Chapter 3

Introduction of scFv antibody fragments into the plant genome

3.1 Organ-specific and cell-compartment-specific accumulation of recombinant antibodies in plants

The choice of the right plant cell organ and cell compartment for the accumulation of recombinant antibodies depends on developmental and/or metabolic events that should be modulated by the antibody expression. The expression of antibodies in the developmental stage or in the cell compartment where the action of an antigen of interest does not take place leads to a failure of this molecular technique. Many attempts have been described for expression and assembly of antibodies constitutively under control of the Cauliflower mosaic virus 35S promoter [Töpfer et al., 1993]. In such case expressed antibodies can be detected in all plant organs from flowers and leaves (for review see [Conrad and Fiedler, 1998]), to roots [van Engelen et al., 1994], hairy roots [Wongsamuth and Doran, 1997] or tubers of potato [Artsaenko et al., 1998]. However, the antibody accumulation exclusively in particular plant organs can be advantageous for control of a certain developmental stage of a plant. The accumulation of antibodies in early stage of seed maturation under control of legumin B4 promoter [Fiedler et al., 1997] or in later stage of seed development controlled by USP promoter [Phillips et al., 1997] has been reported. Antibodies can be potentially expressed in any other plant organ depending on the promoter region of the expression cassette.

Protein trafficking in eukaryotic cells is guided by signal sequences. Proteins without a signal peptide on its amino-terminal side are localized in the cytosol. The presence of a signal peptide specific for the endoplasmic reticulum, nucleus, peroxysomes, mitochondria or chloroplast determines the subcellular compartment where the nascent protein ends up (for review see [Alberts et al., 1994]).

3.2 Recombinant antibodies in the apoplast and in the endoplasmic reticulum of plant cells

The first successful attempts of full-size antibody expression were all ubiquitous and with a murine Ig signal peptide leading to the assembly of the complete antibodies in the endoplasmic reticulum of transgenic plants (for review see [Conrad and Fiedler, 1998]) followed by their secretion into apoplastic space. Antibody secretion across the plant cell wall into the apoplast can be advantageous because this large aqueous space is a stable
environment with minimal hydrolytic processing [Ma and Hein, 1995]. The disadvantage is that apoplast is not the right compartment for modulation of antigens with an intracellular localization and action. Although the maximal level of the antibody accumulation depends on the plant species and on the intrinsic properties of the antibody itself, generally the accumulation level of secreted antibodies is highest for full-size antibodies and Fab fragments [De Jaeger et al., 2000].

If the plant expression cassette contains a signal peptide on an N-terminal side of the antibody-fragment coding sequence and a retention signal on a C-terminal side the antibody retains in the endoplasmic reticulum. The ER contains a number of molecular chaperones, which assist the folding and maturation of nascent protein (for review see [Galili et al., 1998]). Moreover this oxidizing environment facilitates the formation of intra- and inter-chain disulfide bonds necessary for the correct folding of proteins. The retention signal can be the tetrapeptide HDEL or KDEL. Soluble reticuloplasmins are thought to be retained in the ER by membrane receptors that recognize the H/KDEL sequence [Pagny et al., 1999]. These features make the ER a convenient cell compartment for stable antibody accumulation. Indeed many laboratories showed a correct cleavage of the ER signal sequence and an assembly of a functional antibody fragment in the ER (summarised in [Conrad and Fiedler, 1998]). For the immunomodulation in the ER, scFv fragments are the best choice of a format [De Jaeger et al., 2000]. The scFv proteins have been detected in the endoplasmic reticulum, ER-derived vesicles and in the nuclear envelope.

3.2.1 Cloning of anti-jasmonate scFv genes into the vector pRTRA 7/3 for retention in the endoplasmic reticulum of plant cells

Jasmonic acid-specific scFv antibodies and 12-oxo-phytodienoic acid-specific scFv antibodies, collectively named anti-jasmonate scFv antibodies, were selected by A. Hunger [Hunger, 2002] from a screening of Tomlinson’s Human synthetic VH + VL scFv phagemid libraries A + B (for detailed protocol see web site http://www.mrc-cpe.cam.ac.uk/~phage/*g1p.html). Both libraries contain 10⁹ clones with scFv coding sequences inserted into phagemid pIT1. Phagemids as vectors offers some advantages, such as monovalent display of scFv (or Fab) fragments for selection [Hoogenboom, 1997]. They favour the display of less then a single antibody fragment and thereby the avidity effect in the selection process is minimised [Winter, 1998]. Phagemids also enable production of soluble antibodies without another subcloning. The pIT1 vector, illustrated in Fig. 3.1, contains an inducible lacZ promoter. The scFv gene is inserted between SF11 – NotI restriction sites and targeted into the periplasm by the pelB leader. The c-myc-tag sequence is recognized by the 9E10 antibody and used for the detection of secreted scFv antibodies. Between tag-sequence and a gene coding for gIII minor phage coat protein is introduced an amber codon. The scFv can be produced either for display on phage particle from infected amber suppressor strain (TG1 suppressor strain was used in this study) or secreted into the periplasm as a soluble fragment from infected non-suppressor strain (HB2151 strain has been used in this study).
Three obtained jasmonic acid-specific clones (namely, B11, C4 and F8) and three 12-oxo-phytodienoic acid-specific clones (namely D3, E10 and F2) were selected for further cloning. Single-stranded DNA of these clones has been used for radioactive sequencing with the primers LMB3 and pHEN seq. The sites of their matching to pIT 1 vector are shown in the Fig. 3.1. The nucleotide sequences of scFv genes have been compared with the nucleotide sequences of brassinosteroid-specific scFv [Fecker L.F., unpublished], with ABA-specific scFv [Artsaenko, 1996] and with anti-cytokinin – meta topolin – scFv [ten Hoopen P., unpublished]. The comparison done by ClustalW Multiple Sequence Alignment (http://dot.image.bcm.tmc.edu:9331) via the program Vector NTI Deluxe, v.4.0.1, allowed an identification of CDR’s and framework regions and revealed differences between individual scFv’s only in CDR1 and CDR3 of heavy chains and CDR1 of light chains. Table 3.2 summarises this differences on deduced amino acid level.

The anti-jasmonate scFv genes were inserted into the expression cassette for their retention in the ER of plant cells. The vector pRTRA 7/3, Fig. 3.3, containing anti-ABA scFv gene-c-myc fusion under control of the CaMV35S promoter, with the legumin B4 signal sequence and the KDEL retention sequence has been used [Artsaenko, 1996]. The vector was cleaved with BamHI, dephosphorylated and 3200bp fragment was ligated with anti-JA and/or anti-OPDA scFv gene. The BamHI restriction sites were incorporated into the scFv gene inserts by PCR with FORTOM and BACKLEGTOM primers. The PCR product, 800bp long, was cleaved with BamHI prior the ligation. XL1 Blue strain of E.coli was heat shock-transformed with the ligation product and ampicilin-resistant transformants were tested for the presence of scFv insert in the right
orientation by NcoI-NotI cleavage. The positive clones were sequenced with M13 Universal (MU) and M13 Reverse (MR) primers and also with LEG73 and TAG73 primers. The matching positions of all four primers to vector pRTRA 7/3 are depicted in the Fig. 3.3. The results of sequencing confirmed correct orientation and correct reading frame of the cloned scFv genes. Fig. 3.4, page 31, illustrates the schematic overview of the cloning procedure and the final expression cassette for retention of anti-jasmonate scFv antibody fragments in the endoplasmic reticulum of plant cells. The expression cassette was inserted between HindIII restriction sites.

Table 3.2 Differences on deduced amino acid level in CDR regions of chosen scFv antibodies selected against different plant hormones by phage display.

<table>
<thead>
<tr>
<th>phytohormone specific clone</th>
<th>CDR region</th>
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<tr>
<td></td>
<td>CDR I - VH</td>
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<tr>
<td>JA</td>
<td>B11</td>
</tr>
<tr>
<td>JA</td>
<td>C4</td>
</tr>
<tr>
<td>JA</td>
<td>F8</td>
</tr>
<tr>
<td>OPDA</td>
<td>E10</td>
</tr>
<tr>
<td>OPDA</td>
<td>F2</td>
</tr>
<tr>
<td>BRAS.</td>
<td>A1</td>
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<tr>
<td>mTR</td>
<td>A1</td>
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Fig. 3.3 Scheme of the vector pRTRA 7/3. For details to the expression cassette see text of Chapter 3.2.1.
3.3 The accumulation of functional antibodies in the cytosol of tobacco cells

The expression cassette without signal peptide allows the retention of recombinant antibodies in the cytoplasmic space. The accumulation of functional antibodies in high concentration in the cytoplasm is of highest interest because an action of scores of proteins and signalling molecules takes place in this cell compartment. For example most plant viruses are RNA viruses that replicate in the cytosol and an assembly of antiviral antibodies in this compartment has highest impact on the reduction of the viral infection [Tavladoraki et al., 1993], [Zimmermann et al., 1998]. Unfortunately the cytosol is the compartment in which high antibody accumulation levels are the most difficult to obtain because of the lack of chaperones and the reducing environment of the cytosol, which prevents the formation of disulfide bridges [Biocca et al., 1995]. For this reason antibodies were in some studies expressed at low level and the visualization of the immunomodulation effect was prevented. Unlike full-size antibodies or Fab antibody fragments, the scFv antibody fragments have a higher chance of being correctly folded in the cytosol because flexible linker connects light and heavy chains and there is no need for the formation of inter-chain disulfide bonds. Indeed, several examples of sufficient accumulation of functional scFv antibodies in the cytosol have been reported [Owen et al., 1992], [Tavladoraki et al., 1993], [Zimmermann et al., 1998]. However, even scFv has at least one intra-chain disulfide bridge which is essential for correct folding. This is probably the reason why some scFv’s can not be detected in the cytosol in spite of normal level of corresponding mRNA.

It was unexpectedly discovered that the addition of the ER-retention signal at the C-terminus of scFv fragment significantly improves expression level [Schouten et al., 1997]. The KDEL peptide has been suggested to protect sterically the scFv region susceptible to proteolysis. However even this tetrapeptide does not improve the cytosolic accumulation level of every scFv protein and the expression level of a particular scFv is also coding-sequence dependent.

3.3.1 Cloning of anti-jasmonate scFv genes into the vector pRTRA 7/3 for retention in the cytosol of plant cells

The cloning strategy for targeting of three anti-JA and three anti-OPDA scFv genes into the cytosol was the same as described in Chapter 3.2.1 with only one exception. The final expression cassette lacks the LeB4 signal peptide. Therefore, PCR amplification with FORTOM and BACKLEGTM primers was omitted and the anti-JA and anti-OPDA scFv genes were cleaved from pIT1 phagemid by double restriction NcoI-NotI and directly ligated behind the promoter region of the pRTRA 7/3 vector. Fig. 3.5 illustrates the schematic overview of the cloning procedure and the final expression cassette for retention of anti-jasmonate scFv antibody fragments in the cytosol of plant cells. The expression cassette was inserted between HindIII restriction sites.
Fig. 3.4 Schematic overview of cloning of anti-jasmonate scFv genes into the pRTRA 7/3 vector (a) and the final expression cassette for retention of the scFv antibody fragments in the endoplasmic reticulum of tobacco cells (b).

Fig. 3.5 Schematic overview of cloning of anti-jasmonate scFv genes into the pRTRA 7/3 vector (a) and the final expression cassette for retention of the scFv antibody fragments in the cytosol of tobacco cells (b).
3.4 The accumulation of scFv antibody fragments in the stroma and on the outer envelope membrane of tobacco chloroplasts

Chloroplasts are semiautonomous organelles. The consequence of the evolution of the chloroplast from an original prokaryotic endosymbiont to a cellular organelle was the transfer of the vast majority of genes encoding chloroplast polypeptides to nuclear chromosomes (approximately 90%). The chloroplast genome of vascular plants contain only about 100 genes, most of which encode components of the photosynthetic electron transport machinery and elements of the transcriptional and translational apparatus [Stern et al., 1997]. Proteins destined for the internal compartments of the chloroplast (stroma, thylakoid and inner membranes) carry N-terminal transit sequences, which are necessary and sufficient to direct the import of a polypeptide into the chloroplast [Fuks and Schnell, 1997]. The import process of proteins with this chloroplast-targeting signal engages a recognition and translocation machinery at the chloroplast envelope called general import machinery. In contrast, chloroplast outer envelope membrane proteins appear to use an alternative mechanism of integration that does not involve the general import machinery and this alternative mechanism is up till now poorly understood. The envelope translocation does not require a fully unfolded polypeptide chain even though polypeptide-unfolding activity associated with the outer membrane has been identified. Both, outer and inner chloroplast envelope membranes contain also molecular components and chaperones involved in the import process.

Reports concerning the import process into thylakoid membranes [Henry et al., 1997], into the stroma [Filho et al., 1996], or into the outer chloroplast envelope membrane [Li and Chen, 1996] recently emerged. Even though the chloroplast translocation process is far from being understood there have already been some attempts to utilize the chloroplast transport machinery for accumulation of foreign proteins. While the targeting of a foreign enzyme into the chloroplast has been successful [Creissen et al., 1995] the attempt to accumulate a single-domain antibody fragment into the chloroplast had less success. Camel single-domain antibodies (VHH) fused to tetanus toxoid have been targeted into chloroplasts of *Nicotiana tabacum*, however, no VHH proteins were found in the chloroplast extracts [Vû, 1999].

3.4.1 Cloning of anti-12-oxo-phytodienoid acid scFv genes into the pRT103 vector for retention in the stroma and on the outer envelope membrane of chloroplasts

In this study three different transit sequences have been used for the accumulation of anti-12-oxo-phytodienoi acid scFv antibodies in chloroplasts. First, the transit sequence of the small subunit of ribulose-biphosphate-carboxylase, targeting precursor of this enzyme into the stroma of chloroplasts, has been used and called ST3TP [Waegemann and Soll, 1996]. Second, the stromatal transit sequence of the protein ferredoxin has been utilized and called ST5TP [Van’t Hof, and De Kruijff, 1995]. For targeting of scFv into the outer chloroplastic envelope membrane the transit sequence of the outer chloroplast membrane protein OEP14 was used and called OCMTP [Li and Chen, 1997]. There are several lines of evidence supporting the idea that the stroma of chloroplasts and the chloroplastic envelope membranes are the sites where the biosynthesis of 12-oxo-phytodienoic acid – precursor of jasmonic acid biosynthesis – takes place (see
Chapter 1.2.3). Therefore only the anti-OPDA scFv antibodies were candidates for cloning into the chloroplastic stroma and into the outer envelope membrane of chloroplasts. The F2 – anti-OPDA scFv gene has been utilized.

The coding sequences of all three above discussed transit peptides (ST3TP, ST5TP and OCMTP) were available in plasmid pRT103 and scFv expression was detected in regenerated transgenic plants [Rosso M., unpublished]. The pRT103 vector, depicted in Fig. 3.6, contains an expression cassette with anti-ABA scFv and a transit sequence for stromatal targeting (ST3TP or ST5TP) or a transit sequence for outer envelope membrane targeting (OCMTP) under the CaMV35S ubiquitous promoter.

![Fig. 3.6 Scheme of the vector pRT103. For details see text of Chapter 3.4.1.](image)

Cloning of the anti-OPDA scFv gene for the retention in the stroma of tobacco chloroplasts consisted of only one cloning step. The anti-OPDA scFv-F2 gene was double digested with SfiI-NotI enzymes and cloned into the SfiI-NotI site of the pRT103 vector with ST3TP or ST5TP transit sequence. The presence of anti-OPDA scFv gene in the ampicillin resistant clones was confirmed by control restriction and by sequencing with primers BACKLINK (BL) and FORLINK (FL). Matching sites of these primers are included in the Fig. 3.6. Fig. 3.8, page 34, shows the schematic overview of the cloning procedure and the final expression cassette for retention of anti-OPDA scFv antibody fragments in the stroma of tobacco chloroplasts. The expression cassette was inserted between HindIII restriction sites.

The expression cassette for the retention of anti-OPDA scFv antibody F2 gene in the outer envelope membrane of tobacco chloroplasts was designed with utilization of two vectors. The pRT103 vector with the OCMTP transit sequence, Fig 3.6, and vector pRTHook which contains the 237bp long insert of the c-myc-tag sequence fused to the transmembrane domain sequence (Hook domain). This domain should anchor the scFv antibody in the outer membrane of the chloroplast. Fig. 3.7 shows a scheme of the
pRTHook vector. First, the anti-OPDA scFv gene has been inserted into SfiI-NotI site of the pRT103 vector with the OCMTP transit sequence. Afterwards the c-myc-Hook domain fusion has been ligated into the NotI-restricted and dephosphorylated vector pRT103 with the anti-OPDA scFv gene.

Fig. 3.7 Scheme of the pRTHook vector. For details see text of Chapter 3.4.1.

Fig. 3.8 Schematic overview of cloning of the anti-OPDA scFv gene into the pRT103 vector (a) and the final expression cassette for retention of the scFv antibody fragments in the stroma of tobacco chloroplasts (b).
The correct orientation of the c-myc-Hook domain fusion has been checked by XbaI digestion followed by SalI restriction and has also been confirmed by sequencing with primers BACKLINK and FORLINK, whose matching sites are shown in the Fig. 3.6. The correct orientation of the c-myc-Hook domain fusion is illustrated in Fig. 3.9 together with the schematic overview of the cloning procedure and the final expression cassette for the retention of anti-OPDA scFv antibody fragments in the outer envelope chloroplastic membrane. The expression cassette was inserted between HindIII restriction sites.

Fig. 3.9 Schematic overview of cloning of the scFv gene into the pRT103 vector (a) and the final expression cassette for retention of the scFv antibody fragments in the outer envelope chloroplastic membrane (b).
3.5 *Agrobacterium tumefaciens*-mediated gene transfer

Various techniques have been used in the last decades to introduce foreign genes into plants to achieve an alteration of the properties of the recipient. These transformation techniques include transformation of protoplasts by electroporation [Lindsey and Jones, 1987], particle bombardment [Klein et al., 1987], microinjection [Reich et al., 1986] and above all transformation mediated by *Agrobacterium tumefaciens* [Zambryski et al., 1983], reviewed extensively for example in [White, 1993]. The principle of *Agrobacterium*-mediated gene transfer is based on two regions essential for the transfer, the vir region and the T-DNA region. The vir region is not transferred itself; genes of the vir region are induced by phenolic compounds produced after wounding of a plant. The T-DNA region is defined by T-DNA border repeat sequences, which are highly conserved and made up of 25bp DNA sequences. Any DNA placed between the borders will be transferred into the plant. The right border (RB) seems to play a more essential role than the left border (LB) because the T-DNA transfer starts always from RB and only around RB has always been found „overdrive“ DNA sequence that functions as T-DNA transfer enhancer. Vir region gene products recognize the border sequences, cleave T-DNA from the plasmid and direct the T-DNA, which is mostly linear single-stranded DNA, to the nucleus of the plant cell. The T-DNA covalently integrates into plant nuclear DNA and transcription is done by RNA polymerase II. The majority of T-DNA insertions by *Agrobacterium*-mediated transfer are stably inherited in normal Mendelian fashion.

3.5.1 Cloning of the expression cassettes into binary vector pBIN19

Fig. 3.10 shows an overview of four expression cassettes for targeting of anti-jasmonate scFv antibody fragments into the ER (a), into the cytosol (b), into the chloroplastic stroma (c) and on the outer envelope chloroplastic membrane (d) of tobacco cells.

![Expression cassettes](Fig 3.10)

HindIII fragments of size 1.5 kbp containing the expression cassettes were isolated from plasmids pRTRA 7/3 and pRT103 and cloned into the binary vector pBIN19 restricted
with HindIII enzyme and dephosphorylated. The scheme of the pBIN19 vector is shown in Fig. 3.11.

The plasmid contains a kanamycin bacterial selection marker and replicons for replication in both E.coli and A. tumefaciens. Between left and right border of T-DNA is a multiple restriction site including a HindIII site and the neomycin phosphotransferase II gene enabling selection of transgenic plants on kanamycin. The binary vector pBIN19 with the expression cassette was electroporated into the Agrobacterium tumefaciens that provides vir gene products in trans. A. tumefaciens strain 2260 has been used for the transformation of tobacco cells, Chapter 7.2.11. The confirmation of stable incorporation of the expression cassettes in Agrobacterium was done by the extraction of the vector, HindIII restriction and separation of 1% agarose gel followed by southern blot analysis. The HindIII fragment was hybridised with the 800bp long scFv gene-c-myc fusion restricted BamHI from the pRTRA 7/3 vector. As an example, the result of the southern blot analysis with some of the analysed agrobacterium-clones containing different scFv’s in different expression cassettes is summarised in Fig. 3.12. The selection and characterisation of transgenic plants is discussed in details in the following chapters.