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Competence for Regeneration during Tobacco Internodal Development[1]

Involvement of Plant Age, Cell Elongation Stage, and Degree of Polysomaty

Luud J. W. Gilissen*, Marjo J. van Staveren, Johanna C. Hakkert, and Marinus J. M. Smulders

DLO-Centre for Plant Breeding and Reproduction Research, Droevendaalsesteeg 1, P.O. Box 16, NL-6700 AA Wageningen, The Netherlands

This study deals with internodal development in vegetative plants of *Nicotiana tabacum* cv Samsun NN and its reflection in changes of the cellular competence for regeneration. During elongation of the internodes, the cells of the epidermis, subepidermis, and cortex exclusively expanded and increased their DNA content cell type specifically, generally from 2C to 4C. Cells with the 8C DNA content were found mainly among the cortex cells of mature internodes. The frequency of shoot regeneration (directly from subepidermal and epidermal cells together) on thin cell layer explants increased to an optimum along with elongation of the internodes and decreased in mature internodes along with aging. The frequencies of diploid shoots among the regenerants from elongating and mature internodes were high (88 and 75% on the average, respectively), indicating that most cells that had achieved the 4C DNA content generally retained the G2 phase of the diploid cell cycle. Shoots regenerated from explants of young plant material mainly had a vitrified appearance. The occurrence of this type of malformed growth was already determined by the physiological state of the cells in the internode and did not interfere with their acquisition of competence. Vitrification was unrelated to the degree of polysomaty of the internodal tissue. Using the occurrence of tetraploid root regenerants (from intermediate cortex-derived callus), up to a frequency of 50%, we show that the position in the plant where a majority of the 4C cortex cells switched to the G1 phase of the tetraploid cell cycle was at the transition from the elongation phase to the mature phase.

Beyond the apical meristem, cells pass through a complex developmental program that regulates cell and tissue differentiation gradually toward maturation and aging. Simultaneously, the apex continues its production of an increasing number of structures. The polar direction hinders the distinction of temporal, spatial, and quantitative factors in the development of the shoot and its component cells (Poethig, 1990). Submission of defined tissue parts at a specific developmental stage to external stimuli can be applied to reveal the physiological properties at the cell and tissue level. This has been demonstrated for root tips using gravi- and thigmostimulation (Ishikawa and Evans, 1995) and for shoot explants using qualitative and quantitative responses to hormones (Van der Krieken et al., 1990) and competence for regeneration (Smulders et al., 1990).

Competence for regeneration is defined as the capacity of cells to respond with morphogenesis to externally provoked inductive effects (Potrykus, 1990). The factors that determine the competence for regeneration include biochemical gradients and positional control factors at the tissue level, as well as cell type, cell size, state of differentiation, and C value at the cellular level, in relation to developmental stage and age (see relevant examples in Croes et al., 1985; Rajasekaran et al., 1987; Taylor and Vasil, 1987; Barcelo et al., 1991; Karunaratne et al., 1991; Sawhney and Applewhite, 1993; Gilissen et al., 1994). Increased knowledge of this complex of endogenous factors, their mutual relationships, or their autonomy will be highly valuable for a better understanding of the processes of cell and tissue differentiation, shoot development, and the acquisition and loss of competence for regeneration.

In the present paper, the modular construction of the *Nicotiana* plant during its vegetative growth phase has been used as an experimental tool to investigate in detail the development of several endogenous factors in various stages of plant (module) development as reflected by changes of various aspects of the competence for regeneration of shoots and roots from TCL explants (Tran Thanh Van et al., 1973). Either shoot or root regeneration from these explants can be reproduced quantitatively, simply by culturing on a medium containing specific ratios of NAA and BAP (Creemers-Molenaar et al., 1994; Gilissen et al., 1994). The regeneration frequency determined at the tissue level is often used as a parameter to express acquired competence. This parameter should be used with care, since in explants excised from young donor plant tissues many more cells will be exposed to optimum regeneration conditions as compared with equally sized explants from fully expanded tissues. Therefore, knowledge of the initial number and dimensions of the cells involved in the actual regeneration process, in relation to the total number of

[1] This paper is dedicated to Professor Dr. H.F. Linskens on the occasion of his 75th birthday.

* Corresponding author; e-mail l.j.w.gilissen@cpro.dlo.nl; fax 31-317-418094.

Abbreviations: BAP, 6-benzylaminopurine; C value, DNA content of a cell expressed in singular or in multiples of the basic DNA content (C) of the haploid genome; CLSM, confocal laser scanning microscope; NAA, naphthaleneacetic acid; TCL, thin cell layer.

relevant cells in the explant, is essential for correct quantitation of the regeneration frequency and interpretation of cellular competence for regeneration.

The development of polysomaty in plant organs is generally caused by endoreduplication and is related to growth (especially cell expansion) and differentiation (Nagl, 1978; Galbraith et al., 1991; Gilissen et al., 1993, 1994; Melaragno et al., 1993; Smulders et al., 1994, 1995). Explants from polysomatic organs may also be polysomatic, e.g. in tobacco (*Nicotiana tabacum*; Gilissen et al., 1994). Cells in the tetraploid cell cycle will produce tetraploid regenerants if competence for regeneration has been maintained. In mature leaf tissue of Napier grass, changes in the cell cycle were not responsible for the loss of embryogenic competence (Taylor and Vasil, 1987). One question to be answered in the present paper is where in the tissue during shoot development the C values and the cell cycle phases change and how this relates to the competence for the regeneration of shoots or roots.

Qualitative aspects of regeneration may also reveal the physiological properties of the developing internode. A striking qualitative characteristic of the regeneration process is the occurrence of vitrification. Vitrified shoot regenerants generally develop as malformed, translucent structures. Vitrification was frequently found among plantlets regenerated from TCL explants (Gilissen et al., 1994, 1995). The explant position and the age of the donor plant seemed to be involved in the occurrence of vitrification, as was suggested from a preliminary study (Gilissen et al., 1995). Basic knowledge of the origin and the development of the phenomenon is scarce (Pâques and Boxus, 1987; Ziv, 1991). Thus, a next question in this paper concerns the relationship between the physiological state of the internodal tissue and the occurrence of vitrification. Finally, the degree of interdependence of the various changes of physiological factors related to development in their effects on the determination of competence for regeneration is established.

MATERIALS AND METHODS

Plant Material and Tissue Culture

Plants of *Nicotiana tabacum* cv Samsun NN were grown from seed in a climate room at 21°C, 80% RH, and 13 h/d light (type MGR 102 and SON-T, 400 W, at 2 m distance; Philips, Eindhoven, The Netherlands). Plant growth was followed by regular measurement of the lengths of the stem and the individual internodes. Internodal length increased from the plant base to the fourth to sixth internode (Fig. 1). Above the basal internodes, approximately 15 vegetative internodes developed with equal ultimate lengths, generally ranging between 23 and 35 mm, depending on the individual plant. When the total plant length was greater than 30 cm, the growth phase at the top of the plant changed from vegetative to generative. For the internodes of vegetative plants, two main developmental phases were distinguished (Fig. 1): (a) the elongation phase, including the dark-green upper internodes with a length up to 10 mm and the green internodes with a length between 11 and approximately 30 mm, and (b) the mature phase, including the fully elongated, pale green internodes with lengths of approximately 23 mm and longer and the shorter basal internodes. When more plants were used in one experiment, the lengths of elongating and mature internodes partly overlapped because of natural variation of internodal lengths among individual plants. The elongation phase or the mature phase of an individual internode at the overlap was identified with certainty by comparing its length to those of the neighboring internodes in relation to its relative position within the plant.

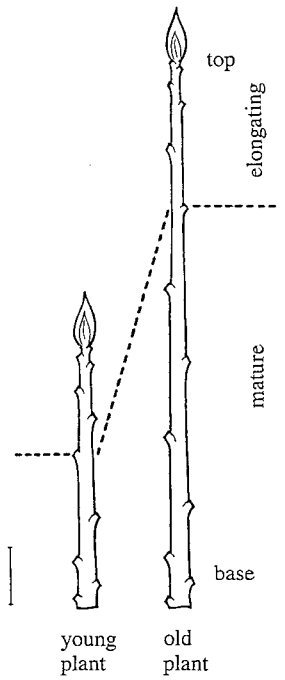

Figure 1. Stems of a representative young and old vegetative plant of *N. tabacum* cv Samsun NN with their mature and elongating internodes. Scale bar, 25 mm.

For tissue culture, stems were taken from young (mean plant length of 10–12 cm) and old (mean plant length of approximately 23 cm) vegetative plants (Fig. 1). After the leaves were excised, the stems were washed for 2 min in 0.01% Teepol HB6 (Sigma), rinsed in running tap water for 5 min, surface-sterilized for 10 min in 2.5% sodium hypochlorite, and finally washed three times in sterile water. Successive internodes were used for the excision of TCL explants, with the explant length ranging from 3 mm (in the case of the shortest [3 mm] internodes) to a maximum of 10 mm (in the case of internodes with a length of 10 mm or longer). Each explant had a maximum thickness of 6 to 10 cell layers, irrespective of the internodal length. The lengths of each internode and the TCL explants derived therefrom were measured at the onset of culture. Since internodes generally consisted of a constant number of cells in the longitudinal direction (see "Results"), the ratio of internodal length to TCL explant length was used to normalize the shoot and root regeneration frequencies of TCL explants from the successive internodes. Ten to 15 explants were placed on a 9-cm Petri dish containing Murashige-Skoog culture medium (Murashige and Skoog, 1962), solidified with 0.8% (w/v) agar (Imperial Laborato-

ries, Andover, UK), and supplemented with 3% Glc and either 0.5 μM NAA and 1.5 μM BAP for shoot regeneration or 5 μM NAA and 0.05 μM BAP for root regeneration (Creemers-Molenaar et al., 1994; Gilissen et al., 1994). The media were autoclaved for 20 min at 110°C. The Petri dishes were sealed with a double layer of Parafilm M (American National Can, Greenwich, CT)and placed in a culture room at 25°C and 16 h/d light (fluorescent type TDL, 50 W, 84HF, Philips), at a 20-cm distance under a white cotton cloth. After 2 weeks, the frequencies of shoot regeneration were determined. Roots were counted after 4 to 5 weeks.

Flow Cytometry

For flow cytometric analysis, samples consisting of three TCL explants, leaf material of individual 3- to 5-week-old regenerated shoots, or single regenerated roots were chopped in 2 mL of nucleus isolation buffer as described previously (Gilissen et al., 1993). For calibration, the 2C peak from nuclei of expanded leaves of *N. tabacum* was adjusted as the reference. The data are presented as percentages of the total number of nuclei in all peaks in the histogram.

Light and Confocal Microscopy

The length of 10 individual cells from the epidermis, subepidermis, and middle cortex of successive internodes (n = 18, with lengths ranging from 4 to 37 mm) from three individual plants of different age classes was measured by light microscopy, using a calibrated eyepiece. Vitrification was determined at low magnification under the binocular microscope. The frequency of vitrification was quantified as the percentage of vitrified regenerants in five explants from the five upper internodes each of a single plant; six plants were involved per experiment. Analysis of the DNA content of individual cells in epidermal, subepidermal, and cortex tissue in median longitudinal sections of internodes of various length was carried out by cytometric fluorescence measurements using confocal microscopy as described previously (Gilissen et al., 1994).

RESULTS

Internodal Growth and Cell Number

Length measurements of individual tobacco internodes at several times during the growth period showed that the ultimate internodal length depended on the internodal position in the plant. This length was approximately constant for the vegetative internodes of an individual plant but varied (generally 23–35 mm) among plants. The actual internodal lengths depended on the plant age (Fig. 1). Figure 2 is a series of photographs that show that internodal growth is based essentially on cell expansion. This was confirmed by measuring in successive internodes from various individual plants the number of cell layers between epidermis and vascular tissue and the cell lengths of different cell types. The number of cell layers was constant (approximately 13). Cellular length in the epidermis, the subepidermis, and the middle cortex was linearly related to the length of the internode. In young and old vegetative plants with internodal lengths ranging from 4 to 37 mm, the mean cell lengths for epidermal, subepidermal, and middle cortex cells were 5.1 (r = 0.96), 4.3 (r = 0.92), and 6.9 μm (r = 0.94) for each millimeter of internodal length, respectively. Each internode thus contained a constant number of approximately 200 epidermal, 230 subepidermal, and 140 middle cortex cells in the longitudinal direction. In the short basal internodes, the number of cells in the longitudinal direction was reduced, but similar cell lengths were found (result not shown). Based on these results, the repetitive internodes were used for further comparative investigations of regeneration frequencies of TCL explants, corrected for the number of cells.

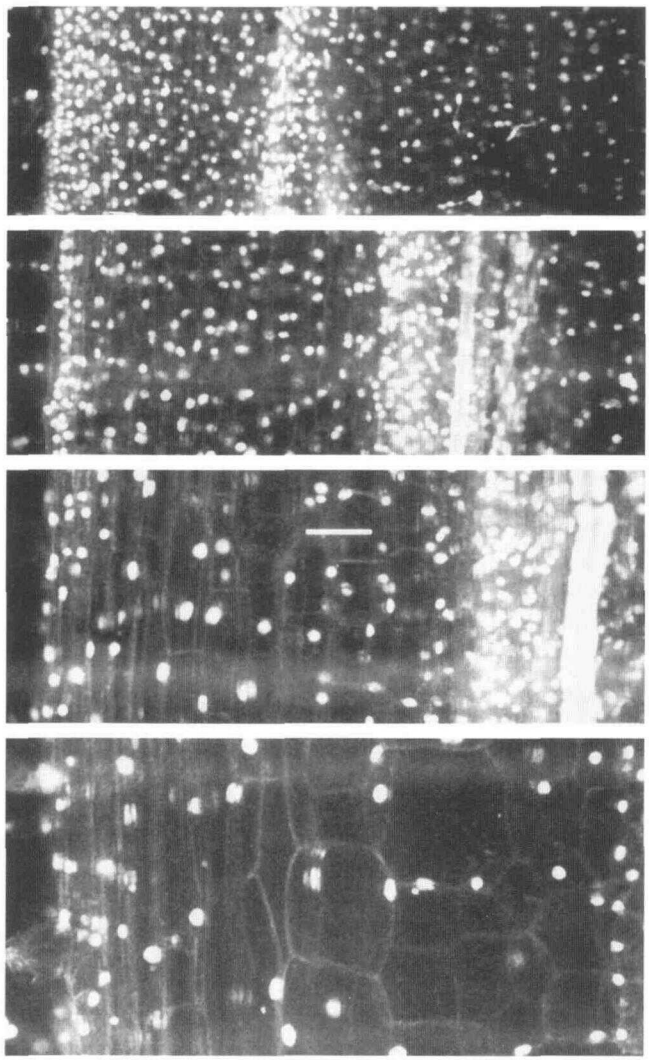

Figure 2. CLSM photographs of ethidium bromide-stained longitudinal sections of the outer cell layers (epidermis, subepidermis, and cortex) from internodes at various stages of elongation of a vegetative plant of *N. tabacum* cv Samsun NN. Internodal lengths were approximately 2, 7, 14, and 28 mm from top to bottom, respectively. All photographs were taken at the same magnification. Scale bar, 100 μm. The small nuclei had the 2C DNA content, medium-sized nuclei were 4C, and the largest nuclei were 8C.

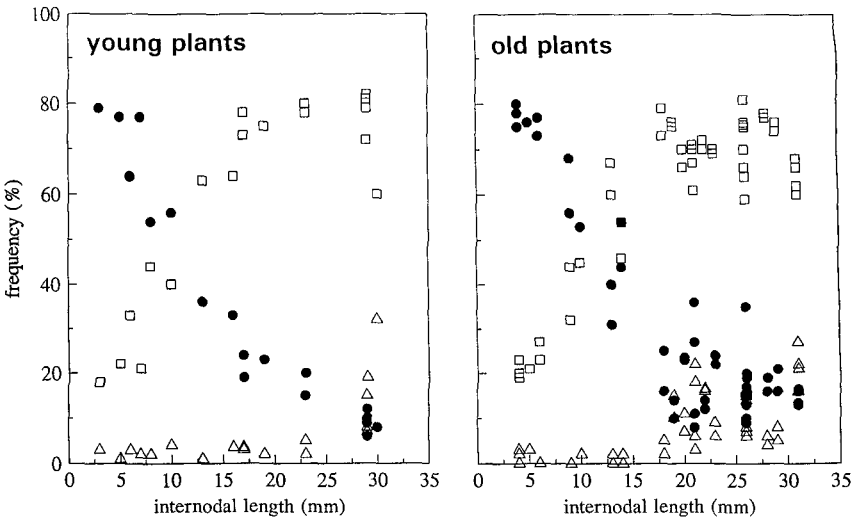

Figure 3. Changes in the frequencies of 2C (●), 4C (□), and 8C (△) cells as analyzed by flow cytometry. Three TCL explants per internode from three young and three old vegetative plants of *N. tabacum* cv Samsun NN with mean plant lengths of 11 and 22 cm, respectively, were used.

Polysomaty in Internodal Tissue

Figure 2 also shows increased nuclear volumes in expanded cells. Flow cytometric analysis revealed that TCL explants, freshly excised from upper internodes of approximately 5 mm in length from both young plants and from old vegetative plants, contained 2C, 4C, and 8C cells at frequencies of approximately 78, 20, and 2% (Fig. 3). With increasing internodal length up to 20 mm, the frequency of 2C cells in the TCL explants decreased almost linearly to approximately 20%, and the frequencies of 4C cells simultaneously increased to about 75%. In explants from mature internodes, the frequency of 8C cells increased up to approximately 25%. Explants from the shorter basal internodes gave frequencies for the various levels of DNA content similar to the other mature internodes.

The shift in the DNA content at the C-value level (based on cytometric fluorescence measurements using CLSM) in epidermis plus subepidermis cells, which are involved in shoot regeneration, and in the cortex cells from which roots generally develop (Creemers-Molenaar et al., 1994; Gilissen et al., 1994) is demonstrated in Table I. The pooled data from elongating internodes showed a high frequency (approximately 80%) of 2C cells in the epidermis plus subepidermis and almost equal frequencies (nearly 50%) of 2C and 4C cells in the cortex. The pooled data from the mature internodes revealed that 70% of the epidermis plus subepidermis cells had the 4C DNA content, whereas similar frequencies (i.e. 50 and 39%, respectively) were found for 4C and 8C cells in the cortex. The above results were obtained from a young plant with a length of 13 cm. In a preliminary experiment on an old plant, similar data were obtained (results not shown).

Internode Developmental Factors and Competence for Regeneration

Of the internode developmental factors described above, i.e. elongation stage and age of the explant source internode, and the related changes of DNA content of the cell types relevant to shoot and root regeneration, the role of competence for regeneration of shoots and roots was studied in various aspects. These aspects concerned the regeneration frequency, the ploidy level of the regenerants, and the occurrence of vitrification. For comparison, the experiments were carried out on both young and old vegetative plants.

Regeneration Frequencies

In general, the shoot regenerants developed from a narrow region along the longitudinal edges of the TCL explant, i.e. the region where the subepidermal cells at the cut surface of the explant are in direct contact with the culture medium (Gilissen et al., 1994). Figure 4 illustrates that 10 to 20 shoot primordia (numbers normalized according to the ratio of internodal length to explant length) were found per explant from the uppermost internodes. The number of primordia on the explants increased linearly with the increase of internodal length, and thus with the length of the internodal cells, up to 90 shoot primordia in explants from the later stages of elongation in young plants as well as in old plants. Numbers lower than 50 primordia were generally obtained in explants from fully elongated, mature internodes. In some explants from the oldest, mature basal internodes, no regeneration was observed.

Table I. *DNA content (C value) of cells in epidermis plus subepidermis and in cortex*

Frequencies (%) of C values (obtained from cytometric fluorescence measurements of individual nuclei using CLSM) of cells relative to shoot regeneration (epidermis plus subepidermis) and root regeneration (cortex-derived callus) in a young vegetative plant of *N. tabacum* cv Samsun NN, with a length of 13 cm and consisting of four elongating and two mature internodes. *n*, Number of cells.

Internodal Category	Epidermis + Subepidermis[a]				Cortex				
	2C	4C	8C	*n*	2C	4C	8C	16C	*n*
Elongating	82	18	0	120	45	49	6	0	180
Mature	8	70	22	60	4	50	39	7	90

[a] Equal numbers of both cell types were measured.

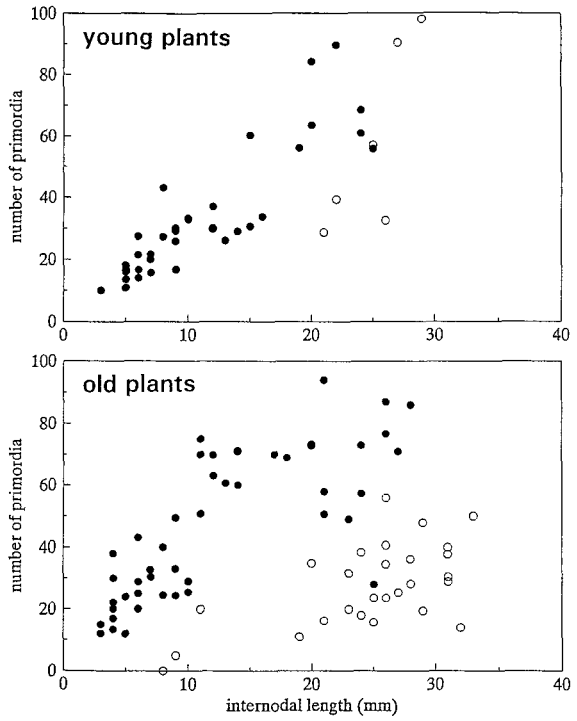

Figure 4. Number of shoot regenerants (primordia) of TCL explants from internodes of various lengths in nine young (mean plant length of 11 cm) and nine old (mean plant length of 20 cm) vegetative plants of *N. tabacum* cv Samsun NN, normalized according to the ratio of internodal length to explant length. Each point represents the mean number of regenerants from five TCL explants per internode. Solid symbols refer to explants from elongating internodes and open symbols refer to explants from mature internodes.

In explants from upper internodes, the development of the regenerants was more pronounced at the apical part of the explant. Some regenerants even appeared at the apical edge. In explants from fully elongated internodes, there was a tendency for increasing shoot regeneration toward the basal part and edge of the explant (results not shown).

Similar to the shoot regeneration frequency, but only in old plants, the frequency of root regeneration increased with increasing internodal length to a maximum of approximately 20 roots in the explants from recently elongated, mature internodes and decreased in explants from older, mature internodes. In contrast, in young plants, relatively high root regeneration frequencies were found in TCL explants taken from the upper elongating internodes and from the mature internodes (Table II).

Ploidy Level of Regenerated Shoots and Roots

Table III shows the frequencies of occurrence of mixoploid and tetraploid shoot regenerants of TCL explants from elongating and mature internodes in young and old plants. A decrease of the frequency of diploid shoot regenerants from 88 to 75% on the average for young and old plants together was observed in explants from elongating internodes as compared to explants from mature internodes. This small decrease did not parallel the shift in the DNA content of the majority of epidermal and subepidermal cells from 2C to 4C (Table I), indicating that most of the competent 4C cells in the mature internodes were in the G2 phase of the diploid cell cycle.

In the case of root regeneration, tetraploid roots developed only on explants from mature internodes, where they appeared at high frequency (approximately 50%). Here, this change did coincide with the shift in the cortex cells from the 2C/4C to the 4C/8C level.

Vitrification

Vitrified shoot regenerants with rapidly growing, pale green, oblong or malformed, translucent leaves, frequently developed on the TCL explants. As shown in Figure 5, the frequency of occurrence of vitrified regenerants on the explants was related to plant age and internodal length. Nearly all regenerated shoots on explants from the successive internodes of the young plants had a vitrified appearance. However, in the old plants, up to 85% of the regenerants were vitrified on explants from upper internodes, but hardly any vitrification was observed on explants from elongating and mature internodes. No vitrified roots were observed.

Ploidy Level and Vitrification

The nDNA content in the cells of the explant source increased along with advancing elongation and aging (Figs. 2 and 3; Table I). Vitrification was highly characteristic of young explant source tissues only. In young plants, vitrification occurred independently of internodal length (Fig. 5). These results suggest that the DNA content of the explant cells and the development of vitrification in the regenerants were unrelated. This suggestion was confirmed by the average distribution frequencies of diploids, mixoploids, and tetraploids among the shoot regenerants in young and old plants according to their vitrified and nonvitrified appearance; these frequencies were highly similar among the vitrified and nonvitrified regenerants (Table IV).

DISCUSSION

The *N. tabacum* plant is constructed of a number of repetitive modules in its vegetative part, sequentially generated by the apical meristem. These modules exhibit a high degree of uniformity during their development and in

Table II. *Root regeneration from TCL explants*

Number and SE of root formation per TCL explant from the upper and the lower elongating internodes and the upper and the lower mature internodes in young and old vegetative plants of *N. tabacum* cv Samsun NN with mean plant lengths of 12 and 23 cm, respectively. Five TCL explants were measured per internode. *n*, Number of internodes.

Internodes	Young Plants	(*n*)	Old Plants	(*n*)
Upper elongating	6.2 ± 1.2	(8)	0.4 ± 0.4	(4)
Lower elongating	2.3 ± 1.3	(4)	10.8 ± 5.0	(4)
Upper mature	8.5 ± 1.4	(4)	20.1 ± 4.9	(6)
Lower mature			1.2 ± 0.8	(6)

Table III. *Ploidy levels of regenerated shoots and roots from TCL explants*

Frequency (%) of ploidy levels among regenerated shoots and roots from TCL explants of successive internodes of young and old vegetative plants of *N. tabacum* cv Samsun NN with mean plant lengths of 11 and 21 cm, respectively. The internodes were grouped according to the elongation stage (Elongating and Mature) and plant age (Young and Old). *n*, Number of regenerants.

Internodal Category	Shoots				Roots		
	Diploid	Mixoploid	Tetraploid	*n*	Diploid	Tetraploid	*n*
Elongating							
Young	92	8	0	60	100	0	20
Old	84	12	4	60	100	0	22
Mature							
Young	75	15	10	20	48	52	40
Old	74	23	3	30	55	45	31

their mature shape. For example, the outer cell layers of the internodes, consisting of epidermal, subepidermal, and cortical cells, contained a constant number of cells in longitudinal and radial directions. These cells expanded exclusively during internodal elongation. The internodes of the repetitive modules were therefore used here as an experimental tool for comparative investigation, on the basis of equal cell numbers, of the effect of various developmental factors on the competence for regeneration of shoots and roots from internodal TCL explants.

Competence for shoot regeneration in tobacco has to be considered as a phenomenon that goes beyond the level of the individual cell, since both epidermal and subepidermal cells are simultaneously involved in this process of direct morphogenesis (Creemers-Molenaar et al., 1994; Gilissen et al., 1994). Nevertheless, the three phases that characterize morphogenesis are still appropriate: acquisition of competence, induction of morphogenesis, and further development (Christianson and Warnick, 1985, 1988).

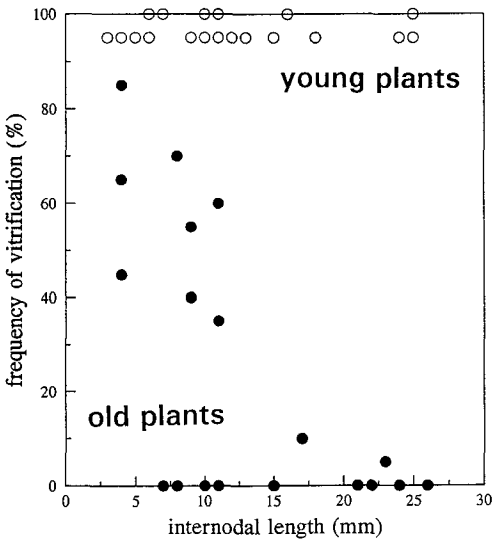

Figure 5. Frequency of vitrification in regenerants on TCL explants from internodes of various lengths in six young (open symbols) and six old (solid symbols) vegetative plants of *N. tabacum* cv Samsun NN with mean lengths of 11 and 23 cm, respectively. Each point represents the mean frequency of vitrified regenerants on five explants per internode.

1. Concerning the acquisition of competence in explants from the youngest internodes, several cells from (one or) both cell types might not have reached or exceeded sufficiently a certain physiological or volume threshold to acquire competence completely. The almost linear relationship between the regeneration frequency of explants and the internodal length during elongation suggests a prominent role for the cell growth during this phase of morphogenesis. Because of the high response of the direct shoot regeneration in TCL explants from internodes at the later stages of elongation, the state of acquired competence in the epidermal and subepidermal tissue has to be considered here as the default state.

2. Regarding the induction of organogenesis, shoot meristem development is limited to the longitudinal edges of the explant, where intact subepidermal cells at the cut surface of the explant were in direct contact with the culture medium. In the other explant regions, notwithstanding their acquired competence, shoot induction was blocked (Gilissen et al., 1994). Meristem development also was found to be reduced in the basal part of the regenerative edge but only in explants from the youngest internodes. Explants from mature internodes often showed increasing shoot regeneration toward the base of the explant. These polarities in regenerant development might be the consequence of auxin accumulation at the basal part of the explant due to polar transport (Smulders et al., 1988). Since auxin concentrations are the highest in the upper plant

Table IV. *Ploidy levels of vitrified and nonvitrified shoot regenerants from TCL explants*

Frequencies (%) of ploidy levels among vitrified and nonvitrified shoot regenerants from TCL explants of stem internodes of *N. tabacum* cv Samsun NN. Data obtained from regenerants of explants of successive internodes in young and old plants are summarized. *n*, Number of regenerants.

Appearance	Diploid	Mixoploid	Tetraploid	*n*
Vitrified				
Young	90	2	0	20
Old	61	28	11	18
Average	76	19	5	38
Nonvitrified				
Young	85	10	5	39
Old	88	11	1	72
Average	86	11	3	111

parts and low in the basal plant parts (Lozhnikova et al., 1989; Heylen et al., 1991), further accumulation of exogenously supplemented auxin to explants from these plant parts will exert relatively the greatest effects in the basal part of the explant.

3. During the phase of further development of the induced meristems, vitrified phenotypes occurred at high frequency from explants of young plant material (young plants as a whole and top internodes of old plants). This result indicates that the occurrence of vitrification must already have been determined by processes in the cells of the donor tissue. These processes apparently do not interfere with the acquisition of competence and the capacity of cells to initiate shoot regeneration. They denote malfunctioning of the newly formed apical meristem (Gaspar et al., 1991), probably because of the maintenance of mineral imbalances from the donor tissue (Lardet et al., 1994). The high reproducibility of the vitrification response makes the TCL explant system suitable for further fundamental research into this matter.

4. Loss of competence, probably at the acquisition level, was found in explants from the mature internodes. Smulders et al. (1990) showed in tobacco TCL explants from pedicels that preincubation during increasing time (up to 10 d) on auxin-free medium resulted in decreasing numbers (down to zero) of flower buds per explant. This effect could not be reversed any more by application of auxin. The reduced shoot regeneration frequencies in explants from the mature internodes as shown in the present paper may have been caused by an analogous process, i.e. (auxin) deprivation of the tissue in the plant (see also paragraph 2 above). The fact that the lowest regeneration frequencies were observed in explants from the oldest internodes, which are the most remote from the apex in space and time, supports this view. The existence of a relationship between the developmental stage of the tissue and its competence for regeneration has also been found among various monocot species, e.g. in leaves of the coconut (Karunaratne et al., 1991), tritordeum (Barcelo et al., 1991), and Napier grass (Rajasekaran et al., 1987). In the latter species, the developmental gradient and the related competence for embryogenesis were found to be associated with endogenous levels of the growth regulators IAA and ABA.

5. The occurrence during elongation and aging of the explant source of endoreduplication and the switch from the diploid to the tetraploid cell cycle without intervening mitosis was expected to have an effect on the ploidy level of the regenerants. In the epidermis and subepidermis, the switch from the 2C to the 4C DNA content mainly occurred at the end of the elongation stage and continued during the maturation stage. This switch did not parallel the rapid increase of the regeneration frequency in explants from internodes of increasing stages of elongation. Most of the 4C cells in the mature tissue must have been in the G2 phase of the diploid cell cycle, resulting in normal diploid regenerants. The limited increase (from 12 to only 25%, on the average) of the frequency of nondiploid regenerants but at a decreased regeneration frequency in the explants from the mature internodes, as compared with elongating internodes, may be the result of two concomitantly operating but autonomous processes, i.e. gradual loss of competence of all cells (see paragraph 4 above) on the one hand and the switch from the G2 phase to the G1 phase of the tetraploid cell cycle in a limited number of the cells on the other. According to these results, the competence for shoot regeneration appeared as a phenomenon independent of the cellular ploidy level and cell cycle phase. In a study of the relationship between leaf age and embryogenic competence in Napier grass, Taylor and Vasil (1987) came to a similar conclusion: loss of competence was not caused by changes in the cell cycle or in the DNA content.

Competence for root regeneration had to be acquired in cortex-derived callus cells, since the default state of the cortex cells refers to acquired competence for cell division, which during culture of the explants resulted in callus formation (Creemers-Molenaar et al., 1994; Gilissen et al., 1994). Because no chimeric root regenerants were found by flow cytometric analysis, roots are suggested to originate from single initial callus cells. In elongating tissue, cortex cells with the 4C DNA content were especially prone to undergo cell division immediately and thus to maintain the diploid level in the resulting callus and roots (Gilissen et al., 1994). The frequent occurrence (up to 50%) of tetraploid roots from explants of mature internodes (at approximately equal total numbers of root regenerants as compared with explants from elongating internodes) indicates normal competence for root regeneration at the tetraploid level. The sudden appearance of tetraploid roots characterizes the location in the plant where a majority of the cortex cells underwent the switch from the diploid to the tetraploid cell cycle, i.e. at the transition from the elongation stage to the mature stage. In the explants, the absence of octoploid root regenerants can presumably be ascribed to a relatively low frequency of 8C cells in the G1 phase of the octoploid cell cycle.

Polysomaty in tobacco has thus far been described only roughly for pith tissue (Murashige and Nakano, 1967; Brossard, 1975) and leaf tissue (Galbraith et al., 1983). In the tobacco TCL explants, the present results on polysomaty revealed an almost linear relationship between internodal elongation (up to a length of 20 mm) and the overall transition from the 2C to the 4C DNA content. In addition, cells with the 8C and 16C DNA content (the latter only in the cortex) were found mainly in explants from mature internodes. This indicates that endoreduplication is regulated at two different levels: first, it occurred above a critical length (volume) of the cells, and second, it was found to be related to aging. Similar types of regulation were found in Arabidopsis (Melaragno et al., 1993) and in tomato (Smulders et al., 1994).

In summary, we studied the development of internodal cells in relation to the changes in cell size, DNA content, and physiological age from its reflection in internodal explants in the regeneration frequency, the ploidy level of the regenerants, and the occurrence of vitrification. The regeneration frequency of shoots elucidated the physiological changes related to cell expansion and distance from the apex. The occurrence of vitrified regenerants revealed the changes of the physiological age of the tissue during plant development, and the ploidy level of shoot and root regen-

erants enabled us to localize the transition from diploidy to tetraploidy in the various cell types. In the explants, plant age and cell expansion, but not the related changes in the DNA content, appeared to determine the competence for regeneration of shoots and roots.

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