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Transglutaminase, an enzyme involved in flower senescence and developmental cell death

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Abstract — To acquire further knowledge on flower senescence and developmental cell death (DCD), we examined protein-protein and protein-polyamine covalent interactions mediated by transglutaminase (TGase) in the tobacco corolla model. In tobacco, corolla senescence proceeds acropetally and is characterised by the formation of a basal abscission zone (AZ) and by changes in the orientation of corolla teeth. TGase, present in various cell compartments, was analysed by using the *Arabidopsis thaliana* TGase antibody. A 58-kDa form was immunorecognised in microsomal, plastidial (together with a 38-kDa band) and cell wall fraction; a 52-kDa protein was found only in the soluble fraction. The activity reached the maximum at the "no-return point" of senescence corresponding to AZ formation. TGase enzyme activity was also detected in microsome, soluble, plastid, and cell wall fractions. Our results suggest that TGase may be released in the cell wall through the Golgi vesicles. In particular, the cell wall TGase activity could be involved in cell wall strengthening, being especially active where the corolla teeth curl, and during the formation of the AZ.

Key words: Cell death, Flower, Polyamines, Senescence, Transglutaminase.

INTRODUCTION

Some features of cell death (CD) are different in animals and plants, in particular plant CD is characterised by the presence of specific cell compartments and by the absence of phagocytosis (Greenberg 1996; Pennel and Lamb 1997).

Plant CD is evidenced by nuclear and membrane blebbing and, in some cases, DNA fragmentation and caspase-like activity (Serafini-Fracassini *et al.* 2002; Kusaka *et al.* 2004). The role of mitochondria could be similar in both kingdoms (Desagher and Martinou 2000). In plants, other organelles, such as chloroplasts, vacuoles, and cell walls, play a pivotal role in the induction of CD (Rubinstein 2000).

In animals, a relevant factor in CD is the post-translational modification catalysed by TGases. The activity produces cross-links between proteins involving interactions between glutamyl residues and amine donors, such as lysyl residues or PAs. TGases cross-link specific proteins, including those of the extracellular matrix or cytoskeleton, which are involved in growth and differentiation processes (Folk *et al.* 1980; Ichinose *et al.* 1990). Hence, in several animal models, the presence of TGase antigen and activity are considered markers of CD (Melino and Piacentini 1998; Griffin and Verderio 2000; Nicholas *et al.* 2003).

In our previous work we analysed several morpho-functional parameters which characterise the onset of tobacco corolla senescence and cell death (Serafini-Fracassini *et al.* 2002). In this study, TGase antigen and activity in the whole cell and in four subcellular compartments (microsomes, soluble fraction, plastids and cell walls) were evaluated during the corolla's life-span. The possible role of corolla TGases in DCD as related to wall modifications is discussed.

MATERIAL AND METHODS

Plant system - Plants of *Nicotiana tabacum* L. cv Samsun were grown in a growth chamber at a temperature of 25°C, and a light intensity of 10^{15} quanta cm^{-2} s^{-1} with a photoperiod of 12/12 h light/dark.

Protein extraction - Corollas were homogenised in 1:3 (w/v) 50 mM Tris-HCl pH 7.4 containing 1

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mM DTT, 10 μg ml^{-1} pepstatin, 0.5 μg ml^{-1} leupeptin, 1 mM PMSF and 0.1% Triton X-100, and then centrifuged at 9700 x *g* for 10 min; the supernatants was collected and further analysed. Protein amount was determined by the method of Lowry *et al*. (1951).

Preparation of enriched microsomal, soluble, plastidial and cell wall protein fractions - Corollas were homogenised at 4°C in 1:5 (w/v) 20 mM Hepes KOH pH 7.7 extraction buffer containing 4 mM $MgCl_2$, 330 mM sorbitol, 1 mM DTT, and protease inhibitors as above indicated, filtered through two layers of Miracloth and centrifuged at 100 x g for 1 min.

1) Enriched cell wall fraction - The 100 x *g* pellet was resuspended in 1:5 (w/v) 10 mM Hepes KOH pH 7.5 resuspension buffer containing 1% (v/v) Triton X-100, 500 mM KCl, 1 mM DTT, and protease inhibitors, then homogenised with a potter and incubated for 10 min at 4°C. The samples, after three sonications for 5 sec, were centrifuged at 1000 x *g* for 5 min. After resuspension in 500 μl of resuspension buffer, the pellets where sonicated twice, and centrifuged as described above. The final 1000 x *g* pellet was resuspended in 100 μl of resuspension buffer.

2) Enriched plastidial fraction - The 100 x *g* supernatants were treated as described in Della Mea *et al.* (2004b). The final pellets were resuspended in 200 μl of resuspension buffer.

3) Enriched microsomal and soluble fraction - The 1700 x *g* supernatants were recovered, and microsomal and soluble proteins were separated as described by Della Mea *et al.* (2004a).

Western-blot analyses - One hundred μg of extracted proteins were boiled in SDS loading buffer, and SDS-PAGE separated using a Bio-Rad Mini-protean III apparatus. The gel was blotted onto a nitrocellulose membrane (Amersham). Incubation of membranes with anti-AtPng1p antibody was performed as described in Della Mea *et al*. (2004a). Band densities and their molecular weights were calculated with a FLA3000 Laser System (Fuji) and with the Total Lab (Raytest) software.

Radiometric assay - Fifty μg of total corolla proteins, and of enriched microsomal, soluble, plastidial or cell wall protein fractions were assayed using 0.25 μCi (2.2 nmol) of [^{14}C] spermine (Amersham), as described in Del Duca *et al*. (2000).

RESULTS

Tobacco corolla developmental stages - The following stages were identified: stages 1 to 4, early development; stage 5, maximum corolla development; stage 6, the "no-return point" with the formation of the abscission zone; stage 7, senescence; stage 8, late senescence when the corolla is dying; stage 9 and 10, death (corolla teeth are shown in Fig. 1; see also Serafini Fracassini et al., 2002).

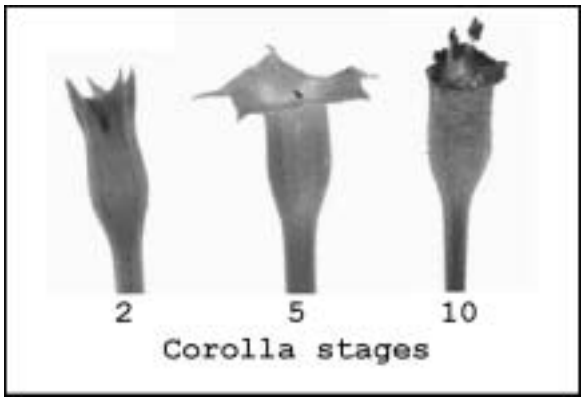

Fig. 1 — **Tobacco flower corolla teeth at different developmental stages.** Stages 2: early flower development; the teeth are closed and parallel to the corolla axis. Stage 5: maximum corolla opening; the distal part of the corolla has an intense pink colour and the teeth are orthogonal to the corolla axis. Stage 10: death, the corolla is dry, brown, papyraceous and teeth are curled inwards.

Immunodetection of TGase - The immunodetection of TGases was obtained using the antibody against *A. thaliana* TGase (Della Mea *et al*. 2004a). The antibody reacted mainly with a 58-kDa protein, and to a lesser extent with 38- and 61-kDa proteins (Fig. 2). The main 58-kDa protein was detected at all stages with a decreasing trend with senescence progression (data not shown). In the microsomal fraction, the anti TGase antibody reacted with the 58-kDa band and also with high-molecular-mass proteins. In the plastid and in the cell wall fractions, the main immunoreactants were the 58-kDa and 61-kDa proteins; a faint 38-kDa protein was present in plastids. In the soluble fraction a unique 52-kDa band was recognised (Fig. 2).

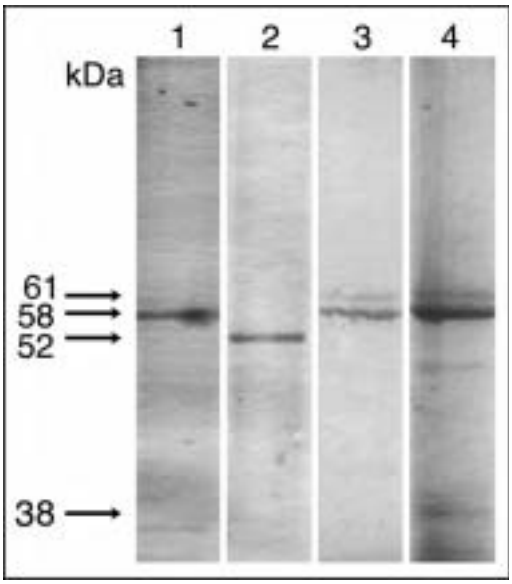

Fig. 2 — **Transglutaminase immunodetection**. Proteins extracted from enriched subcellular fraction of corolla cells at developing stage 5: 1) microsomal, 2) soluble, 3) plastid and 4) cell wall, were analysed using the *A. thaliana* antibody.

TGase activity - The activity of TGase was analysed during the developmental stages of the corolla both in whole cell and in subcellular compartments. The TGase activity of the whole corolla, which is low in the early stages, was found to rapidly increase after stage 4, to double at stage 6, and to decrease at stages 7/8 (Tab. 1).

Microsomal enriched fraction - TGase activity was very low at stages 2-4, with a maximum five-fold increase at stages 5/6, which was followed by a decrease. *Soluble enriched fraction* - the activity was close to zero at stages 2-5; it increased from stage 6 onwards. *Plastid enriched fraction* - the activity increased at stage 4 and remained constant in the following stages, with an increase at stage 7/8. *Cell Wall enriched fraction* - the activity was negligible at stages 2-4, and then increased (five-fold), with two maxima at stages 5 and 7/8 (Tab. 1).

DISCUSSION

Identification of corolla transglutaminases - The anti-TGase antibody recognises some bands, amongst which a main 58-kDa protein with a decreasing trend during the corolla life-span. A protein of this mass has been detected in chloroplasts of many plants, thus it seems to be the most widespread form of enzyme in leaves (DEL DUCA and SERAFINI-FRACASSINI 2005).

Transglutaminase activity - The maximum activity occurred before DNA laddering and protease activation suggesting an active involvement of the enzyme in senescence progression.

Transglutaminase subcellular location - The 58-kDa band is the most represented form, being detected in the plastid, microsomal and cell wall fractions, all fractions where the enzyme is active. The 52-kDa band, which is expressed only in the soluble fraction, might derive from the 58-kDa enzyme by post-translational modifications. The soluble activity is detectable only from stage 6, when cell membranes begin to be degraded (SERAFINI-FRACASSINI *et al.* 2002). As the active 58-kDa TGase form is present also in cell walls, a transport mechanism via microsomal vesicles is possible. We propose that cell wall TGase might have a role in strengthening the corolla cell walls by structural protein cross-linking during the dramatic modifications occurring in the senescent corolla (Fig. 1). TGase might be also involved in the peculiar cell wall modifications of cells located in the abscission zone, e.g. to prevent the release of toxic substances from the scar. The presence of a TGase activity in the cell walls has, to date, been reported only in lower organisms, such as algae (WAFFENSCHMIDT *et al.*, 1999) and fungi (reviewed by DEL DUCA and SERAFINI-FRACASSINI,2005), but never in higher plants.

Table 1 — **Transglutaminase activity** in total extract and in enriched subcellular fraction during corolla life (from stage 1/2 to 10). Values and standard errors are expressed in [^{14}C]Spm nmol/h/mg protein. nd: not detected.

Stage	Total	Microsomal	Soluble	Plastid	Wall
1	0,157 ± 0,001	nd	nd	nd	nd
2	0,123 ± 0,005	0,062 ± 0,006	0,031 ± 0,005	0, 213 ± 0,007	0,070 ± 0,006
3	0,120 ± 0,006	nd	nd	nd	nd
4	0,131 ± 0,013	0,084 ± 0,017	0,040 ± 0,005	0,355 ± 0,007	0,069 ± 0,007
5	0,195 ± 0,001	0,424 ± 0,033	0,010 ± 0,002	0,368 ± 0,013	0,148 ± 0,019
6	0,247 ± 0,017	0,402 ± 0,067	0,127 ± 0,054	0,368 ± 0,017	0,131 ± 0,024
7/8	0,205 ± 0,013	0,110 ± 0,015	0,148 ± 0,067	0,443 ± 0,022	0,266 ± 0,021

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