Cloning and Construction of Single-chain Variable Fragments (scFv) to Cucumber Mosaic Virus and Production of Transgenic Plants

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Keywords: disease resistance, monoclonal antibodies, immunoglobulin genes

Abstract

The variable regions of heavy and light chain genes were cloned from the RNA of mouse hybridoma cells that produce monoclonal antibodies specific to Cucumber mosaic virus (CMV). The cloned genes were constructed into single-chain variable fragments (scFv) in combination with various promoters and transferred into Nicotiana benthamiana leaf tissue using Agrobacterium tumefaciens. Transgenic plants were generated and selected using the bar gene that confers resistance to phosphinothricin. From purified genomic plant DNA, the scFv was re-cloned after PCR and sequenced to verify the presence of the transgene. Resistance to CMV infection was evaluated by mechanically inoculating plants with purified CMV. Several R0 transgenic plants exhibited some degree of disease resistance in the first screening, showing no symptoms, reduced symptoms or delayed symptom development. In subsequent challenge inoculations of R1 plants with lower dilutions of inoculum, occasionally plants showed resistance when compared with non-transformed plants, but, overall, results were variable. For each of the four promoters, three or four transgenic plants were generated exhibiting some degree of disease resistance.

INTRODUCTION

Cucumber mosaic virus (CMV), a broad host range cucumovirus transmitted in nature by aphids, causes economically important diseases in many crops, including cereals, vegetables and ornamentals. Subgroup-specific mouse monoclonal antibodies (Mabs) have been produced to CMV for taxonomic and diagnostic purposes (Hsu et al., 2000). Some of these Mabs were found to neutralize virus infectivity and block virus transmission by aphids (Gera et al., 2000) making them candidates for engineering CMV resistance in plants expressing single-chain variable fragments (scFv) (Fecker et al., 1996, 1997; Tavladoraki et al., 1993; Voss et al., 1995; Zimmermann et al., 1995; Hu et al., in this issue). In the present study, immunoglobulin heavy and light chain variable regions were cloned from hybridoma cells producing these Mabs and were constructed into scFv’s. The constructs were transferred into Nicotiana benthamiana leaf tissue using Agrobacterium tumefaciens, and transgenic plants were produced. Resistance to CMV infection was evaluated by mechanically inoculating the plants with the virus.

MATERIALS AND METHODS

Promoters

The pGPTV-BAR-35S vector contains the GUS gene, nos3’ termination sequences, 35S promoter, and the bar gene, which encodes phosphinothricin acetyltransferase and confers resistance to the herbicide Basta (phosphinothricin) at 5 mg/L. The pBI505 plasmid contains the double 35S promoter with the 28 bp AMV leader peptide. The ubi4 and ubi9 promoter plasmids contain sugarcane polyubiquitin promoters (GenBank accession numbers AF093504 and AF093505), a GUS or npt gene, and nos3’

Proc. XIth IS on Virus Diseases in Ornamentals
Ed. C.A. Chang
Acta Hort. 722, ISHS 2006 129
termination sequences within a pUC vector.

**Hybridomas and RNA Purification**

Antibody production and secretion by the hybridomas was verified in ELISA tests of culture supernatants. RNA from the hybridoma cells was purified using the RNAGents kit (Promega). A DNase treatment was included, and the samples were re-precipitated in ethanol and stored at −70°C.

mRNA was purified using the PolyATtract System III (Promega) from 49-128 ug total RNA and was quantified by measuring absorbance at 260nm. From 1.8 to 3.0 ug of mRNA was purified, representing between 1.5% and 4.4% of the total RNA.

**Cloning immunoglobulin Heavy and Light Chain Variable Regions**

The Access RT-PCR kit (Promega), heavy or light chain specific primers (Table I), and the TOPO TA Cloning kit (Invitrogen) were used to clone the immunoglobulin variable regions from hybridoma RNA or mRNA. Alternatively, the RPAS system (Amersham) was used to clone these regions following manufacturer’s instructions. Sequences were deposited in Genbank with accession numbers AY556402 through AY556407.

**Subcloning and Construction of scFv’s**

Immunoglobulin gene regions were amplified from plasmid vectors by PCR, bands were purified from agarose gel slices using the Gene Clean kit (Bio101) or GenElute Gel Purification kit (Sigma) and, if necessary, concentrated using Microcon YM-100 filters (Millipore). Plasmid DNA was purified using commercial kits (Promega, QiaGen, Sigma). DNA fragments were purified from agarose gels using Gene Clean or GenElute Gel Purification Kit (Sigma).

**Agrobacterium Transformation**

*Agrobacterium tumefaciens* C58PMP90 was maintained at 28°C on LB containing gentamicin at 20 ug/ml. Production of competent *Agrobacterium* and transformation was by the method of An et al. (1988) with some modification including the use of LB media containing kanamycin (50 ug/ml) and gentamicin (20 ug/ml). Gene transfer into *N. benthamiana* was essentially by the method of Horsch et al. (1988) with some modification. Notably, leaf strips were either dipped into *Agrobacterium* for 5 sec (Method A) or fresh cuts with the scalpel were made to the leaf tissue while in the *Agrobacterium* suspension for 5-10 sec (Method B). Initial selection was on plates containing 1000 ug/ml carbenicillin, 100 ug/ml cefotaxime and 5 ug/ml Basta (Hoescht). Subsequent transfers were to plates with reduced antibiotics and then to Magenta jars containing further reduced antibiotics and no Basta.

**Screening for Disease Resistance by Virus Challenge Inoculation**

Transgenic plants rooted in soil were inoculated with either CMV-Fny (serogroup I isolate) or CMV-S (serogroup II isolate) at 0.4 ug/ml in 5 mM borate buffer pH 8 with a little celite added.

**Confirmation of the scFv in Transgenic Plants by Sequencing**

Transgenic plant DNA was purified using either GIBCO Trizol Reagent (Molecular Research Center), DNeasy Plant DNA kit (Qiagen) or GenElute Plant DNA kit (Sigma). Transgenic plant DNA (17-500 ng) was used in standard PCR reactions with AmpliTaq Gold DNA polymerase (Applied Biosystems). The amplified product was purified from PCR reactions using the Qiagen PCR Purification kit and ligated into pGEM-T Easy Vector (Promega) at RT for 1 hr. Following transformation and DNA minipreps, clones were selected for sequencing (Univ. of MD, CAB DNA Sequencing Facility).
Gene Expression in Transgenic Plants

Transgenic plant RNA was purified using either the RNeasy Plant Minikit (Qiagen) or, for more gelatinous plant extracts, Gibco Trizol Reagent. The Access RT-PCR system (Promega) and the appropriate primers were used to amplify the scFv from plant RNA. Total RNA (77–700 ng) or mRNA (24 ng) was added to each reaction, and the annealing temperature was 60°C. In one experiment, a sample of DNase-treated RNA (RQ1 RNase-free DNase, Promega) was included to ensure that no DNA was present in the sample.

RESULTS

Cloning the Heavy Chain Variable Regions

Cloning variable heavy chain regions was achieved with the heavy chain primer set for each of the four Mabs (Table I). The heavy chains were sequenced and compared with immunoglobulin sequences in GenBank by NCBI blast search. The four clones had sequences similar to other immunoglobulin heavy variable regions in GenBank. For 10F that had two amplified products, only the 433 bp fragment resembled an immunoglobulin gene. Clones that had no mutations in the primer-linker region were selected for scFv construction. For all four Mabs, cloning the heavy chain regions was also achieved with the RPAS System (Table I).

Cloning the Variable Light Chain Regions

The Promega Access RT-PCR kit and the light chain primer set were used to attempt to clone the variable light chain regions (Table I). From 1 μg of 10F RNA, a 347 bp DNA fragment was amplified and sequenced. It was found to share homology with other variable kappa light chain gene regions in GenBank. No amplified product, however, was generated from 4H or 62B RNA. A PCR product of a reasonable size was obtained from an RT-PCR reaction with 100 ng of 7B mRNA; however, its sequence data did not identify it as immunoglobulin in structure.

A second set of light chain primers based on sequences used to clone a light chain gene from a monoclonal antibody (Ohshima et al., 1994) was used to successfully clone a 375 bp variable light chain region from 7B mRNA.

For all four Mabs, cloning the light chain regions was achieved with the RPAS system using 250 ng of mRNA in each reaction. From 1.5% LE agarose gels, DNA fragments were excised and further purified using RPAS reagents.

Subcloning the 4H scFv and Transgenic Plant Production

The 4H scFv was subcloned downstream of the ubi9 promoter and ligated into pGPTV-BAR (with its 35S-GUS-nos3' sequences removed). This construct, named p4H10S2 (Fig. 1), was transformed into A. tumefaciens and transferred into N. benthamiana using method B. Twenty-two transgenic plants were produced. One showed delayed symptom development following inoculation with virus (plant #8v), and two showed reduced symptoms (#8a and #8b) (Table II).

The 4H scFv was also subcloned with the double 35S promoter and AMV leader peptide making p4H10S3. This plasmid was transformed into A. tumefaciens and transferred into N. benthamiana using method B. Nineteen transgenic plants were produced. Two plants developed milder symptoms than control plants when inoculated with virus (#6d and #6i) and two exhibited no symptoms (#6p and #6r).

Construction of 7B scFv and Transgenic Plant Production

The 7B variable heavy chain region cloned using the heavy primer set and the variable light chain region cloned using the 2nd light primer set were used to construct the 7B scFv. The heavy chain was ligated in front of the light chain and the entire scFv was inserted downstream from the ubi4 promoter and ligated into pGPTV-BAR (Fig. 1). The plasmid, p7B3S3, was then transformed into A. tumefaciens and transferred into N.
benthamiana using method B. Twenty transgenic plants were produced, and four of them developed no symptoms when inoculated with CMV-S in the first screening (#9's, Table II).

Construction of 10F scFv and Transgenic Plant Production

The variable heavy and light chain regions cloned with the heavy primer and light primer sets, respectively, were used to construct the 10F10 scFv which was subcloned into the pGPTV-BAR-35S making p10F10S2 (Fig. 1). This construct was used to transform A. tumefaciens and was transferred into N. benthamiana using method A in one experiment and method B in another. Method A produced four transgenic plant lines with one plant developing no symptoms (plant #3b), and Method B produced eleven plants, two of which exhibited reduced virus symptoms with recovery or diminishing symptoms with time (#7a and #7b, Table II).

Gene Transfer of pGPTV-BAR-GUS Vector (with no scFv) into N. benthamiana

As a control, transgenic N. benthamiana containing the pGPTV-BAR-GUS vector alone with no scFv were also produced by Method B. Freshly cut transgenic and non-transgenic N. benthamiana roots and leaves were stained for detection of GUS activity (Wilkinson and Lindsey, 1998). Color development was observed in leaves and roots of pGPTV-BAR-GUS-transgenic plants, but not in the leaves or roots of non-transformed N. benthamiana.

Detection and Sequencing of the scFv Gene in Transgenic Plants

The scFvs were amplified from transgenic plant DNA. The presence of a band of the expected size on an agarose gel indicated the plants were transgenic for scFv gene sequences. Non-transformed N. benthamiana did not produce a gel band. The scFv transgene was also amplified in the T2 generation of plant #3b. Two plant lines transgenic for the 10F scFv were selected for sequencing. In one transgenic plant (#3b), sequencing confirmed the presence of the 10F scFv. In the second transgenic plant (#7b), sequencing confirmed the presence of the 10F scFv although several mutations leading to amino acid changes were present. Two plants transgenic for the 4H scFv clones were sequenced in the heavy chain region of the scFv. The sequence was confirmed in the #6p plant. In #8v, sequencing confirmed the presence of the heavy chain although there was one point mutation which causes an amino acid change.

Gene Expression in Transgenic Plants

Access RT-PCR system and heavy chain specific primers were used to amplify the heavy chain of the 4H scFv from plant RNA (100 ng) or mRNA (24 ng) with an annealing temperature of 60°C. Also, a sample of DNase-treated RNA was included to ensure that no DNA was present in the sample. The presence of an appropriate size band on an agarose gel indicated the scFv was expressed as RNA in all eight 4H transgenic plants, but not in non-transformed plants, and not in plants containing the 10F scFv transgene.

Access RT-PCR system and heavy chain specific primers were used to amplify the heavy chain of the 10F scFv from plant RNA. Plant RNA (77-700 ng) was added to each reaction, and the annealing temperature was 60°C. The presence of an appropriate size band on an agarose gel indicated the ScFv was expressed as RNA in the three 10F transgenic plants, but not in non-transformed plants.

Seeds from the 10F transgenic plant #3b were germinated in soil, and, 10 months later, RNA was purified from the leaves. The heavy chain was amplified by RT-PCR indicating scFv was expressed as RNA in the T1 generation.

Virus Resistance

R0 plants that showed any degree of resistance to CMV infection were grown for seeds and/or cuttings were taken. Subsequent virus inoculation onto leaves of the rooted
cuttings and newly germinated seedlings often resulted in infection rates equal to non-transformed *N. benthamiana*. Only a few of these plants demonstrated disease resistance in this second round of inoculations. Transgenic plant #3b (10F scFv) did not exhibit delayed symptom development in subsequent generations even though the gene continued to be expressed. Plants #6d and #6i (4H scFv with double 35S promoter) exhibited some degree of disease resistance in that, at the lowest concentration of inoculum tested, fewer of the transgenic plants developed mosaic symptoms than the non-transformed plants.

DISCUSSION

Cloning and construction of the scFv’s was achieved for three Mabs. Cloning of the variable heavy chain region was successful with either set of primers. Difficulties arose cloning the variable light chain region from the four Mab genes since three different sets of primers were needed. Even though the RPAS system reagents were able to clone a reasonably sized product from all four antibodies, a true kappa light chain was only found for the 4H antibody. Because an scFv was produced with the RPAS system, and this method selects for scFvs which bind the antigen, the prospects for generating virus-resistant transgenic plants were high. But some degree of disease resistance was seen with only seven of the 41 4H scFv plants in the first screening, and none proved to be exceptionally disease resistant in subsequent challenge inoculations.

Problems with mutations being generated were seen throughout this study. The mutations can be easily identified in the linker region because the sequence is known. It is a reasonable assumption that if two mutations appear in the 75 bp linker-primer region, there could be 20 additional mutations in the rest of a 750 bp scFv. Even if a virus-binding scFv is selected early in the study, mutations could alter the protein’s structure and its binding ability later in the transgenic plant.

Our goal was to produce scFv constructs complete with functional promoters that produce a protein in plants which interferes with CMV infection. We were successful in constructing scFvs for three of the four Mabs. Too few plants were produced to judge whether one promoter-scFv combination was superior to another. All four promoters, 35S, ubi4, ubi9, and the double 35S with leader peptide, expressed scFv RNA and generated at least one transgenic plant exhibiting some degree of disease resistance. However, no highly resistant plants were produced.

ACKNOWLEDGEMENTS

We thank Richard W. Jones for use of the Helios Gene Gun System, and Emily Devillier, Laarni Umayag, and Sharon Jhingory for their technical support.

Literature Cited


Hu, J.S., Hsu, H.T., Xu, M.Q., Li, H.P., Wang, M., Wu, Z.C., and Borth, W.B. Transgenic plants expressing a single-chain Fv antibody to Tomato Spotted Wilt Virus (TSWV) are resistant to TSWV systemic infection. Acta Hort. This issue.


**Tables**

Table 1. Primers, source, and sizes of cloned products (approximate size determined by agarose gel electrophoresis, bp).

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Source</th>
<th>4H</th>
<th>7B</th>
<th>10F</th>
<th>62B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heavy primer set</td>
<td>Orlandi et al., 1989; Coloma et al., 1991</td>
<td>450</td>
<td>459*</td>
<td>419 + 433*</td>
<td>480</td>
</tr>
<tr>
<td>RPAS system-heavy</td>
<td>Amersham</td>
<td>350*</td>
<td>340</td>
<td>310</td>
<td>365</td>
</tr>
<tr>
<td>RPAS system-light</td>
<td>Amersham</td>
<td>240* + 325 + 420</td>
<td>340</td>
<td>325</td>
<td>355</td>
</tr>
<tr>
<td>Light primer set</td>
<td>Coloma et al., 1991;</td>
<td>No product</td>
<td>290 + 338</td>
<td>347*</td>
<td>No</td>
</tr>
<tr>
<td>2nd light primer set</td>
<td>Ohshima et al., 1994</td>
<td>N.D.</td>
<td>375*</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

*Nucleic acid sequence is homologous with other immunoglobulins in Genbank
N.D. = not determined
Table 2. Disease resistance in transgenic *Nicotiana benthamiana* plants following the first challenge inoculation with *Cucumber mosaic virus*.

<table>
<thead>
<tr>
<th>Promoter and scFv</th>
<th>Number of transgenic plant lines</th>
<th>Plants exhibiting disease resistance</th>
<th>Delayed symptom development</th>
<th>Reduced symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>ubi9-4H scFv</td>
<td>22</td>
<td>No symptoms</td>
<td>8v</td>
<td>8a,8b</td>
</tr>
<tr>
<td>35S-35S-4H scFv</td>
<td>19</td>
<td>6r,6p</td>
<td>6d,6i</td>
<td></td>
</tr>
<tr>
<td>ubi4-7B scFv</td>
<td>20*</td>
<td>9c,9g,9h,9k</td>
<td>9a,9b</td>
<td></td>
</tr>
<tr>
<td>35S-10F scFv</td>
<td>4</td>
<td>3b</td>
<td>7a,7b</td>
<td></td>
</tr>
<tr>
<td>35S-10F scFv</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*These plants were inoculated with CMV-S, a serogroup II isolate, and infection in *N. benthamiana* was symptomless or very faint. Infection was diagnosed by the ImmunoStrip Test 8 days after inoculation. Four plants were negative.

*These transgenic plants were produced by gene transfer method A. All other plants were produced by gene transfer method B.

Figures

![Fig. 1. Constructs of the three scFv’s and either the ubi9 promoter, double 35S promoter with leader peptide, ubi4 promoter, or 35S promoter. The BAR gene is under control of the Pnos promoter.](image-url)