Effect of Climate Conditions and Plant Developmental Stage on the Stability of Antibodies Expressed in Transgenic Tobacco

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Plants are regarded as a promising system for the production of heterologous proteins. However, little is known about the influence of plant physiology and plant development on the yield and quality of the heterologous proteins produced in plants. To investigate this, tobacco (*Nicotiana tabacum* cv Samsun NN) was transformed with a single construct that contained behind constitutive promotors the light- and heavy-chain genes of a mouse antibody. The in planta stability of the antibody was analyzed in transgenic plants that were grown under high and low irradiation at 15°C and 25°C. High-light conditions favored the production of biomass, of total soluble protein, and of antibody. The plants grown at 25°C developed faster and contained less antibody per amount of leaf tissue than the plants grown at 15°C. Both endogenous protein and antibody content showed a strong decline during leaf development. The heavy chains of the antibody underwent in planta degradation via relatively stable fragments. In vitro incubations of purified plantibody with leaf extracts of wild-type tobacco indicated the involvement of acidic proteases. It is interesting that the same antibody produced by mouse hybridoma cells exhibited higher stability in this in vitro assay. This may be explained by the assumption that the plant type of *N*-glycosylation contributes less to the stability of the antibody than the mouse-type of *N*-glycosylation. The results of this study indicate that proteolytic degradation during plant development can be an important factor affecting yield and homogeneity of heterologous protein produced by transgenic plants.

Crop plants are considered as a potential system for the production of antibodies in bulk amounts at relatively low costs. Since the initial demonstration that transgenic tobacco (*Nicotiana tabacum* cv Samsun NN) is able to produce functional IgG1 from mouse (Hiatt et al., 1989), full-length antibodies, hybrid antibodies, and antibody fragments like Fab and single-chain variable fragments have been expressed in higher plants for a number of purposes. The produced antibodies can serve in health care and medicinal applications, either directly by using the plant as food ingredient or as pharmaceutical or diagnostic reagent after purification from the plant material. In addition, antibodies may improve plant performance, e.g. by controlling plant disease or by modifying regulatory and metabolic pathways (for reviews, see Conrad and Fiedler, 1994; Ma and Hein, 1995; Smith, 1996; Whitelam and Cockburn, 1996).

IgG consists of two identical "heavy" (H) and two identical "light" (L) chains, which are folded in discrete domains that are stabilized by intermolecular disulfide bonds. The four chains are covalently linked by intramolecular disulfide bonds. It has been shown that for a proper assembly of the antibodies in plant cells it is essential that the proteins are targeted to the endoplasmic reticulum (ER), as in mammalian systems (Hein et al., 1991). This requires the presence of a signal sequence fused to the genes encoding the mature H and L chains. The origin of the required signal sequence is not critical, since sequences from plant, mouse, and yeast have been successfully used (Ma and Hein, 1995). Proteins that are cotranslationally inserted into the ER are folded in a specific conformation before they can undergo further downstream transport, glycosylation, and processing (Pagny et al., 1999). In general, IgG1 contains one, highly conserved glycosylation site in the Fc region. Mouse IgG1 produced by transgenic tobacco has been reported to be *N*-glycosylated with plant-specific glycan structures (Cabanes-Macheteau et al., 1999). The glycans attached to antibodies may play a role in structure stability, protection against proteolytic degradation, and recognition by receptors (Dwek, 1996; O'Connor and Imperiali, 1996). The secretory system in principle releases the proteins into the extracellular space, the cell membrane, the vacuole, or the tonoplast (Pagny et al., 1999). It has been experimentally confirmed that in plants the antibodies are excreted into the apoplastic space (Hein et al., 1991; van Engelen et al., 1994; De Wilde et al., 1998).

When plants are commercially used as heterologous system for large-scale production of functional

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antibodies high yields can be crucial. Yield is the net-result of synthesis and breakdown. So far, research has mainly been focused on obtaining balanced synthesis and proper assembly of the individual subunits, the latter being important for both functionality and stability of the antibody. In general, relatively low yields of far below 1% are obtained. Little attention has been paid to proteolytic degradation in planta of the antibodies synthesized. The finding that along with the expression of full-length antibodies considerable amounts of Fab-like (De Neve et al., 1993) and F(ab′)2-like (van Engelen et al., 1994) fragments are formed in transgenic tobacco indicates that degradation may play a significant role. When the protein is produced for pharmaceutical applications, its stability is even more important as a factor that determines product homogeneity. Massive proteolytic degradation occurs in particular during tissue senescence and during stress when nutrients are remobilized for transport to other plant parts or when an increased capacity for synthesis of stress gene products is required. The induction of these processes can be triggered by a number of external (e.g. drought, temperature, mineral deficiency, shading, and pathogen infection) and internal factors (e.g. growth regulators, reproduction, age; for review, see Noodén and Guiamét, 1997; Smart, 1994; Buchanan-Wollaston, 1997). The developmental stage and the environmental conditions of the plant may therefore be important determinants for the proteolytic degradation of the antibodies synthesized.

The objective of the present study was to investigate whether proteolytic degradation in planta is a serious constraint for the production of antibodies by transgenic tobacco. We therefore measured the levels of full-length monoclonal mouse IgG1 (MGR48) in tobacco plants that were grown under four different climate conditions and analyzed the in planta proteolytic degradation of the antibody. This was done by establishing the profile of H-chain content and the relative content of the major H-chain breakdown product present in leaves of different developmental stages. In addition, the relative susceptibility of the antibody produced by the transgenic plants toward the proteolytic activity in tobacco leaf tissue was investigated. For this, the breakdown of MGR48 antibody purified from tobacco and of MGR48 antibody from mouse hybridoma cells was compared in the course of in vitro incubations with crude leaf extract from wild-type tobacco plants.

RESULTS

Expression and Purification of the Antibody

MGR48 monoclonal antibody is an IgG1 type immunoglobulin from mouse directed against subventral gland proteins of the nematode *Globodera rostochiensis*. It contains one glycosylation site, namely in the Fc region of each H chain. The MGR48 cDNAs of H and L chains were fused with a slightly modified antibody signal sequence and cloned into a single T-DNA. The expression of the H-chain gene was under regulatory control of a constitutive cauliflower mosaic virus 35S promotor, and the expression of the L-chain gene was under control of a constitutive TR2′ promotor (van Engelen et al., 1994). The construct was introduced into tobacco by *Agrobacterium tumefaciens*-mediated leaf-disc transformation. The expression of functional antibodies was tested by western blotting (not shown) and binding to *G. rostochiensis* antigen by means of ELISA (not shown). Based on these data the line with highest expression of functional antibodies was selected, propagated in vitro, and transferred to the greenhouse for further experiments.

Immunoblotting of crude leaf extract of the transgenic greenhouse plants after SDS-PAGE under reducing conditions resulted in two major bands that positively reacted with polyclonal sheep-anti-mouse IgG and which corresponded with the H and L chains of the MGR48 antibody of hybridoma cells. In addition, some faint positive bands were observed, all exhibiting higher mobility than the H chain. No positive reaction was found with control extracts from wild-type plants.

The antibody (and antibody fragments) were purified from crude leaf extract by ammoniumsulfate precipitation and subsequent protein G-affinity chromatography. Comparison of immunoblots with Coomassie-stained PAGE gels indicated that all proteins present in the fraction that showed binding affinity to protein G ("total antibody") reacted with sheep-anti-mouse IgG. By means of cation-exchange chromatography the purified antibody could be separated into two fractions, one exhibiting weak binding (fraction I) and one exhibiting stronger binding (fraction II). The results of the successive steps in the purification procedure are depicted in Figure 1, which shows the protein fractions on a SDS-PAGE gel run under reducing conditions. The fraction ob-

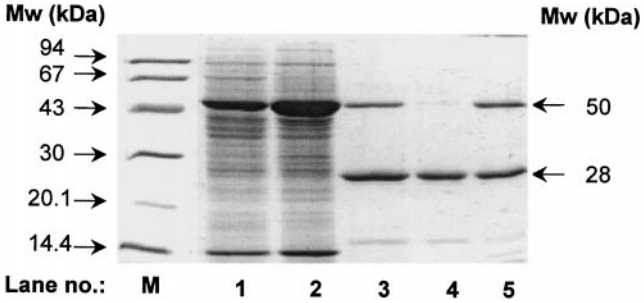

Figure 1. Coomassie-stained 12% (w/v) SDS-PAGE gel (reducing conditions) showing the proteins from the subsequent fractions obtained during the antibody purification procedure. Lane 1, Crude leaf extract (7 μg); lane 2, 20% to 60% ammonium sulfate saturation fraction (10 μg); lane 3, fraction retained on Protein G column (5 μg); lane 4, cation-exchange peak I (2.5 μg); lane 5, cation-exchange peak II (2.5 μg). M, Molecular mass marker proteins.

tained after protein G-bioaffinity chromatography mainly consisted of two proteins, a small one and a large one (Fig. 1, lane 3), the latter exhibiting a similar molecular mass as the large subunit of Rubisco (Fig. 1, lane 2). It is interesting that fraction I only exhibited the small band (Fig. 1, lane 4), whereas fraction II exhibited both small and large bands (Fig. 1, lane 5).

Qualitative Analysis of the Plantibody

The purified total antibody (and antibody fragments) and fractions I and II were compared with MGR48 from mouse hybridoma cells by SDS-PAGE under reducing and non-reducing conditions (i.e. with and without β-mercaptoethanol). The small and large protein bands visible under reducing conditions showed identical mobility as the L and H chains of MGR48 from hybridoma cells, exhibiting molecular masses of 28 and 50 kD (Fig. 2, lanes 5–8), which is in fair agreement with the molecular masses calculated from the amino acid sequences (26.8 and 51.3 kD). By means of immunoblotting it was shown that the small monomer band of 28 kD of both hybridoma and plant antibody reacted with antibody specifically directed against Fab fragments of mouse IgG1 (results not shown). In addition, it was found that only the H chain of the plant antibody reacted with antibodies that specifically bind to plant-specific (Xyl and Fuc containing) *N*-glycans, whereas the H chain of the hybridoma antibody did not (results not shown). The fact that the small band of the plant antibody did not react with this glycan-specific antibody indicated that these monomer(s) did not contain the *N*-glycan part of the Fc-fragment. Under non-reducing conditions the antibody from MGR48 hybridoma cells showed only one band, representing the intact tetramer of two H and two L chains with apparent total molecular mass of 182 kD (Fig. 2, lane 1). The purified antibody from tobacco exhibited the same molecular mass, which confirmed the complete assembly of the tetramer in tobacco (Fig. 2, lane 2). In addition, one major band with apparent molecular mass of 125 kD was found and three minor bands corresponding with 160, 65, and 44 kD (Fig. 2, lane 2). SDS-PAGE under non-reducing conditions (Fig. 2, lane 2) did not provide any evidence for the presence of intact monomeric H chain (50 kD) or intact monomeric L chain (28 kD). The fractionation by cation-exchange chromatography had resulted in the separation of the small oligomer of 44 kD (fraction I; Fig. 2, lane 3) and the complexes of 182, 160, and 125 kD (fraction II; Fig. 2, lane 4).

These results showed that the transgenic tobacco plants produced intact MGR48 antibody that contained plant-specific *N*-glycans attached to the H chains. The presence of the discrete extra bands below 182 kD on non-reducing PAGE gels strongly indicated that the produced antibody is broken down via some relatively stable intermediates. Most probably, the prominent protein band of 125 kD represented a F(ab′)2-like fragment, and the band of 44 kD represented a Fab-like fragment, which implies that in tobacco the degradation of intact antibody starts with the proteolytic removal of (part of) the Fc-region. This assumption is supported by the protein pattern on reducing PAGE gels, which showed that the purified antibody mainly consisted of H chain (probably belonging to the intact antibody) and of protein with approximately the same molecular mass as the L chain (belonging to the intact antibody and to antibody fragments). Furthermore, the observation that the monomer(s) of 28 kD derived from tobacco did not exhibit any Fuc and Xyl containing *N*-glycans indicated that the breakdown intermediates were devoid of the *N*-glycan part of the Fc-region.

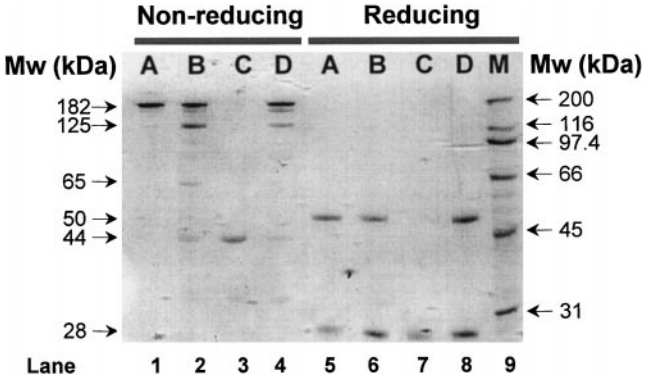

Figure 2. Coomassie-stained 10% (w/v) SDS-PAGE gel showing mouse hybridoma MGR48 antibody (A), purified total plantibody (fragments; B), cation-exchange fraction I of total plantibody (fragments; C), and cation-exchange fraction II of total plantibody (fragments; D) run under non-reducing and reducing conditions (i.e. without and with β-mercaptoethanol, respectively). Each lane was loaded with approximately 3 μg of protein. M, Molecular mass marker proteins.

Effect of Growth Conditions and Developmental Stage on Antibody Levels

To find out whether climate conditions affect the net level of antibodies, the described transgenic tobacco plants were grown at low and high temperature (15°C and 25°C) under high and low irradiation (75 and 275 μmol m^{-2} s^{-1} during one continuous light period of 16 h d^{-1}), giving four groups of plants: (a) 15°C/high light; (b) 15°C/low light; (c) 25°C/high light; and (d) 25°C/low light. The plants were harvested and analyzed after approximately 4 weeks when the first plants started to flower.

The treatments resulted in large differences with respect to biomass, plant length, and number of leaves. Temperature strongly affected plant development. Plants grown at 25°C developed faster than plants grown at 15°C; the plants were taller, and more leaves were produced (Fig. 3A). At the time of harvest the plants grown at 25°C and high light had reached the stage of flowering whereas the plants

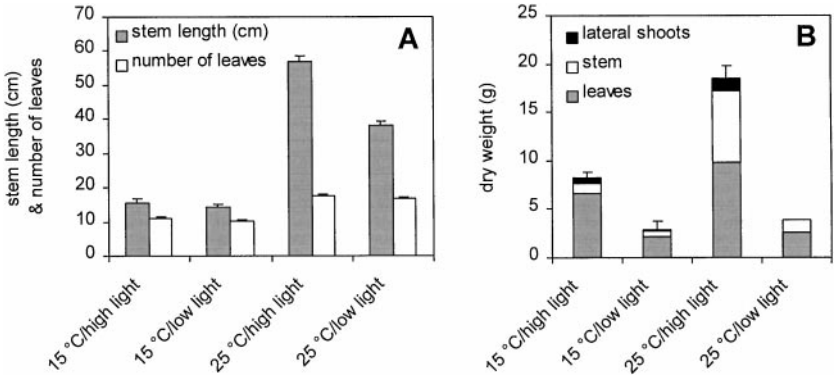

Figure 3. Number of leaves and stem length (A), and production of dry weight biomass as lateral shoots, stems, and leaves (B) of the transgenic plants grown under four different climate conditions, i.e. at 15°C/high irradiation, 15°C/low irradiation, 25°C/high irradiation, and 25°C/low irradiation. Bars show SD ($n = 3$).

grown at 15°C only showed a nearly visible developing flower. The amount of applied light showed a positive correlation with biomass production (Fig. 3B); plants grown at 15°C and high light showed even a higher production of dry weight then plants grown at 25°C and low light (Fig. 3B).

To obtain an insight in the possible relationship between antibody content and developmental stage of the plant tissue, leaves of three different ages were analyzed separately for the four groups of plants. These were young, growing leaves at the top of the plant (top leaves), mature, fully expanded leaves at the middle of the plant (middle leaves), and yellowing, old leaves at the plant bottom (base leaves).

Expressed on fresh weight basis, the top leaves contained more or less twice the amount of total soluble protein of the middle leaves, and the middle leaves in turn contained about twice the amount of the base leaves. This general profile of dramatically decreasing protein content from young to old leaves was observed for all four growth conditions tested (Fig. 4A). The plants grown at 25°C contained less protein per amount of leaf tissue than the plants grown at 15°C, in particular with respect to the base leaves. In fact this reflects the differences in rate of plant development at different temperatures that has been reported above. Also the amount of applied irradiation affected protein content. Leaf tissue contained more protein when grown under high light conditions. This effect was most pronounced for the plants grown at 25°C.

The qualitative analysis of the plantibody and the plantibody fragments strongly indicated that the initial steps of IgG degradation comprised breakdown of the H chain resulting in some relatively stable F(ab′)2-like and Fab-like fragments. This suggests that intact H chain only was present as part of intact IgG, and possibly also as intact free monomer. Since no monomeric H chain could be detected by SDS-PAGE under non-reducing conditions (Fig. 2, lane 2), we used the total amount of intact H chain as a measure for intact IgG content. This was done by densitometric quantification of the H chain on immunoblots after SDS-PAGE under reducing conditions. Highest antibody levels were found in the top leaves (approximately 30–60 $\mu g\ g^{-1}$ of fresh weight; Fig. 4B) and lowest levels were found in the base leaves (approximately 5–15 $\mu g\ g^{-1}$ of fresh weight; Fig. 4B). However, since the profiles of IgG content (Fig. 4B) essentially matched the profiles of protein content (Fig. 4A), the amount of IgG expressed as percentage of total soluble protein in top (0.15%–0.24%), middle (0.13%–0.19%), and base (0.14%–0.21%) leaves were rather similar. The plants grown at 25°C contained less antibody per amount of leaf tissue than the plants grown at 15°C. High-light conditions favored antibody content. In conclusion, highest levels of antibody per amount of fresh weight were found in the plants that were grown at 15°C and under high light.

Immunoblots of SDS-PAGE performed under reducing conditions resulted in two main protein bands of which the one of 50 kD represented the

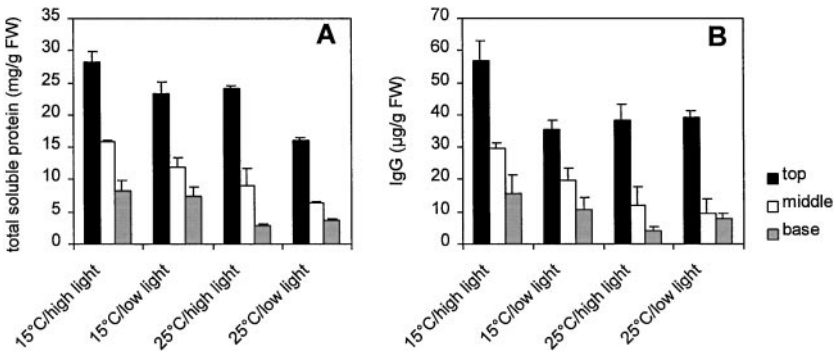

Figure 4. Total soluble protein content (A) and IgG content (B) in top, middle, and base leaves of the transgenic tobacco plants grown at 15°C/high irradiation, 15°C/low irradiation, 25°C/high irradiation, and 25°C/low irradiation. The IgG content was determined by densitometric quantification of the intact H chain on immunoblots after SDS-PAGE under reducing conditions, using polyclonal sheep anti-mouse IgG that was directed against total mouse IgG (H + L chain). Bars show SD ($n = 3$).

intact H chain (H) and the other one represented the main H-chain fragment together with L-chain protein (H′). The ratio between these two bands (H/H′) can thus be regarded as indicator for the proteolytic degradation of the antibody in the different leaf tissues. Therefore, we determined also the relative amount of protein present in the bands of putative H-chain fragment, using the same quantification procedure as applied for the detection of H chain. The affinity of the polyclonal sheep-anti-mouse antibody to the H chain and to the protein present in the bands of putative H-chain fragment may differ; the H/H′-ratios presented here should therefore not be interpreted as molecular ratios. The results showed that the H/H′-ratios of the top leaves were 3 to 7 times higher than the H/H′-ratios of the middle and base leaves (Fig. 5). This large difference indicated that a substantial part of the H chain was broken down during the development of the leaf.

In Vitro Degradation of Plantibody MGR48 and Mouse Hybridoma MGR48

To corroborate the proteolytic potential of tobacco leaf tissue toward the plantibody we incubated crude protein extracts from top, middle, and base wild-type tobacco leaves with antibody purified from the transgenic tobacco plants (fraction II in Fig. 1). The incubations were performed at pH 4.5 and 7. The reaction mixtures were analyzed by immunoblotting after SDS-PAGE performed under reducing conditions. The band patterns of the immunoblots did show only little proteolytic degradation of the antibody at pH 7

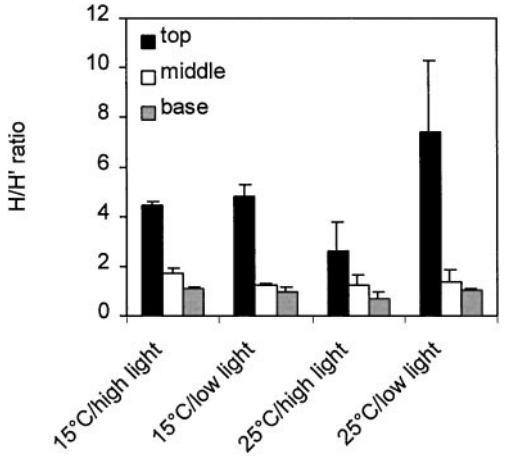

Figure 5. Ratio between relative amount of H chain and relative amount of protein that exhibits L-chain mobility (i.e. L-chain and putative H-chain fragment; H′) in top, middle, and base leaves of the transgenic tobacco plants grown at 15°C/high irradiation, 15°C/low irradiation, 25°C/high irradiation, and 25°C/low irradiation. The H-chain and the protein-exhibiting L-chain mobility were quantified by densitometric quantification on immunoblots after SDS-PAGE under reducing conditions, using polyclonal sheep anti-mouse IgG that was directed against total mouse IgG (H + L chain). Bars show SD ($n = 3$).

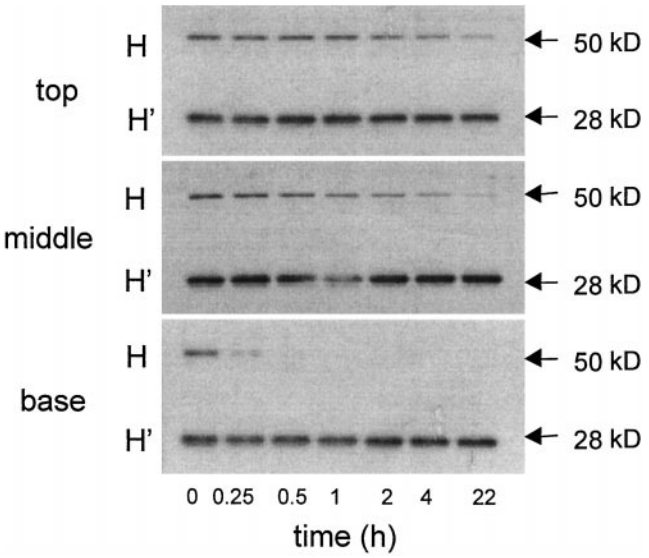

Figure 6. Immunoblots after SDS-PAGE under reducing conditions, showing the H chain (H) and the protein band that exhibits L-chain mobility (i.e. L-chain and putative H-chain fragment; H′) of the MGR48 plantibody in the course of its incubation with crude protein extracts of top, middle, and base leaves from wild-type tobacco. The incubations were performed at pH 4.5 with 25 μg mL^{-1} of purified plantibody and 58 μg mL^{-1} of leaf protein. The protein bands were probed with polyclonal sheep anti-mouse IgG that was directed against total mouse IgG (H + L chain). Similar results were obtained in two independent experiments.

(data not shown). However, in the course of the incubations at pH 4.5 the H chain disappeared; the proteins that exhibited L-chain mobility (i.e. the L-chain and the putative H-chain fragment) were relatively stable (Fig. 6). This protein pattern on gel, obtained after incubation in vitro confirmed the conclusion that in tobacco the antibody is broken down by cleavage of (part of) the Fc region, resulting in a relatively stable intermediate that consists of subunits that exhibit the same electrophoretic mobility on SDS-PAGE gels as the L chain. Separate incubations with equal amounts of crude leaf extract instead of equal amounts of leaf protein showed that the proteolytic activity per amount of leaf tissue was significantly higher in the base leaves than in the top and middle leaves (Fig. 7). Although the middle leaves exhibited virtually the same proteolytic activity per amount of leaf tissue (Fig. 7) as the top leaves, they contained substantially less intact antibody (Fig. 4B). It is presumed that breakdown increases with time of residence of the antibody in the leaf tissue.

Antibodies are relatively stable proteins. The observed breakdown during leaf development prompted us to investigate whether the antibody produced by the plants is less stable than the antibody produced by the hybridoma cells. We therefore followed proteolytic degradation of equal amounts of hybridoma MGR48 and plantibody MGR48 in separate incubations with equal volumes of the same crude leaf extract from wild-type tobacco. The immunoblots

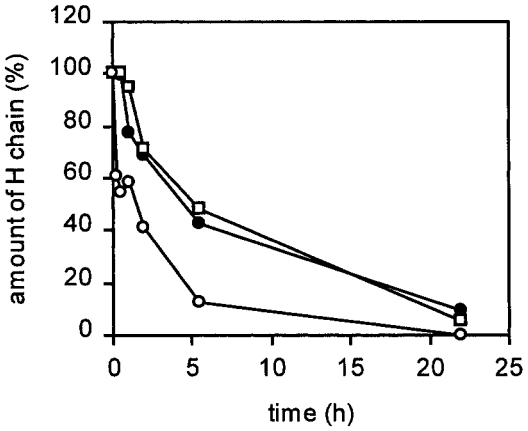

Figure 7. Proteolytic degradation of the MGR48 plantibody in the course of separate incubations with crude extracts of top (□), middle (●), and base (○) leaves from wild-type tobacco. The incubations were performed at pH 4.5 with 25 μg mL^{-1} of purified plantibody and equal volumes of leaf extract, the latter corresponding with equal amounts of fresh weight. The amount of H chain is expressed as percentage of the initial amount and was quantified by densitometry of the H bands on immunoblots after SDS-PAGE under reducing conditions, using polyclonal sheep anti-mouse IgG that was directed against total mouse IgG (H + L chain).

showed that in the course of the incubations the H chain of MGR48 antibody produced by the tobacco plants disappeared with a higher rate than the H chain of MGR48 from hybridoma cells (Fig. 8). Since the *N*-glycans attached to glycoproteins are assumed to play a role in folding, quaternary structure, and stability of the protein (Dwek, 1996; O'Connor and Imperiali, 1996), the vulnerability of the plantibodies to proteolytic degradation may be due to the fact that they contain plant-specific *N*-glycans instead of mouse *N*-glycans.

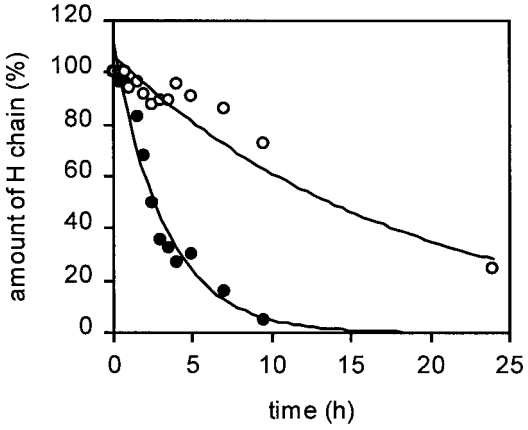

Figure 8. In vitro proteolytic degradation of MGR48 antibody from tobacco (●) and of MGR48 antibody from mouse hybridoma cells (○) at pH 4.5. The parallel incubations were performed with an equal amount of antibody (31 μg mL^{-1}) and an equal volume of the same crude leaf extract from wild-type tobacco. The amount of H chain is expressed as percentage of the initial amount and was quantified by densitometry of the H bands on immunoblots after SDS-PAGE under reducing conditions. Similar results were obtained in three independent experiments.

DISCUSSION

The here reported general profile of decreasing total soluble protein content from young to old leaves is an obvious characteristic of senescence. Essentially, senescence functions as a recycling system of nutrients, which are translocated from the senescing tissue to young plant parts and reproductive organs. Therefore senescence normally occurs in correspondence with plant maturation and transition to the reproductive phase. This is perfectly illustrated by our observation that the plants that developed faster and started to flower consistently exhibited lower protein levels than the plants that were less developed (Figs. 3A and 4A).

The results showed that the antibody content kept close pace with the level of total soluble protein, as is illustrated by the ratio between the relative amount of IgG1 and total soluble protein, which was constant throughout the developmental stage of the leaf tissue (Fig. 9). This remarkable correlation makes it easy to predict changes of antibody levels from changes in amount of total protein. In theory however, there is no evident necessary link between both parameters. During senescence the changes in synthesis and breakdown are not the same for all proteins. Several enzyme activities are known to increase in particular enzymes, which are involved in nitrogen metabolism. Other proteins, such as chlorophyll-binding proteins, ribulose 5-phosphate kinase, and Rubisco show a clear decrease (Smart, 1994; Buchanan-Wollaston, 1997; Noodén and Guiamét, 1997). Furthermore, the vast majority of soluble protein in green leaf tissue, being Rubisco, is localized in the chloroplasts, whereas after proper assembly and

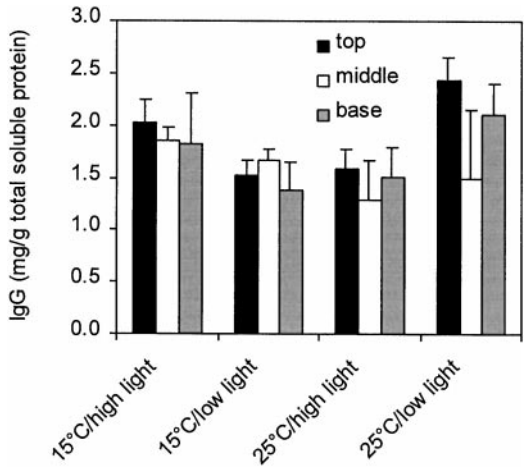

Figure 9. Amount of IgG expressed per amount of total protein in top, middle, and base leaves of the transgenic tobacco plants grown at 15°C/high irradiation, 15°C/low irradiation, 25°C/high irradiation, and 25°C/low irradiation. Bars show SD (n = 3).

maturation in the ER and Golgi apparatus the antibodies are excreted into the apoplastic space (Hein et al., 1991; van Engelen et al., 1994; De Wilde et al., 1998). Therefore the antibodies probably are exposed to different pools of proteases.

Only few data are available about protease activity present in the apoplastic space. In developing oat leaves 16% of total acidic protease, active at pH 4.5 could be washed out from the intercellular space (van der Valk and van Loon, 1988). Significant endopeptidase activity was observed at acidic pH in the extracellular fluid from etiolated hypocotyls of *Phaseolus vulgaris* (Gomez et al., 1994). In the process of tracheary element differentiation of *Zinnia elegans* a Ser protease, active at pH 5 is secreted during secondary cell wall formation (Groover and Jones, 1998). The acidic pH optima of these enzymes are consistent with the apoplastic pH, which may vary between pH 4 and 7 and for most plant species ranges between pH 5 and 6.5 (Grignon and Sentenac, 1991). The in vitro incubations of the antibody with crude enzyme preparations of wild-type tobacco at pH 4.5 and 7 indicated that the in planta degradation is catalyzed by acidic proteases. This is in agreement with the assumption that the antibodies are degraded in the apoplast.

The band patterns obtained by SDS-PAGE and immunoblotting indicated that the antibody was degraded via some relatively stable intermediates, which probably were F(ab′)2-like and Fab-like fragments. Similar results have been reported before (De Neve et al., 1993; van Engelen et al., 1994). This means that the proteolytic cleavage occurs between the Fab and Fc domains of the H chain, which is not unlikely, since this hinge region is susceptible for proteolytic cleavage by pepsin and papain.

One may only speculate about the possible causes of the difference in susceptibility to proteolytic degradation exhibited by the MGR48 antibody from the plant and the MGR48 antibody from the mouse. The finding that removal of saccharides has been shown to result in 60-fold increase in the rate of C_H2 cleavage by trypsin (Dwek et al., 1995) points at a role of the *N*-glycans. Plants and mammals differ in type of *N*-glycosylation. Plants have neither sialic acid nor Gal-β1–4 residues on their glycoproteins and exhibit carbohydrate motifs in their *N*-glycans that are not found in mammals (Lerouge et al., 1998). The glycan chains are N-linked on the inner face of the C_H2 domain and therefore are more or less buried inside the Fc region of the IgG molecule. From x-ray crystallography and NMR studies it is known that the nature of the sugar residues partly determines noncovalent binding interactions between the surface of the protein and the *N*-glycan chain; in particular Gal is associated with restricted motion of the *N*-glycans that fill the volume between the C_H2 domains (Dwek, 1996; O'Connor and Imperiali, 1996). The lack of a terminal Gal residue on the *N*-glycan but also differences in absolute volume of the *N*-glycans may affect the conformation of the protein and consequently the accessibility to proteolytic cleavage.

The main conclusion of this study is that proteolytic degradation in planta can be a serious obstacle for the production of antibody in tobacco. It negatively affects yield as well as product homogeneity. The results strongly indicated that the major portion of the proteolytic degradation is part of the natural process of senescence, which starts when the plant tissue is mature and completely developed. Regulation of climate conditions does not offer a real solution to this problem. Temperature affected in particular the timing of antibody decline by controlling the rate of plant development. The final amount of antibody in mature leaf tissue could be slightly upregulated by the application of high light conditions during growth; however, the antibody level per amount of total soluble protein was less sensitive to the amount of light, since total soluble protein content also increased with higher irradiance. Senescence-associated processes including protease expression also occur under stress conditions (Huffaker, 1990). This has implications for post-harvest handling and processing of the plant material. Furthermore, the presence of proteolytic activity in the source material may be a disadvantage for purification processes which make use of protein based bioaffinity techniques. The question remains whether these conclusions drawn for tobacco and IgG1 can be extended to other crops or tissues and to other heterologous proteins. The process of senescence is a general phenomenon. It would be interesting to make an inventory of the main production crops with respect to their expression of protease patterns and substrate specificity, since this enables to make ideal combinations of production plant and product of interest.

MATERIALS AND METHODS

Vector Construction, Tobacco Transformation, and Selection of Antibody Producing Line

The IgG1 antibody was directed against subventral gland proteins of the nematode *Globodera rostochiensis*. The mouse hybridoma cell lines from which cDNAs of the MGR48 H and L chains were derived have been described by De Boer et al. (1996). The isolation of the cDNAs of H and L chains by means of PCR amplification, the vector construction, and the observation that the construct encodes antigen binding antibody have been described elsewhere (A. Wilmink, J. Molthoff, A. Schouten, J. Roosien, A. Schots, J. Bakker, W.J. Stiekema, and D. Bosch, unpublished data). Expression of the H chain is driven by the cauliflower mosaic virus 35S promoter with duplicated enhancer (Kay et al., 1987) and the expression of the L chain by the TR2′ promoter (van Engelen et al., 1994). Tobacco (*Nicotiana tabacum* cv Samsun NN) leaf discs were transformed essentially according to the method of Horsch et al. (1985). Stable transformed plants were maintained under sterile conditions on Murashige and Skoog

(Murashige and Skoog, 1962) agar medium (Duchefa, Haarlem, The Netherlands) containing 3% (w/v) Suc and subsequently were transferred to soil in the greenhouse. From leaves of 33 independent greenhouse-grown transgenic plants, protein extracts were prepared and antibody expression levels were estimated by SDS-PAGE followed by immunoblot analysis using sheep-anti-mouse antibodies as described by van Engelen et al. (1994). Based on these data, extracts of seven plants were selected for ELISA analysis. Microtiter plates were coated overnight with 200 ng per well of *G. rostochiensis* homogenate proteins in 50 mM sodium carbonate, pH 9.6 at 4°C. Wells were washed with 0.1% (v/v) Tween 20 in phosphate-buffered saline (PBS), pH 7.2, blocked for 2 h with 5% (w/v) non-fat dry milk powder in PBS, and washed twice with 0.1% (v/v) Tween 20 in PBS. Serial dilutions of extracts of the seven transgenic lines were added to the wells and incubated for 2 h. Hybridoma-produced MGR48 antibodies were used as a standard. After washing three times with 0.1% (v/v) Tween 20 in PBS, sheep-anti-mouse alkaline phosphatase was added in PBS with 1% (w/v) non-fat dry milk, and incubated for 1 h. Plates were washed five times with 0.1% (v/v) Tween 20 in PBS before adding 150 μL of substrate buffer (0.75 mg mL^{-1} *p*-nitrophenylphosphate in 0.1 M Tris [tris(hydroxymethyl) aminomethane]/HCl, pH 9.8, and 5 mM $MgCl_2$) was added and the A_{405} was measured. One line (line 31), showing the highest expression of antigen binding MGR48 (0.3%) was selected for all further experiments.

Plant Growth Conditions

The transgenic tobacco plants were propagated in tissue culture on Murashige and Skoog medium (Murashige and Skoog, 1962) containing 2% (w/v) Suc, at 20°C and under light/dark cycles of 14 h of continuous light (60 μmol m^{-2} s^{-1}) per day. Plants of approximately 5 cm in length were allowed to adapt to climate room conditions for 1 week at 18°C, under light/dark cycles of 14 h of continuous low light per day, and relative humidity gradually declining from 97% to 70%. The plants were then grown on potting compost in climate rooms, either under low or high temperature (15°C and 25°C), and low- or high-light conditions (75 and 275 μmol m^{-2} s^{-1} during one continuous light period of 16 h d^{-1}), which resulted in four groups of nine plants. Each group was subdivided in three subgroups of three plants, which were analyzed separately. The night temperatures were kept 3°C lower than the day temperatures. Relative humidity was 70%. Of every plant three portions of leaves were harvested, namely the top, the middle, and the basal leaves. The top leaves are defined as the youngest leaves of at least 5 cm in length; the basal leaves are the first leaf at the bottom of the plant of at least 15 cm in length together with the first one in succession; the middle leaves are defined as the three leaves in the middle between the top and the basal leaves. The rest of the leaves were not analyzed with respect to IgG and protein content. Immediately after harvest the plant material was frozen in liquid nitrogen and stored at −70°C.

Total Soluble Protein Extraction

Leaves were ground in a precooled mortar under liquid nitrogen. To 1 g of powdered tissue was added 5 mL of ice-cold protein isolation buffer (60 mM Tris, pH 8.0, containing 500 mM NaCl, 10 mM EDTA, 30 mM β-mercaptoethanol, and 0.1 mM phenylmethanesulfonyl fluoride). This was thoroughly mixed and centrifuged (12,000*g*, 0°C, 3 min). The supernatant was stored at −80°C before further analysis. The safety of the isolation procedure with respect to stability of the plantibody was checked by separate incubations at 4°C of two types of total soluble protein extracts, i.e. (a) extracts prepared of base leave tissue from the transgenic plant and (b) extracts prepared of wild-type base leave tissue to which purified plantibody was added (approximately 0.4% of total protein). At different time points (0, 15, 31, 64, and 94 min) samples were taken and analyzed on immunoblots for intact H-chain content by the standard densitometric method described below. The results of these control experiments showed that the applied isolation procedure did not induce any detectable breakdown of plantibody.

Purification of IgG1 from Tobacco

Transgenic tobacco plants propagated in tissue culture and adapted to climate room conditions as described above, were grown on potting compost in the greenhouse under normal daylight conditions at 21°C (7 AM–11 PM) and 18°C (from 11 PM–7 AM) and 75% relative humidity. After a growth period of approximately 4 weeks in the greenhouse the total leaf tissue of the plants was harvested and immediately frozen in liquid nitrogen. The frozen leave tissue was powdered in a stainless steel blender, which was precooled with liquid nitrogen. To 200 g of powdered plant material was added 600 mL of 5 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride, 20 mM sodium bisulfite, and 10 g of polyvinylpolypyrrolidone in 150 mM sodium phosphate, pH 7.0. The mixture was thawed and subsequently clarified by centrifugation (10,000*g*, 10 min, 4°C). From this homogenate a protein precipitate was prepared by ammonium sulfate precipitation (20%–60% ammonium sulfate saturation). This was resuspended in 90 mL of 100 mM NaCl in 50 mM sodium phosphate, pH 7.0 and after clarification by centrifugation (20 min, 10,000*g*, 4°C) applied on a HiTrap Protein G bioaffinity column (column volume 5 mL; Amersham-Pharmacia Biotech, Uppsala), which was equilibrated with 50 mM sodium phosphate, pH 7.0. Non-binding protein was washed off with 10-column volumes of the same buffer. Bound protein was subsequently eluted with 0.1 M Gly, pH 2.7, and immediately brought to neutral pH by mixing with 1 M Tris, pH 9.0 (50 μL mL^{-1} of eluate). By means of buffer exchange on Sephadex G25 (PD-10 columns; Amersham-Pharmacia Biotech) this protein fraction was brought in 50 mM MES (2-[*N*-morpholino]ethanesulfonic acid), pH 6.0, and applied on a cation exchange column (Mono S HR 5/5; Amersham-Pharmacia Biotech), which was equilibrated with the same buffer. Protein sep-

aration was performed with a linear 0 to 0.3 M NaCl gradient over 17.5 mL in 50 mM MES, pH 6.0.

Electrophoresis, Immunoblotting, and Quantification of IgG1

SDS-PAGE was performed as described by Laemmli (1970) on minigels of 10% or 12% (w/v) acrylamide and 0.32% (w/v) bisacrylamide. The samples were prepared by mixing the protein extracts with loading buffer (4:1, v/v), which contained either 0 or 30 mM β-mercaptoethanol, and subsequent heating on a boiling water bath for 2 min. The loading buffer consisted of 8% (w/v) SDS, 40% (v/v) glycerol, and 0.1% (w/v) bromphenol blue in 200 mM Tris, pH 6.8. After separation the proteins were either stained in the gel with Coomassie Brilliant Blue (R250) or immediately western blotted. Blotting was performed by electrophoretic transfer of the protein bands onto nitro-cellulose membranes for 1 h at 50 V in 1 mM Tris and 10% (v/v) ethanol in 10 mM 3-cyclohexyl-amino-1-propane sulfonic acid, pH 11, at room temperature. The membranes were blocked for 2 h at room temperature with 2% (w/v) bovine serum albumin and 0.2% (v/v) Tween 20 in PBS. Xyl- and Fuc-containing *N*-glycans were detected by incubating the blots directly with anti-horseradish peroxidase antibodies (Rockland, Gilbertsville, PA). Antibody (and fragments) were detected by incubating the blots with anti-mouse IgG antibodies either conjugated with alkaline phosphatase or, for densitometric quantification, conjugated with horseradish peroxidase. The alkaline phosphatase reaction was performed with 0.1 mM 4-nitroblue tetrazolium (prepared from 92 mM stock solution in dimethylformamide) and 0.1 mM 5-bromo-4-chloro-3-indolyl-phosphate 4-toluidine in 100 mM Tris, pH 9.5, containing 100 mM NaCl and 10 mM $MgCl_2$ until the bands of the positive controls were clearly visible. For densitometric quantification of the protein bands of the H chain and H-chain breakdown product, the blots were incubated for 2 h at room temperature with polyclonal sheep-anti-mouse IgG antibodies conjugated with horseradish peroxidase in 1% (w/v) bovine serum albumin, 0.2% (v/v) Tween 20, and 2% (v/v) protein isolation buffer in PBS. The blots were washed five times with 0.2% (v/v) Tween 20 in PBS, pH 7.2, and subsequently incubated with enhanced chemiluminescence western blotting detection reagent (Amersham-Pharmacia Biotech). Films were exposed to the blots (1–10 min) and subjected to densitometric analysis using Scion Image software (release Beta3B). A concentration range of polyclonal mouse IgG (Sigma, St. Louis) in crude protein extract of wild-type tobacco leaves was used as standard.

In Vitro Study of Proteolytic Activity

Crude protease extracts were prepared from top, middle, and base leaves of wild-type Samsun NN plants, which were at the start of flowering. The leaf tissue was ground in a precooled mortar under liquid nitrogen. Three milliliters of ice-cold phosphate/citrate buffer, pH 6.0 (0.4 M Na_2HPO_4:0.2 M citric acid, 1:0.58, v/v), containing 30 mM β-mercaptoethanol was added per gram of tissue powder. The suspension was gently mixed by using a tube pestle and centrifuged (12,000*g*, 0°C, 3 min). The supernatants served as crude protease preparation and were stored at −80°C before use. To compare the proteolytic capacity of the three leaf tissues either 15 μL of each leaf preparation or 7 μg of leaf protein was mixed with 3 μg of purified MGR48 IgG1 from tobacco in a total volume of 120 μL of the phosphate/citrate buffer (titrated to either pH 4.5 or 7.0 with 1 M citric acid and 1 M Na_2HPO_4, respectively), and incubated at 30°C. At different time intervals samples were taken, immediately mixed with SDS-PAGE loading buffer (4:1 v/v) containing 30 mM β-mercaptoethanol, and subsequently heated on a boiling water bath for 2 min. The samples were stored at −80°C before analysis. The susceptibility of MGR48 IgG1 from the plant was compared with the susceptibility of MGR48 IgG1 from mouse hybridoma cells by incubating these antibodies with a crude protease preparation from wild-type tobacco. The MGR48 IgG1 from mouse hybridoma cells was a kind gift of Dr. Arjen Schots (Department of Nematology, Wageningen University, The Netherlands). Pure antibody (7.5 μg) was mixed with 120 μL of crude leaf extract in a total volume of 240 μL of the phosphate/citrate buffer titrated to pH 4.5 with 1 M citric acid and put in a closed tube on a 30°C water bath. At different time intervals samples were taken, immediately mixed with SDS-PAGE loading buffer (4:1, v/v) containing 30 mM β-mercaptoethanol, and subsequently heated on a boiling water bath for 2 min. The samples were stored at −80°C before analysis. The samples were analyzed by electrophoresis on 15% (w/v) SDS-PAGE gels and subsequent western blotting as described in the previous section. The lanes were loaded with a mixture of 4 μL of sample, 4 μL of loading buffer, and 7 μL of water. Development of the blots and subsequent densitometric quantification of the H chain were performed as described in the previous section.

Protein Determination

Protein concentrations were determined according to Bradford (1976) using the Coomassie plus protein assay reagent from Pierce Chemical (Rockford, IL) with bovine serum albumin as standard protein.

ACKNOWLEDGMENT

The authors thank Dr. Arjen Schots (Department of Nematology, Wageningen University, The Netherlands) for the kind gift of MGR48 antibody purified from mouse hybridoma cells.

Received January 3, 2000; accepted May 22, 2000.

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