and 2 mg/l NAA. Five days later, emerging roots were removed. The immature embryo tissue was transferred monthly to fresh medium. Emerging shoots were excised and transferred to rooting medium (MS with 1% sucrose and 0.1 mg/l NAA).

Agrobacterium transformation

Internodal explants (in some experiments, leaf explants and decapitated seedlings as well) of 10-days-old axenic seedlings were inoculated with *Agrobacterium* suspension. Bacterial cells were suspended (10^8 cells per ml) in liquid minimal A medium (Shahin et al., 1986) and incubated for 16–18 hours on shaker. One ml of bacterial suspension (10^8 cells) was applied to the fresh cut basal surface of stem explant. The apical ends of stem explants were immersed in agarized MSO. Leaf explants were incubated in twice diluted bacterial suspension for 2–3 hours, dried and transferred to solid MSO medium. Explants were incubated with bacteria for 2 days and transferred to MSO medium containing claforan (500 mg/l). Result assessment (30 days after inoculation) was accomplished according to conventional procedures described earlier (Lutova and Sharova, 1993).

Phytohormone analysis

We used a "Beckman" HPLC unit to determine cytokinin content in 20 days old axenic seedlings of cv. *Sparkle* and lines Sprint-2, 32, 9 and 4. Characteristics were: Column: "Beckman", ultrasphere OPS 5 m 4,6 mm × 25 cm; pre-column: ultrasphere OPS 5m 4.6 mm × 5 cm; conditions: Gradient (system % B, MeOH and bidistil H_2O); l = 280 nM.

Time	Function	Value	Duration
0.00	Flow	0.400 ml/min	0.00
0.00	Curve	0	
0.00	Solvent A	1	
0.00	Solvent B	1	
0.00	% B	40.00	5.00
5.00	% B	80.00	20.00
25.00	% B	80.00	5.00
30.00	% B	40.00	10.00
40.00	END		

Cytokinin detection in green parts of plant was performed by means of procedures described earlier (Kislin, 1992).

We used the following phytohormones (from Serva Co., St. Louis) as standard: zeatin (Z), zeatin-ribozide (ZR), dihydrozeatin (DZ), dihidrozeatin-ribozide (DZR), 6-(g,g-dimethylallylamino)-purine (DAP), 6-(g,g-dimethylallyladenosin (DAPR). The hormones moved in the following order: ZR, DZR, Z, DZ, DAP, DAPR.

3. Results and Discussion

Analysis of pea regeneration capacity

To study the variability of pea morphogenetic responses to wounding (explantation), a hormone-free medium was used (Lutova and Zabelina, 1988; Lutova et al., 1994). The pea lines showed diversity in callus and root formation capacities (Table 2).

Table 2. Callus and root formation of pea lines on hormone-free medium.

Number of		Stem explants			Leaf explants	
Line	Explants	Callus formation		Root formation	Callus formation	
		%	size	%	%	size
9	105	81.9		43.8	19.1	
		78.0–85.5*	3**	38.9–48.6	18.1–27.0	1
32	309	93.8		43.0	17.2	
		92.8–94.2	3	42.9–46.0	10.8–24.7	1
4	451	84.8		9.68	0	
		82.7–85.9	2	7.9–11.4	0–0.25	–
26	47	100		2.19	0	
		96–100	1	0.52–5.5	0–1.8	–
29	688	86.4		0.87	0	
		88.1–94.8	1	0.49–1.4	0–0.15	–

Note: No shoot formation was observed in this experiment.
*Confidence interval; **Size of callus: 1 = callus on the cut surface does not cover the whole surface, 2 = callus fully covers the cut surface of explant, 3 = callus exceeds the cut surface of explant.

The regeneration characteristics depended on the plant organ: internodal (stem) explants showed greater regeneration capacity than leaf explants. These results are consistent with those obtained by others (Ezhova et al., 1985; Mallick and Rashid, 1988).

The results of studying pea morphogenetic responses to wounding suggest that intraspecific variability and organ-specificity of pea regeneration characteristics on hormone-free medium reflects the differences in endogenous hormonal balance (or hormone sensitivity or hormone degradation mechanisms) (Hussey and Gunn, 1984; Lutova and Zabelina, 1988; Lutova et al., 1994). Lines 4, 26 and 29 showed low frequency of root formation from stem explants, and their leaf explants did not form calli or roots at all. Lines 9 and 32 with high frequency of root formation from stem explants formed calli from leaf explants as well.

Cytokinin supplement (1 mg/l BAP) did not stimulate regeneration in the tested pea genetic lines and even suppressed callus formation of some lines (especially in lines 4 and 3) (Table 3). NAA (1 mg/l) induced root formation in all pea lines analyzed; but the lines showed diversity in root formation frequency and vigour. Lines 32 and 3 showed the highest root formation frequencies (Table 3). On the medium with both cytokinin BAP (1 or 5 mg/l) and auxin NAA (2 mg/l), callus as well as root formation were observed (Table 4). Such concentrations of exogenous phytohormones decreased the differences between lines in callus formation, as all analyzed lines formed large calli with high frequency in the first culture passage. However, intraspecific variability in shoot formation capacity was revealed, but the frequency of shoot formation was low even in regenerating lines (32, 29, 15). Shoots could be rooted though with low frequency on rooting medium.

Callus formation capacity and especially shoot formation and rooting capacities decreased greatly after subculture (in the passed calli). The dependence of regeneration characteristics on pea genotype became more noticable. Lines 32 and 29 showed the highest shoot formation capacities (Table 4). Indeed, line 32 showed the highest regeneration capacity in all the experiments on media with different hormone constitutions (Tables 2–4).

Induction of organogenic calli from immature pea embryos

Since the regeneration capacity of stem explants of the analyzed pea lines was not high and decreased after subculture, we tried to increase it using a modified procedure (Schroeder et al., 1993). Use of the immature pea embryos as the source of explants allowed an increase of pea regeneration capacity. Organogenic calli were obtained from immature embryos of pea lines 3 and 4. Immature embryo tissue formed numerous shoots on the medium containing phytohormones (5 mg/l BAP and 2 mg/l NAA) (Fig. 1). After shoot removal, calli formed new shoots (on the medium with 1 mg/l BAP and 2 mg/l NAA, callus necrosis was observed). Lines 3 and 4 did not differ in their shoot responses. The well-developed organogenic calli were maintained for six

Table 3. Response of pea internodal explants to exogenous phytohormones.

Medium		Hormone-free MSO			MS + 1 mg/l NAA				MS + 1 mg/l BAP			
Line	n	Callus %	size	Roots %	n	Callus %	size	Roots %	n	Callus %	size	Roots %
32	20	70.0 55–80*	1	0	26	46.0 28–65	1	100 95–100	28	47.0 26–69	1	0
9	13	92.0 71–100	2	0	20	40.0 20–61	1	30.0 12–51	25	24.0 9–47	1	0
3	12	67.0 39–82	1	0	15	45.0 22–70	1	100 94–100	20	5.0 0–19	2	0
4	17	100 87–100	1	0	13	31.0 9–58	1	23.0 5–48	16	0 0–7	–	0

Note: No shoot formation was observed; *Confidence interval; n = Number of explants.

Table 4. Regeneration capacity of pea lines in 0 and 1st passages on media with auxin and cytokinin supplement.

Line	Null passage (1 mg/l BAP + 2 mg/l NAA)						First passage (1 mg/l BAP + 2 mg/l NAA)				(5 mg/l BAP + 2 mg/l NAA)			
	Callus			Shoots		Rooting of primary regenerants	Callus			Shoots	Callus			Shoots
	n	%	size	%	n	%	n	%	size	%	n	%	size	%
9	87	98.9 95.6–100**	3	1.2 0–4.5	8	12.5 0–42.3	55	23.6 13.4–35.6	2	0 0–1.7	24	70.8 51.0–87.0	3	0 0–3.9
32	128	99.2 96.2–100	3	13.3 7.6–19.4	20	45.0 24.4–66.6	168	23.4 17.0–29.4	1	0.6 0–2.3	72	76.4 66.0–85.0	3	5.6 1.5–12.0
15	136	98.5 95.8–99.8	3	2.9 0.7–6.4	24	20.8 7.3–38.9	178	27.0 20.7–33.7	1	0 0–0.5	55	52.7 40.0–66.0	2	0 0–1.7
26	187	98.9 96.9–99.9	3	0.5 0 – 2.1	15	40.0 17.5–65.0	202	12.4 8.2–17.3	1	0 0–0.5	119	36.1 28.0–45.0	1	0.8 0–3.3
29	241	99.6 98.4–100	3	3.7 1.7–6.5	29	37.9 21.4–56.0	167	21.0 15.2–27.5	1	0 0–0.6	67	46.2 35.0–58.0	2	4.5 0.9–10.7
3	140	100 92.0–100	2	0 0–5.0	0	–	24	30.0 10.0–50.0	1	0 0–4.0	8	100 90.0–100	2	0 0–10.0

Note: *Number of regenerants, **Interval of confidence. No root formation was observed.

months. Senescence of the embryo tissue culture (older than 2 months) led to quantitative and qualitative changes in shoot formation efficiency. For example, the number of shoots decreased and their phenotype changed (they became small with thick stems and short internodal intervals).

Figure 1. Embryogenic callus from pea immature embryos (line 3) on the MS medium with BAP (5 mg/l) and NAA (2 mg/l) supplement (on 9 cm diameter Petri dishes).

Morphogenetic responses of different pea forms to transformation

Pea genotypes responded in different ways to transformation with some oncogenic strains of *A. tumefaciens* and *A. rhizogenes* (Lutova and Sharova, 1993) (Table 5). Lines differed in responses to transformation greater than in responses to wounding or exogenous phytohormones. For example, *Sparkle* cultivar and, to a smaller extent, line 15 formed large tumors even after transformation with root-inducing *A. rhizogenes* strain 8196. Line 32, on the contrary, formed roots even after transformation with tumor-inducing *A. tumefaciens* strain A281 (Table 5). These results of transformation are consistent with the phenotypic responses to wounding and exogenous phytohormones (without transformation). According to the data mentioned above, line 32 showed the highest root formation capacity (Tables 2–4). These responses to wounding, exogenous phytohormones and *Agrobacterium* transformation may be connected with the higher concentrations of endogenous auxin or higher sensitivity to it in tissues of line 32 than in the other lines used

in this study. In general, lines 3, 4, 9 and Sprint-2 demonstrated typical response to transformation :tumor formation after inoculation with *A. tumefaciens* strain A281 and root formation after inoculation with *A. rhizogenes* strain 8196 (Fig. 2).

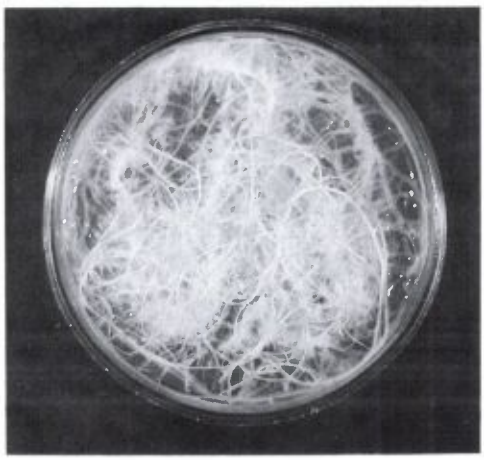


Figure 2. Root cultures of pea line 4 transformed by *A. rhizogenes* 8196 strain.

In this experiment we did not reveal the differences between Nod$^-$ mutants and their original parents. Nodulation, tumor- and root formation processes are believed to be determined mostly by cytokinins and auxin (for example, Hirsch et al., 1989; Shoichiro et al., 1991).

Mutation sym8 is considered to affect the early steps of plant-rhizobia interaction and not to affect the plant hormonal status since exogenous hormones and their inhibitors do not restore nodulation in these mutants (Fearn and LaRue, 1991).

Mutation sym5 shows early steps of nodule development hypersensitive to ethylene level normal for cv. *Sparkle*. Ethylene inhibitors and low temperature decreasing ethylene synthesis restore nodulation in sym5 mutants and do not increase nodulation in the original cultivar *Sparkle* (Fearn and LaRue, 1991; Juinel and LaRue, 1991).

Cultivar *Sparkle* and its Nod$^-$ mutants may contain higher levels of ethylene or may possess higher susceptibility to ethylene than most pea genotypes. Ethylene is known to inhibit nodulation. So perhaps any mutation in *Sparkle* genotype leading to even insignificant increases of ethylene sensitivity or ethylene content in roots may affect nodulation.

Table 5. Phenotypic characteristics of the transformed tissues of different pea genetic forms.

Strain	Lines	3	4	9	32	15	a	b	c	d	e
A. tumefaciens											
	A281	Large tumors			Large tumors rare roots	Very large tumors					
	A277	Tumors	Not tested	Tumors	Tumors roots	Tumors				Not tested	
A. rhizogenes											
	8169	Roots				Tumors roots	Large tumors			Roots	
	15834	Roots				Tumors roots	Large tumors			Roots	
Untransformed control * –		Small calli on explant surfaces rare roots									

*–Untransformed control was treated with medium without bacteria. a = *Sparkle*; b = *Sparkle* sym5; c = *Sparkle* sym8; d = Sprint-2; e = Sprint-2 Nod$^-$-2.

Cultivar *Sparkle* and its Nod$^-$ mutants differed significantly from the other pea lines in phenotypic responses to transformation with *A. rhizogenes* strains. Cv. *Sparkle* and its sym5 and sym8 mutants formed large tumors without roots after inoculation with strains A281 (*A. tumefaciens*) and 8196 (*A. rhizogenes*). The tumors induced by *A. rhizogenes* strain 8196 approached the size of those induced by supervirulent strain A281, though some explants inoculated with strain 8196 formed rare non-hairy roots. The same phenotypic effect (tumor formation instead of "hairy root") was observed by other authors in some pea lines transformed by *A. rhizogenes* strain R1000 (Robbs et al., 1991). These data may suggest a changed auxin/cytokinin ratio (may be lower auxin concentration) or changed sensitivity to these hormones (may be lower sensitivity to auxin) in these lines.

The intraspecific variability of pea transformation capacity was mentioned by other authors as well (Hobbs et al., 1989; Robbs et al., 1991). The absence of teratoma formation in the transformed tissues is consistent with the low shoot formation capacity of the lines in response to wounding and exogenous

phytohormones. So the regeneration characteristics *in vitro* in general are consistent with phenotypic responses to *Agrobacterium* transformation. It must reflect the influence of endogenous hormone status (such as hormone content or tissue sensitivity to phytohormones).

Genetic forms of pea, which displayed contrasting morphogenic responses to transformation, differed in the set of main endogenous cytokinins (zeatin Z, dihydrozeatin DZ, zeatin riboside ZR, dihydrozeatin riboside DZR, N^6-(2-(isopentenyladenine)) DAP, N^6-(2-(isopentenyladenosine)) DAPR), as was shown by HPLC analysis (Table 6). Line 32 and Sprint 2 showed less variability in cytokinin content than the other forms analysed.

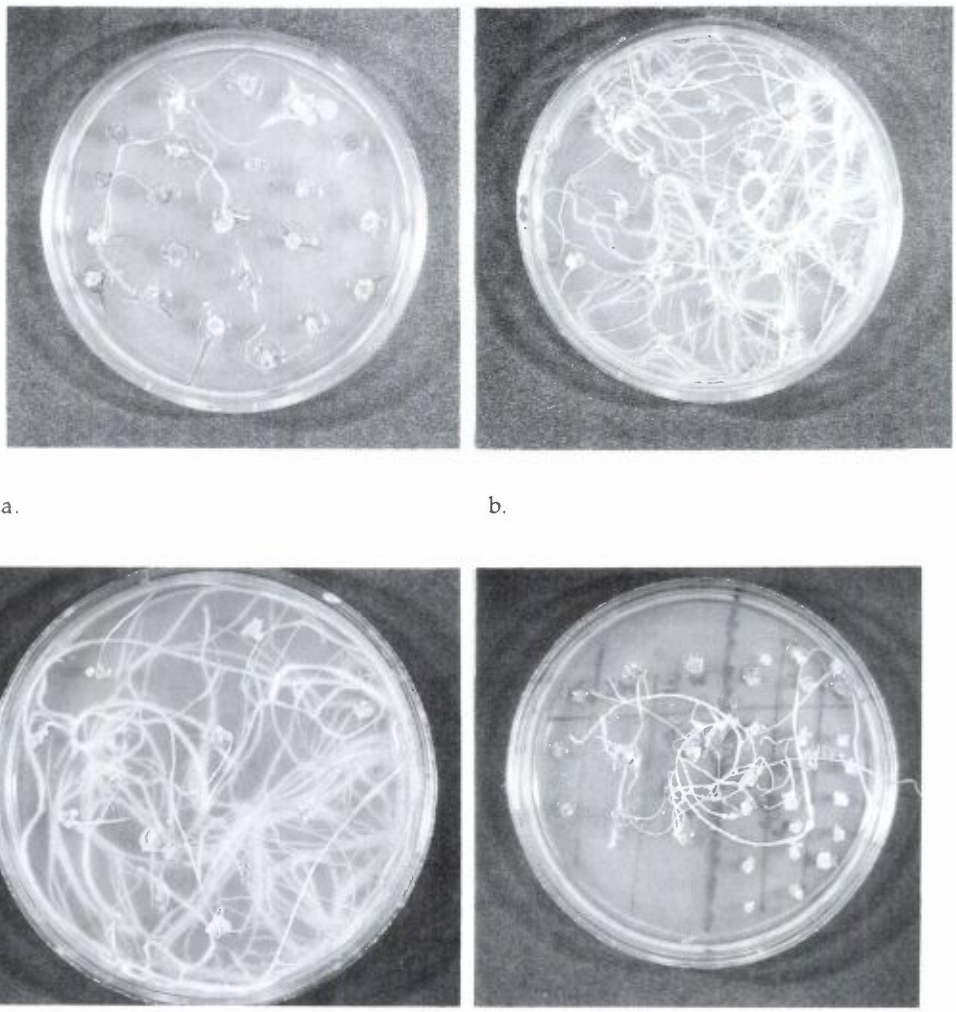
Table 6. Differences between pea genetic forms in the set of main endogenous cytokinins shown by HPLC analysis.

Pea genetic form	Cytokinin					
	ZR	DZR	Z	DZ	DAP	DAPR
cv. *Sparkle*	–*	+	+	–	+	+
Sprint-2	+	–	+	–	–	+
32	–	+	+	–	–	+
9	–	+	–	+	+	+
4	+	+	–	+	–	+

*Minimum detection level is 0.5 ng aliquote.

Accordingly, transformation provides a good inducing condition to reveal pea genotypes contrasted in traits which characterize phytohormonal status and can be used to study the genetic control of these traits.

The existing differences between the pea genotypes for root and tumor formation allowed us to analyze these traits genetically. F_1 hybrids between root forming line 9 (Fig. 3a) and cv. *Sparkle* which is not able to form roots (Fig. 3b), were all root forming (Fig. 3c) (Table 7). These results suggest that the capacity to form roots is a dominant trait. F_1 hybrids between tumor-forming plants and non-tumor forming ones were all no tumor forming suggesting that tumor formation is a recessive trait (Table 7). We studied only root formation and did not analyze tumor formation in F_2 hybrids since vigorous root formation disturbed analysis of tumors. In F_2 generation segregation in root formation



a. b.

c. d.

Figure 3. Root formation capacities of pea lines with contrast differences and their F_1 and F_2 hybrids after transformation by *A. rhizogenes* 8196 strain. a) Non-root forming cv. *Sparkle*; b) root forming pea line 9; c) F_1 hybrids (9xSp): an intensive root formation; d) F_2 hybrids (Spx9): a segregation within plants according their root forming capacity.

capacity was revealed (Fig. 3d). The results obtained to date do not allow us to discuss segregation in F_2. The results of reciprocal crossing, complete cyclic

crossings, analysis of F_3 and F_b progenies are needed to make conclusions about the inheritance of root formation in response to *Agrobacterium* transformation. To confirm and refine the role of endogenous plant hormones in morphogenetic response to transformation we studied the responses of the transformed tissues to exogenous phytohormones. For this purpose we used long-term cultivated transformed tumors and roots from the hormone-free medium. These tissues, maintained for 0.5–2 years, preserved their primary characteristics described a month after *Agrobacterium* inoculation. Two to four weeks after transferring of root cultures to the medium with cytokinin BAP the root dedifferentiation became visible: root tissues got replaced by green callus. The rate of this process increased directly with the exogenous cytokinin concentration (data not shown). The long-term maintenance (not less than 2 months) of the root tissues on medium with high BAP concentration (5 mg/l) resulted in virtually total replacement of roots by green calli. Further transferring of such cultures (for example, 32-8196) on hormone-free medium led to the reverse process – root differentiation from callus tissues.

Table 7. Phenotypes of tissues of pea forms and F_1 and F_2 hybrids transformed by *A. rhizogenes* 8196 strain.

Plant genotype	Number of plants	Tumor formation	Root formation
Sparkle (Sp.)	45	+	–
9	58	–	+
32	66	–	+
4	29	–	+
F_1			
32xSp.	32	–	+
9xSp.	56	–	+
Spx4	26	–	+
F_2			
32xSp.	110	not tested*	66(+) : 44(–)
9xSp.	110	not tested	64(+) : 36(–)
Spx4	100	not tested	77(+) : 23(–)

Note: + = All plants tested possessed this trait; – = all plants tested did not possess this trait; * = we did not analyse tumorformation in F_2 hybrids since vigorous rootformation disturbed analysis of tumor.

The phenotype of the tumorous transformed culture (for example, Sprint2-A281) was not changed by transferring to the medium with BAP.

NAA induced root formation in the tumorous cultures (*Sparkle*- 8196), but the effect of the NAA was less than the influence of BAP on the root transformed cultures. NAA at concentrations of 1 mg/l did not change the phenotype of the root tissues (32-8196). However, increasing exogenous auxin concentration resulted in insignificant suppression of root formation.

Exogenous phytohormone supplements made it possible to obtain phenocopies of typical morphogenetic responses of plant tissues to transformation with certain *Agrobacterium* strains (for instance, NAA caused root formation in the tumorous culture *Sparkle*-8196), and to obtain the phenocopies of the atypical response to transformation in genetic forms responding typically (BAP caused callus formation in root cultures 32-8196 and Sprint 2-8196). These results can be considered as confirming the suggestion made above concerning the hormonal balances of the pea genetic forms used in this work. Plant genotype controlling the plant hormonal status determines the morphogenetic responses to any environmental influence.

4. Conclusion

The complex assessment of transformation capacity (on hormone-free medium) revealed intraspecific variability as well as the assessment of separate transformation characteristics. Line 32 showed the highest transformation as well as regeneration capacity. These results suggest that line 32 responds actively to any stress (wounding, or inoculation with *Agrobacterium*).

Comparison of the complex assessments of regeneration and transformation capacities suggests the existence of a relationship between these processes, that is consistent with the results of the other authors (for example, Gresshoff et al., 1979). The existence of such a relationship is consistent with the proposal that genotype-determined differential susceptibility to transformation reflects the endogenous phytohormone content and susceptibility of plant tissues to phytohormones. Our results do not contradict the opinion that cells competent to transformation are also competent to regeneration (Potrykus, 1992). The pea genotypes with high regeneration potential usually possess high transformation capacity, and the characteristics of morphogenesis *in vitro* correspond to the phenotypic characteristics of transformation.

We suggest that endogenous plant hormonal status (hormone content, tissue sensitivity to phytohormones, degradation, metabolic antagonists) determined

by the plant genotype may influence plant morphogenesis processes to a greater extent than exogenous phytohormone supplemented *in vitro* or introduced by the bacterial phytohormonal genes.

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