Pergamon

PII: S0031-9422(97)00171-4

Phytochemistry, Vol. 45, No. 7, pp. 1337–1341, 1997

0031-9422/97 $17.00+0.00

PROPERTIES OF PLASMA MEMBRANES OF P*hsp* 70-*ipt* TRANSFORMED TOBACCO (*NICOTIANA TABACUM*)

LIEVE BULTYNCK, JAN M. C. GEUNS,*† GIJS VAN GINKEL‡ and ROLAND J. CAUBERGS

Ruca, Groenenborgerlaan 171, 2020 Antwerp, Belgium; †KULeuven, Laboratory of Plant Physiology, Kard Mercierlaan 92, 3001 Heverlee, Belgium; ‡Utrecht University, Department of Molecular Biophysics, Princetonplein 5, PO Box 80000, 3508 TA Utrecht, Netherlands

(*Received* 5 *December* 1996)

Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; plasma membrane; fluidity; sterols; fatty acids; *ipt*; cytokinin.

Abstract—Application of 10 successive daily heat shocks reduced the growth of control tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) plants by about 15%; for P*hsp* 70-*ipt* transformed plants this is about 48%. The shoot diameter of these *ipt*-transformed plants increased by about 75%. In addition, in heat shock treated *ipt*-plants (IPT-HS) the upper lateral buds grew out due to a reduction of apical dominance. The older leaves of IPT-HS plants had a higher chlorophyll content. In spite of the observed effects due to a higher endogenous cytokinin content in the IPT-HS plants, no significant changes were observed on the plasma membrane fatty acid composition, nor on its fluidity as determined from the steady-state fluorescence anisotropy of DPH. Only a minor change in the plasma membrane free sterol composition was found as evidenced by a 20% decrease in the stigmasterol to sitosterol ratio in IPT-HS, indicative for a possible anti-senescence effect of enhanced endogenous cytokinins, but without significant effects on the plasma membrane function.

INTRODUCTION

Cytokinins are involved in a variety of plant growth and developmental processes. Natural or synthetic cytokinin effects were mostly studied by external application to plants or parts. Cytokinins affect the release of auxillary buds from dormancy [1], retard leaf senescence [2], and stimulate the development of chloroplasts [3]. Root initiation and growth is inhibited at a relative high concentration of cytokinins [4].

A promising approach to study the role of cytokinins in whole plants during their development is the use of stable genetically transformed plants [5]. To this end the T-DNA *ipt* gene of *Agrobacterium tumefaciens* has been inserted into the plant genome under control of its own promoter or other plant promotors. This gene encodes an isopentenyl transferase that catalyses the conversion of 5′-AMP and isopentenyl pyrophosphate into isopentenyl-adenosine-5′-monophosphate [6]. According to many authors this is the first rate limiting step in the biosynthesis of cytokinins.

Constitutive overexpression of the *ipt* gene from its own promotor results in several abnormalities in transgenic plants, e.g. a complete inhibition of root formation. Therefore, many investigators have tried to solve this problem using regulatable promoters to control the expression of the *ipt* gene. The gene was fused with a heat regulated promotor [7], while some authors preferably used a light induced promotor [8]. A correlation between endogenous cytokinin levels and developmental phenomena in transgenic plants is demonstrated by several investigators [7–11].

Although a correlation between an endogenous change in cytokinin content and a physiological response was observed, the mode of action of cytokinins remains obscure [12]. Cytokinins are known to delay senescence [13]. In plant leaves senescence manifests itself as a clearly observable phenomenon; the amount of chlorophyll, DNA, RNA and proteins decreases whereas the amount of hydrolytic enzymes and growth inhibitors increases. The cellular membranes are also affected by the ageing process: the structure of the lipid bilayer changes and membrane permeability increases. These effects are brought about by different reactions, e.g. the oxidation of membrane lipids by oxygen radicals and changes in the

* Author to whom correspondence should be addressed.

Abbreviations: *ipt*: isopentenyltransferase; ODS: octadecyl silica; RiTL-DNA: root inducing left hand transfer DNA, DPH: diphenyl-1,3,5-hexatriene, HS: heat shock, BHT: butylated hydroxytoluene.

phospholipid to sterol ratio. The hypocotyl tissue of Mung bean seedlings shows for example an increase in stigmasterol to sitosterol ratio upon ageing [14]. Ageing of the hypocotyl segment membranes is at the same time accompanied by a change in the physical properties of these membranes as observed with fluorescence depolarization techniques. It was suggested [14] that membrane deterioration in etiolated Mung bean seedlings due to ageing may be caused by two sequential events: an early decline of the membrane phospholipid content, which causes a relative increase in the sterol to phospholipid ratio resulting in a change in the physical properties of the membrane. In a more advanced stage of ageing, the sterol content declines which brings about a decrease in sterol to phospholipid ratio and this in turn leads to a progressive leakiness of the membranes and finally a loss of membrane function.

Cytokinins delay plant senescence by an up to now unidentified mechanism. There are indications that the cytokinins have an effect on the rate of synthesis of unsaturated membrane lipids as well as on the rate of scavenging of oxygen radicals. The amount of experimental data with regard to the action mechanism of the senescence delay by cytokinins is very limited and investigation of this mechanism is not straightforward. However, the availability of IPT transformed plants gives a good opportunity for a study of the mode of action of cytokinins on senescence delay since these transformed plants have a strongly increased cytokinin production [15]. In the present study we have investigated leaves of different ages of normal plants compared with those of IPT transformed plants. Since senescence has profound effects on plant membranes we focused our investigation on plasma membranes from leaves of different physiological age of control and *ipt*-transformed tobacco plants. In these membranes we have investigated the sterol and fatty acid content. Moreover, we have employed fluorescence depolarization measurements on 1,6-diphenyl-1,3,5-hexatriene (DPH), a very frequently used rod-shaped apolar fluorescent membrane probe, embedded in the plasmamembranes. These measurements yield information on the physical properties of the investigated membranes in the form of structural parameters (order parameters of the fluorescent probes) and dynamic parameters (rotational diffusion coefficients of the membrane probes).

RESULTS AND DISCUSSION

Growth characteristics and pigment content

The HS itself caused a slight decrease in shoot length (Table 1). After 10 HS the SR1-HS plants were about 14% shorter than the SR1-C plants. In IPT plants about 48% reduction of shoot length was observed in IPT-HS as compared to the corresponding IPT-C. The reduction of shoot length in IPT-HS plants was due to the inhibition of the uppermost internodes, that had a larger diameter instead (about 11 mm against 6.3 mm). In the IPT-HS plants apical dominance was reduced and the upper lateral buds developed into side-shoots. No effects were observed on root development. These results are in agreement with previous work [8, 10] except for the lack of visible effect on root development.

The lower leaves of SR1-C, SR1-HS and IPT-C became yellow, whereas those of IPT-HS plants still contained more pigments at the end of the growth period (Table 1). Comparing the total chlorophyll content in the three younger and in the three older leaves of HS treated plants it was obvious that mainly in the three older leaves of IPT-HS plants the pigment content remained much higher than in the corresponding control leaves. Senescence in these older IPT-HS leaves is much delayed due to a higher cytokinin content. The cytokinin content in different levels of the same progeny of SR1 and of IPT plants was analysed [15]. The sum of all the zeatin compounds decreased sharply in the SR1 plants from 462 ng g^{-1} fresh weight in the upper leaf to about 13 ng g^{-} fresh weight in the lower leaf. However, in IPT plants the total sum decreased from 775 in the upper to about 40 ng g^{-1} fresh weight in the middle leaves, and increased again to 345 ng g^{-1} fresh weight in the lower leaves.

Anisotropy measurements

We have measured the steady-state fluorescence anisotropy of DPH in plasma membrane vesicles

Table 1. Growth (in cm) of SR1 and IPT-transformed plants with (-HS) of without (-C) 10 heat shocks ($n = 6$); chlorophyll content (in $\mu g\ g^{-1}$ fr. wt.) in the three youngest (y) or the 3 oldest (o) leaves of the SR1 and IPT-transformed plants after 10 heat shock treatments (1 hr daily at 40°); anisotropy (r_{ss}) measured at 18° of plasma membranes prepared from the three youngest or three oldest leaves of these plants

Treatment 1	Length	Treatment 2	Pigment			Anisotropy	
			Series 1	Series 2	Series 3	Series 1	Series 2
SR1-C	89.4±4.2	SR1-HS y	474±22	893±33	1022±190	0.217±0.007	0.197±0.001
SR1-HS	77.6±6.8	IPT-HS y	496±60	551±19	1044±91	0.232±0.029	0.213±0.016
IPT-C	96.5±5.9	SR1-HS o	198±19	314±10	502±18	0.229±0.050	0.198±0.004
IPT-HS	50.6±8.2	IPT-HS o	441±24	493±28	767±33	0.201±0.001	0.200±0.011

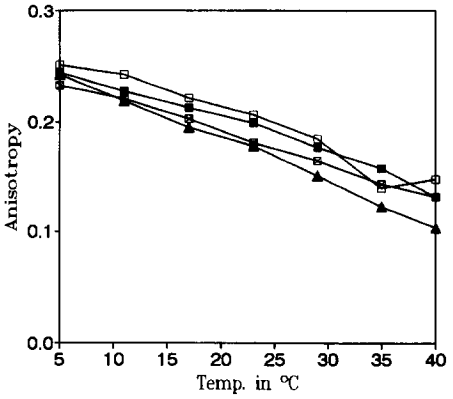

Fig. 1. Anisotropy in function of temperature. (■) SR1-C (control without heat shocks), (▲) SR1-HS (control with heat shocks), (□) IPT-C (IPT plants without heat shocks), (⊠) IPT-HS (IPT plants with heat shocks). The s.e. is not given for clarity, but is similar to that of Table 1.

made of leaves from the SR1-C, SR1-HS, IPT-C and IPT-HS plants of different physiological age, in a temperature range of 6–40° with temperature intervals of 6°. The temperature dependence of the fluorescence anisotropy of DPH in plasmamembrane vesicles of the SR1-C, SR1-HS, IPT-C and IPT-HS plants of a first harvest, is illustrated in Fig. 1. As can be expected the anisotropy parameter values gradually decrease with increasing temperature, since at higher temperatures the lipid chains become much more disordered and the dynamics increase. We have repeated these experiments with young and old leaves from two additional plant series, now at a temperature of 18°. The results are grouped in Table 1. No significant differences in fluorescence anisotropy of DPH are found in the different plasma membranes of young and old control tobacco plants and IPT transformed plants. This indicates that enhanced cytokinin levels in IPT transformed plants have no significant physical effects on plasma membrane fluidity. Moreover, the fluorescence anisotropy of DPH in the plasma membranes of young and old leaves does not change within experimental error.

Sterols and bound fatty acid analysis

The fatty acid fraction of the plasma membranes consisted of palmitic acid (22.5%), stearic acid (11%), oleic acid (17.1%), linoleic acid (19.7%) and linolenic acid (29.5%). No significant changes in fatty acid composition were found in any of the different treatments, nor were differences found in the α-tocopherol content of the membranes (results not shown).

The free sterol composition of plasma membranes from leaves of SR1 and *ipt*-transformed plants is given in Table 2. The cholesterol content in isolated plasma membranes is very high (around 17% in SR1-C) as compared to the sterol content of whole leaf extracts in which its part is only between 6 and 10% [16]. The different treatments have only minor effects on the sterol composition. The stigmasterol to sitosterol ratio decreases by about 20% in IPT-HS plants, which may be an indication of a (small) delay of membrane deterioration. The change in this stigmasterol to sitosterol ratio in function of tissue age in tobacco has also been observed by [16–18]. Enhanced cytokinin levels in the leaves of *ipt* transformed tobacco (Pssu-promotor) delayed the increase of the stigmasterol to sitosterol ratio [16]. Free sterols are membrane components and the sterol composition is very important for the maintenance structure, function and integrity. An enhanced stigmasterol to sitosterol ratio enhances membrane leakage and reflects membrane senescence. Changes in sterol and fatty acid composition of membranes do influence membrane permeability and enzyme activity (e.g. ATPase, whose activity is influenced by lipid environment, [19–21]).

Our results reveal that enhanced cytokinin levels have nonsignificant influence on membrane fatty acids or membrane fluidity and only a minor influence on plasma membrane sterols. This might be an indication that the plasma membrane is not the primary site of cytokinin action or that the endogenous cytokinin levels are sufficient to maintain the plasma membrane integrity. The difference in pigment content is not a good indication for the senescence or deterioration of all the cell membranes and certainly not the plasma membrane, that seems to withstand senescence at the furthest.

EXPERIMENTAL

Plant material. Seeds of tobacco plants (*Nicotiana tabacum* L. cv. Petit Havana SR1) transformed with the P*hsp*70-*ipt* gene construction were obtained from Schmülling *et al.* (1989). This chimeric gene consisted

Table 2. Percentual sterol composition of plasma membranes prepared from leaves of SR1 and IPT-transformed plants with (HS) or without (C) 10 heat shocks

Sterol	SR1-C	SR1-HS	IPT-C	IPT-HS
Cholesterol	16.7±2.03	19.7±2.47	19.2±3.58	19.7±3.8
Campesterol	14.9±1.27	17.6±3.43	14.0±1.74	13.4±2.17
Stigmasterol	43.5±1.02	47.7±5.2	42.7±1.08	40.2±1.77
Sitosterol	23.3±1.32	26.3±4.39	21.8±1.68	24.8±2.36
28-Isofucosterol	2.0±0.8	2.8±0.8	2.3±0.32	2.7±1.15
Stigmasterol:sitosterol	1.86	1.81	1.96	1.62

of the gene 4 (*ipt*) from *Agrobacterium tumefaciens* coupled to the heat-shock promotor of the *hsp*70-gene of *Drosophila melanogaster*. This promotor is heat inducible (e.g. 1 hr at 40°).

Seeds were germinated in peat and after about 22 days transferred to larger polyethylene pots containing 1 plant per pot. Plants were grown in a growth room at 25°, 80% RH and under a 12 hr light/12 hr dark cycle (10 W m^{-2}). Fifty-five-day-old plants were given daily heat shocks (40°) during 1 hr per day and under illumination of 20 W m^{-2}, each day starting 4 hr after the beginning of the daily light period. Up to 10 consecutive daily heat shocks were applied. After the heat shocks, the plants were grown for a further 5 to 10 days. In each experiment 4 series of plants were used: SR1-C (controls without heat treatment), SR1-HS (control plants with HS), IPT-C (*ipt* transformed plants without HS) and IPT-HS (*ipt* transformed plants with HS).

Preparation of plasma membranes. Plant leaves were collected on ice. Generally 100–150 g tissue fr. wt. were homogenized in 250 ml of ice-cold homogenization buffer. The plasma membranes were prepd and their purity confirmed as described [22].

Protein and pigment content. Protein concs were determined according to ref. [23] and pigments to ref. [24].

Fluorescent labelling of the membranes. Plasma-membrane frs of the SR1-C, SR1-HS, IPT-C and IPT-HS plants were diluted ×15 with Hepesbuffer. The appropriate amount of an ethanolic DPH soln was added to these membranes to yield a final lipid to probe ratio 250:1. The membrane were kept in the dark for 2 hr to allow a good incorporation of DPH. For all the samples, steady state fluorescence depolarization was measured over a temp. range of 5–40°, and was expressed as the fluorescence anisotropy, r_{ss}, defined as : $r_{ss} = I_{vv} - I_{vh}/(I_{vv} - 2I_{vh})$ where I stands for light intensity and v and h for horizontally and vertically polarized light, respectively.

Steady-state fluorescence depolarization measurements were performed using an SLM Aminco SPF 500C spectrofluorimeter equipped with a set of polarization filters. Excitation was set at 350 nm and emission was selected at 430 nm. The temp. was controlled by a thermostat (±1°). I_{vh} and I_{vv} were determined in the excitation wavelength range 280–400 nm at a fixed emission wavelength of 430 nm. The measurements were repeated ×5. The anisotropy is calcd from these 5 values at 350 nm. The technique has been described by ref. [25].

Sterol analysis. Membrane frs (2 to 3 mg of proteins) were extracted in glass test tubes with 4 × 2 ml Me_2Co containing 20 μg 5α-cholestanol as int. standard. The free sterol fr. was prepd and analysed essentially as described by ref. [16].

Fatty acid analysis. Base-catalysed transmethylation of the fatty acids bound to phospholipids (and glycerides) was done in 5% methanolic KOH, and extraction and analysis were essentially as described by ref. [26].

Chemicals. DPH (1,6-diphenyl-1,3,5-hexatriene) was purchased from Molecular Probes (Eugene, Oregon, U.S.A.). Stock sols of 5×10^{-4} M in pure EtOH were stored under N_2 in the dark at 4°. All other chemicals were PA or HPLC grade.

Acknowledgements—The authors acknowledge the skilfull technical support by Hilde Verlinden, Colette Wellens, Hilde Van Hemelrijk and Jan Neefs. The authors also acknowledge the financial support by the Belgian F.K.F.O. and N.F.W.O. (grant 2.0049.93)

REFERENCES

1. Cline, M. J., *Physiologia Plantarum*, 1994, **90**, 230.
2. Van Staden, J., Cook, E. L. and Noodin, L. D., In *Senescence and Ageing in Plants*, ed. L. D. Noodin and A. C. Leopold. Academic Press, San Diego, 1988, p. 281.
3. Chory, J., Reinecke, D., Sim, S., Washburn, T. and Brenner, M., *Plant Physiology*, 1994, **104**, 339.
4. Wightman, F., Scheider, E. and Thimann, K. V., *Physiologia Plantarum*, 1980, **49**, 304.
5. Klee, H. and Estelle, M., *Annual Review of Plant Physiology and Plant Molecular Biology*, 1991, **42**, 529.
6. Akiyoshi, D. E., Klee, H. Amasino, R. M., Nesder, F. W. and Gordon, M. P., *Proceedings of the National Academy of Science of the U.S.A.*, 1984, **82**, 5994.
7. Schmülling, T., Beinsberger, S., De Greef, J., Schell, J., Van Onckelen, H. and Spena, A., *FEBS Letters*, 1989, **249**, 401.
8. Beinsberger, S. E. I., Valcke, R. L. M., Deblaere, R. Y., Clijsters, H. M. M., De Greef, J. A. and Van Onckelen, H., *Plant Cell Physiology*, 1991, **32**, 489.
9. Smigocki, A. C., *Plant Molecular Biology*, 1991, **16**, 105.
10. Van Loven, K., Beinsberger, S. E. I., Valcke, R. L. M., Van Onckelen, H. A. and Clijsters, H. M. M., *Journal of Experimental Botany*, 1993, **44**, 1671.
11. Li, Y., Hagen, G. and Guilfoyle, T. J., *Developmental Biology*, 1992, **153**, 386.
12. Binns, A. N., *Annual Review of Plant Physiology and Plant Molecular Biology*, 1994, **45**, 173.
13. Richmond, A. E. and Lang, A., *Science*, 1957, **125**, 650.
14. Stallaert, V. and Geuns, J. M. C., *Phytochemistry*, 1994, **36**, 1177.
15. Kudoyarova, G. R., Veselov, S. Y., Van Onckelen, H. and Valcke, R., *Russian Journal of Plant Physiology—English Translation*, 1995, **42**, 617.
16. Geuns, J. M. C., Van Loenhout, H. E. M., Valcke, R. L. M., Van Loven, K., Redig, P., Veselov, S.

Y., Kudoyarova, G. R., Van Onckelen, H. A. and Vendrig, J. C., *Phytochemistry*, 1997, **44**, 797.
17. Davis, D. L., *Phytochemistry*, 1972, **11**, 489.
18. Grunwald, C., *Phytochemistry*, 1975, **14**, 79.
19. Ros, R., Cooke, D. T., Burden, R. S. and James, C. S., *Journal of Experimental Botany*, 1990, **41**, 457.
20. Ros, R., Cooke, D. T., Martinez-Cortina, C. and Picazo, I., *Journal of Experimental Botany*, 1992, **43**, 1475.
21. Stallaert, V., Plasma membrane biochemistry and whole plant physiology of etiolated mung bean seedlings as affected by ageing and different water regimes. Ph.D. thesis, KULeuven, 3000 Leuven, Belgium, 1993, p. 184.
22. Asard, H., Venken, M., Caubergs, R. J., Reijnders, W., Oltmann, F. L. and De Greef, J. A., *Plant Physiology*, 1989, **90**, 1077.
23. Markwell, M. A. K., Haas, S. M., Bieber, L. L. and Tolbert, N. E., *Analytical Biochemistry*, 1978, **87**, 206.
24. Inskeep, W. P. and Bloom, P. R., *Plant Physiology*, 1985, **94**, 1233.
25. Levine, Y. K. and Van Ginkel, G., in *The Molecular Dynamics of Liquid Crystals*, ed. G. R. Luckhurst and C.A. Veracini. Kluwer, Netherlands, 1994, p. 537.
26. Krebsky, E. O., Geuns, J. M. C. and De Proft, M., *Phytochemistry*, 1996, **43**, 747.