Summary of Ph.D. thesis

Expression of dsRNA-specific monoclonal antibodies in transgenic plants

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Introduction

Antibodies have attracted researchers' attention for many years and much data on antibody sequences, folding, affinities, and transgenic expression have been obtained. However, the problem of how to express active antibody molecules in various subcellular compartments of heterologous systems has not yet been solved. The most critical location for antibody expression remains the cytoplasm, the replication site of many plant RNA-viruses.

A number of mouse monoclonal antibodies, named J2, K1, K2, P6, were produced by Noémi Lukács’s group in earlier work (Schönborn et al., 1991). Antibodies J2, K1 and K2 specifically recognize structural features of double-stranded RNA (dsRNA) independent of the nucleotide sequence of the antigen, and their binding to single-stranded RNA, double-stranded DNA or RNA-DNA hybrids is below detection levels. Although the P6 antibody also reacts with dsRNAs independently of their sequence, it also interacts with structured single-stranded RNAs such as viroid- or ribosomal RNA. Double-stranded RNAs are known to play an important role in several basic biological processes, among other things in replication of ssRNA-viruses and in dsRNA-based gene silencing. Since more than 90% of all plant viruses are RNA-viruses, dsRNA, which necessarily arises during replication, offers a potential target through which multiplication of most plant viruses might be influenced. Viral dsRNAs are also the targets of dsRNA-mediated gene silencing, which helps to protect plants against virus multiplication. We hypothesized, that when expressed in the right intercellular compartment in transgenic plants, dsRNA-specific antibodies may interfere with the replication process and/or with the dsRNA-mediated silencing mechanism of the host. It was shown in earlier experiments that the antibody J2 indeed prevents replication on partially double-stranded alfalfa mosaic virus template in vitro (de Graaff et al., 1995).

Aims of Study

Considering the scientific and possible practical potential of dsRNA-specific antibodies to interfere with dsRNA-mediated viral and/or host processes within the plant cell, we set the following aims for the current doctoral studies:

(i) to establish strategies for the expression of correctly folded dsRNA-specific antibodies or antibody fragments inside the cytoplasm as well as in other cellular compartments in *N. tabacum*;

(ii) to identify antibody frameworks that can be stably expressed in the cytoplasm;
to construct single-chain antibody Fv fragments from full-length monoclonal antibodies using the phage display technique;

(iv) to investigate the physiological effects of dsRNA-specific antibodies and antibody fragments on the transgenic plants in which they are expressed.

Results

First we investigated the stability and the assembly of intact J2 (IgG2a) antibodies expressed in the cytosol. We found that while the H-chain of J2 could be easily detected by ELISA, the L-chain concentration remained at background levels. The H:L ratio was estimated to be ≤10:1, in contrast to the 1:1 ratio of hybridoma-produced J2. After affinity purification on Protein A the eluted fraction weakly bound dsRNA. Infection of transgenic tobaccos with CMV or PVY viruses did not reveal any significant protection or tolerance against these viruses.

The low degree of assembly prompted us to express single-chain antibody fragments, which may fold more efficiently and which do not need the assembly of independent polypeptide chains to form the antigen binding site. Using the phage display technique four scFv fragments specific to dsRNA were constructed and expressed in E. coli. scFvs J2 and K1 originated from cloned IgG cDNAs, while scFv P6 and K2 were directly amplified from mRNA purified from the corresponding hybridoma lines. All four scFvs were stably expressed in E. coli periplasma. Their antigen binding activity was demonstrated by ELISA: scFvs J2, K1 and K2 bound dsRNA, whilst scFv P6 preferentially bound rRNA. We found that the ELISA-activity of some scFvs varied widely from batch to batch, possibly because of the instability of the scFv proteins.

The most active clones, namely J2.104, K1.21 and P6.84, were selected for expression in higher plants. Expression studies were completed for J2- and P6-scFVs, which possess highly homologous VH domains and dissimilar VL domains. For both scFv J2 and P6 five types of constructs were made to express them in the cytosol (2 constructs), inside the endoplasmic reticulum, in the apoplast or to anchor them at the cytoplasmic side of the plasma membrane. The constructs were transferred from E. coli into the co-integrative plant expression vector pGEJAE1 and transgenic Nicotiana tabacum L. cv. Xanthi plants were generated by Agrobacterium tumefaciens-mediated transformation of leaf disks.

mRNA-expression was observed in all types of transformants. Investigating the expression of scFv proteins in the cytoplasm we found, that scFv P6 was stably expressed, but scFv J2 could not be detected in any of the transformants. To increase the stability of antibody fragments in the cytoplasm and to protect them against proteolytic degradation we fused the ER retention signal KDEL to the C-terminus, because this
sequence was shown to stabilize some scFvs in the cytoplasm (Figure 6-24 B). Close proximity to the plasma membrane has also been thought to stabilize antibody fragments and to elevate their concentration at the site of virus replication. To anchor scFv to the plasma membrane and possibly to other intracellular membranes we used the N-terminally linked fatty acylation signal of the Fyn kinase (Figure 6-24 E). Neither of these strategies resulted in the accumulation of detectable amounts of scFv J2. Nonetheless, the stably expressed P6 might provide the antibody framework in which antigen binding CDR-loops of other antibodies could be grafted for cytoplasmic expression in future experiments. In a control experiment using Green Fluorescent Protein (GFP) to evaluate the performance of Fyn signal, which is responsible for polypeptide myristoylation and palmitoylation and such for membrane targeting, membrane association of the reporter protein in tobacco plants could be clearly detected.

The oxidative environment inside the endoplasmic reticulum is optimal for formation and accumulation of correctly folded scFvs. Therefore we directed J2 and P6 scFvs into the ER lumen with the murine N-terminal IgG J2 leader peptide and made them ER-resident by adding the C-terminal KDEL retention signal. For both antibodies, the highest expression levels and the highest dsRNA-binding activity was achieved by using this strategy. When, however, the ER-retention signal was removed to achieve scFv-secretion into the apoplast, none of the scFvs could be detected by Western-blotting. This finding indicates that although the scFvs seem to fold correctly in the apoplast, they are probably unstable in the intercellular space and quickly become degraded.

To analyze the effect of RNA-specific antibodies on virus replication we infected transgenic plants with potato virus Y (PVY). PVY is a relatively slow virus, which induces well-defined symptoms and which is known to be subject of dsRNA-mediated gene silencing. Although we could not detect scFv-protein in all types of transformants, we inoculated all of them at three different virus concentrations. The rationale behind this decision was that in several cases in the literature the biological effect of a transgene has been observed even when the protein concentration was below detection level. No significant tolerance or resistance to PVY was found among the transgenic tobacco plants. However, one significant difference between transgenic lines was observed: After infection with PVY all transgenic lines behaved like the control, except the line expressing ER-resident J2-scFv. Not only were pathogenic symptoms in this line seen earlier than in the others, but there was also an obvious increase in the severity of the disease caused by the virus. Beginning with the third leaf above the infected leaf very severe necrosis was observed and some leaves wilted and died. Such symptoms occasionally occurred in other, control or transgenic lines, but they appeared later and were less severe. ER-resident P6 did not induce this kind of pathogenesis. It should be emphasized that although P6 binds to dsRNA, it was originally produced against viroid
RNA and reacts with ssRNA as well. J2 is strictly specific for long, completely base-paired dsRNA species. The effect could only be observed when the scFv was targeted to the ER lumen, because J2 IgG expressed in the apoplast did not give rise to such necrotic symptoms.

The most plausible conclusion to be drawn from these results is that J2-scFv confined to the ER cannot protect the plant against PVY multiplication but may influence the dsRNA-mediated silencing of virus replication by protecting viral dsRNA against fragmentation by the dicer enzyme. It is still difficult to understand how an ER-resident protein may influence a process thought to take place in the cytoplasm. Potyviruses are known to replicate in association with large vesicular structures derived from endoplasmic reticulum. For tobacco etch virus (TEV) it was shown that on infection with TEV the ER-network collapses into aggregated structures and this process probably begins with the binding of the 6 kDa virus protein to the ER-membrane (Hull, 2002). We believe that when PVY is replicating in association with the ER, the chances are relatively good that the highly concentrated scFv comes in contact with the replication complex. It is also possible that during the fragmentation of the ER, scFv is being set free and may act also outside the ER. Whichever explanation is correct; our results demonstrate that not only hapten- or protein-specific antibodies but also nucleic acid specific scFv may efficiently influence in vivo equilibria.

Thus, the strategies we developed for expression of correctly assembled antibodies or antibody fragments inside the cytoplasm of higher plants have been successfully executed. Single-chain antibody fragments were developed in E. coli and then expressed as plantibodies. The accumulation of scFvs was observed in the cytoplasm and in the ER. In addition, the physiological effects of the ER-localized expression of two scFv fragments as well as of the dsRNA-specific IgG J2 were investigated in N. tabacum in vivo upon PVY infection and in the case of J2-scFv a clear effect on viral pathogenesis was found.

Conclusions

1. The assembly and activity of intact IgG J2 H- and L-chains expressed in the cytoplasm of N. tabacum cv. Xanthi was investigated. We found that although some association between H- and L-chains could be detected in ELISA by using γ- or κ-specific antibodies, after ammonium sulphate precipitation and affinity chromatography on Protein A only the heavy chains could be seen by gel electrophoresis and silver staining. We estimate that the H:L ratio of cytoplasmically expressed J2 in N. tabacum was at most 10:1 in contrast to the 1:1 ratio of hybridoma-produced antibodies. Since a similar difference was not observed in the ratio of H- and L-chain mRNAs we conclude, that the spontaneous assembly of the two chains is very inefficient and that the isolated H-chains are probably more stable
in the cytoplasm than the L-chains. Virus challenge of transgenic plants with CMV or PVY viruses did not reveal any influence of J2 gene expression on virus replication or symptom development.

2. Four single-chain Fv fragments specific to dsRNA were constructed from hybridoma-derived cDNAs using the phage display technique. scFvs J2 and K1 originated from cloned IgG cDNAs, while scFv P6 and K2 from the polyA+ mRNA-pool of hybridoma cell lines. All four scFvs were successfully expressed in E. coli and their RNA-binding activity was demonstrated by AN-ELISA. Some scFv-preparations, especially K1- and K2-scFv showed large variation in the extent of antigen binding, which could not be eliminated by rigorous standardization of cultivation, extracition and ELISA conditions. We suggest that this variability is due to the inherent instability of the structure of bacterially expressed scFvs, which may be inactivated by immobilization on ELISA plates. In the case of scFv P6 a change in fine specificity was observed: the rRNA-binding activity remained high, but dsRNA-binding was much lower than that of the original IgG P6. This change may also be caused by subtle structural changes at the antigen binding site of bacterially expressed scFv P6, because ER-resident scFvs expressed in tobacco did not show this effect (Morgun et al., 2005). Clones J2.104, K1.21 and P6.84, which showed the highest activities and the lowest number of mutations in the given genotype, were selected for expression in tobacco.

3. Five types of gene constructs were designed to target scFv to the cytosol, to the endoplasmic reticulum or to the apoplast. Murine N-terminal leader peptide, C-terminal KDEL ER-retention signal and fatty acylation signals derived from Fyn and Src kinase were fused in different combinations to scFv or GFP sequences. The genes were cloned into the co-integrative plant expression vector pGEJAE1 and transgenic N. tabacum cv. Xanthi plants were generated by Agrobacterium-mediated transformation of leaf discs.

4. The expression of scFv J2 and P6 in transgenic tobacco and its physiological effect on the plant were examined. As shown by Western blotting scFv P6 was stably expressed in the cytoplasm, while none of the two constructs led to any detectable accumulation of scFv J2 in the cytoplasmic compartment. Because of the stable expression of P6 in the cytoplasm, the framework of this antibody may be used in future experiments to express scFvs with different specificities in the cytoplasm by CDR-grafting. The highest levels of both scFvs were observed when the proteins were made resident in the ER. In contrast to the intact J2 IgG molecule neither J2-nor P6-scFvs were detected in the apoplast. Modification of GFP by the fatty acylation signal of Fyn kinase resulted in association of GFP in cellular membranes, but the same modification did not led to detectable accumulation of scFv J2 in the membrane fraction.

5. The expression of dsRNA-specific scFvs does not cause any agriculturally relevant tolerance or resistance to PVY infection in transgenic tobacco. However, it was observed that in plants expressing ER-resident scFv J2 the virus symptoms appeared somewhat earlier and more leaves developed necrotic lesions and died than in any other genotypes. The enhanced necrotic symptoms may indicate the interference of dsRNA-specific scFv J2 with the RNA silencing pathway. Recent results in our laboratory show that 14-20 % of non-infected transgenic plants usually develop
characteristic morphological changes that may also be the result of an interaction
with natural silencing mechanisms. In our studies we demonstrate for the first time
physiological effects caused by expression of RNA-specific antibodies in transgenic
plants.

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List of Publications

The thesis is based on the following articles:

1. Morgun, B.V., Richter, A., Deshmukh, S., Stepanyuk, V., Kálai, K.,


**List of Presentations**

The following are the most important meetings where the topic of the thesis had been introduced:


5. **Morgun, B.V.**, Deshmukh S., Stepanyuk V. Kálai, K., Richter, A. and Lukács, N. Targeting antibodies to different cell compartments in plants. Lippay -


**Declaration**

I hereby declare that the work presented in this manuscript is my own and was carried out entirely with help of literature and aid cited in the manuscript.

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