Targeted expression of single-chain antibody inhibits the accumulation of Beet necrotic yellow vein virus in Nicotiana benthamiana

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ABSTRACT
Rhizomania is one of the most damaging sugar beet diseases and is caused by Beet necrotic yellow vein virus (BNYVV). In order to interfere with viral propagation, the expression of recombinant antibody strategy was employed. The coding sequence of a single-chain variable fragment (scFv) specific to the main BNYVV coat protein, P21, was targeted to the cytosol, apoplast or outer membrane of mitochondrion of Nicotiana benthamiana plants. Also, an endoplasmic reticulum retention signal peptide, KDEL, was added at the C-terminal of the scFv protein which presumably stabilizes it in cytoplasm. Although the accumulation levels of scFv could not be correlated to the levels of the inhibition of viral accumulation, the titer of the virus in all groups of transgenic plants was significantly lower than the wild type ones when grown in BNYVV-infested soil. No significant differences were found in the number of resistant plants targeting P21-scFv in either putative subcellular locations.

Introduction
Rhizomania is the most devastating and prevalent viral disease of sugar beet caused by Beet necrotic yellow vein virus (BNYVV). This virus is classified into the genus Benyvirus and is transmitted by Polymyxa betae Keskin, a soil-borne plasmidiphorid with thick-walled resting spores allowing persistence in the soil for many years (Abe and Tamada 1986). Although a reliable way to keep a profitable yield in BNYVV-infested fields has been achieved through the introduction of resistant varieties, some recent reports have revealed resistance breaks in specific regions (McGrann et al. 2009).

Expression of recombinant antibodies (rAbs) in plants is one of the promising approaches to immunomodulate viral resistance, particularly by using single-chain variable fragments (scFv). Such molecules comprise only the variable domains of heavy and light chains linked by a small flexible peptide. The scFv retains antigen specificity and blocking function of the full-size antibody, with no need for complex protein folding and post-translational assembly.

The rAb potential to prevent plant viral disease was initially revealed in transgenic tobacco plants that accumulated a coat-protein specific scFv against Artichoke mottled crinkle virus (Tavladoraki et al. 1993). Since then, antibody-based resistance has been exploited for immunomodulation of several viral diseases by expressing rAbs raised against structural viral proteins such as coat proteins (Voss et al. 1995; Fecker et al. 1996; Zimmermann et al. 1998; Xiao et al. 2000; Villani et al. 2005; Nölke et al. 2009; Cervera et al. 2010; Ghanam et al. 2013) as well as non-structural viral proteins such as RNA replicase (Boonrod et al. 2004; Gil et al. 2011), Nla, p1 protease (Gargouri-Bouzid et al. 2006; Nickel et al. 2008; Bouaziz et al. 2009; Ayadi et al. 2012) and movement proteins (Prins et al. 2005; Zhang et al. 2008).

Cytosol is a desirable place for targeting viruses where the majority of the viral life cycle occur; however, the stability of antibodies in this cellular compartment has been questioned (Schillberg et al. 1999). Low or undetectable accumulation levels of scFv have often been reported in the cytosol of plant cells (Conrad and Fiedler 1998; Zimmermann et al. 1998; Dejaeger et al. 2000; Fischer et al. 2001). Therefore, targeting scFv to the other specific cellular compartments such as the apoplast where oxidizing environment leads to much more efficient folding and assembling has been of interest.

During the initial stages of infection, BNYVV particles are found transiently anchored to cytosolic surfaces of mitochondria. Assembly of BNYVV particles likely takes place on mitochondria surfaces, recruiting energy supplied by this organelle (Valentin et al. 2005). ORF2 of BNYVV RNA2 encodes a 75-kDa minor coat protein, P75, as a translational readthrough (RTD) product of the major coat protein (P21) stop codon. Bioinformatic and experimental analyses detected a mitochondrial target sequence and several trans-membrane motifs in N-terminal region of the P75 RTD. These sequences are involved in targeting and anchoring of virions to mitochondria (Valentin et al. 2005).

As reported previously, a cDNA encoding scFv antibody derived from a phage display library that specifically interacted with the BNYVV P21 coat protein was engineered and characterized (Jahromi et al. 2009). In this study, the scFv antibody was directed to the cytosol, apoplast or outer membrane of mitochondrion where the viral coat proteins potentially accumulate abundantly. T1 transgenic plants were reproduced and challenged with BNYVV for viral resistance assessments.
Materials and methods

Construction of the scFv-expression vectors

A synthetic sequence comprising chalcon synthase 5'-untranslated region (5'-UTR) (Voss et al. 1995) and a proper Kozak's nucleotide context for translation initiation in plants, the signal peptide of the polygalacturonase-inhibiting protein (PGIP) of Phaseolus vulgaris, a mitochondrial targeting sequences (MTS) as well as three transmembrane regions (TM1, TM3 and TM4) encoded by BNYVV, six histidine and KDEL tetrapeptide together with the appropriate restriction enzyme sites was designed (Figure 1). The synthetic sequence was excised from pGH plasmid by XhoI/ PstI digestion and cloned into the same sites of digested pHANNIBAL (Wesley et al. 2001) to produce pH plasmid. pTZ57R/T (InsT/Aclon PCR product cloning kit; Fermentas) plasmid was used to clone a PCR-product comprising the scFv fused to six-histidine epitope (Jahromi et al. 2009), named pTZ57-scFv. An Ncol/XbaI fragment carrying scFv-His tag sequence was then transferred into the corresponding sites of pPH to generate pHA apoplastic plasmid. To produce cytosolic targeting pHCC construct, pH was digested with HindIII to eliminate signal peptide-encoding sequence. For construction of pHC, the Ncol/NcoI scFv fragment from pTZ57-scFv was inserted at the same restriction sites of pHG. Then, scFv-His-tag fragment of pHCC was replaced by Ncol/XbaI fragment from pGH plasmid carrying scFv-KDEL-His. To construct pHM mitochondrial plasmid, pHG was digested with HindIII and Ncol and the resulting MTS - TM fragment was ligated into the corresponding sites of pHCC. The Salt/XbaI fragments from all four resulting constructs were ligated at the XhoI/XbaI restriction sites of pINT binary vector (Zare et al. 2015) between Cauliflower mosaic virus 35S promoter and octopine synthase terminator to form pLCC, pIC, pIA, pIM constructs containing scFv-His (CC), scFv-KDEL-His (C), SP-scFv-His (A) and MTS + TM-scFv-His (M) cassettes, respectively (Figure 1).

Plant transformation

The above constructs were transferred into Agrobacterium tumefaciens GV3101 cells as described (Holsters et al. 1978). Transgenic N. benthamiana plants were obtained through the leaf disc transformation method (Horsch et al. 1985). The regenerated plants were screened by PCR to confirm transformation. Seeds were collected from self-fertilized T0 plants and germinated on MS medium (Murashige and Skoog 1962) containing 150 mg L\(^{-1}\) kanamycin. Those lines indicating 3:1 inheritance of T-DNA were selected as plants with single locus gene insertion for further analyses.

DNA analysis

To verify the existence of the transgenes in transformed plants, genomic DNA was isolated from leaves as described Edwards et al. (1991) with minor modifications. The integration of the transgenes into the plant genome was revealed through the leaf disc transformation method (Horsch et al. 1985). The regenerated plants were screened by PCR to confirm transformation. Seeds were collected from self-fertilized T0 plants and germinated on MS medium (Murashige and Skoog 1962) containing 150 mg L\(^{-1}\) kanamycin. Those lines indicating 3:1 inheritance of T-DNA were selected as plants with single locus gene insertion for further analyses.

Analysis of the scFv expression

Total soluble proteins were extracted from 1 g of root material pulverized in liquid nitrogen to a fine powder and adding 0.5 ml protein extraction buffer containing 100 mM Tris-HCl (pH 7.4), 500 mM NaCl, 5 mM EDTA, 5 mM DTT, 0.25 mM PMSF, 5% β-mercaptoethanol. Amounts of protein were calculated using the Bradford method (Bradford 1976). Around 40 µg total soluble protein extracts were separated on 12% SDS-PAGE. After the electrophoresis, proteins were electrotransferred onto PVDF membrane (Roche Biochemical). For Immunodetection, the membrane was probed with Penta-His MAb (Qiagen) detected by a goat anti-mouse IgG conjugated with HRP (Jackson Immuno Research) coupled to ECL Western Blotting Detection Kit (Amersham).

Viral inoculations and detection

For inoculation with BNYVV, T1 seedlings were transplanted into a 1:1 mixture of soil and sand composed from rhizomania-infested farms near Shiraz City, I. R. Iran. Plants were kept in the greenhouse at 25°C and a 16-h photoperiod. BNYVV contents were estimated two months after inoculation by DAS-ELISA method (Clark and Adams 1977). In all experiments, BNYVV-infected wild-type N. benthamiana plants and non-infected ones were included as positive and negative controls, respectively. Anti-BNYVV P21-specific antibodies and alkaline phosphatase conjugated to antirabbit antibodies were from Shiraz University (Shiraz, I.R. Iran). The cutoff value was defined as the mean absorbance values at 405 nm plus three standard deviations obtained for the healthy plants. Values below the cutoff were judged as resistance trait for the examined plants.

Statistical analysis

The statistical significance of means of ELISA values indicating the virus contents in each transgenic event or construct was shown by the analysis of variance (ANOVA) using SPSS v. 16 software. Duncan’s multiple range tests at P < 0.05 were used for mean comparisons. Segregation analysis was performed using Chi-square (χ\(^2\)) test in which the observed values were compared to the expected Mendelian 3:1 ratio corresponding to single locus integration.

Results

Generations of transgenic plants expressing scFv protein

scFv cDNAs were fused with four molecular signals targeting to various cellular locations (Figure 1). PGIP signal sequence and BNYVV MTS and TM sequences were used for targeting scFv to the apoplast (A cassette) and mitochondrial outer membrane (M), respectively. Leaderless forms of scFv in CC and C cassettes were made for cytosolic targeting. In the latter, KDEL tetrapeptide was fused at the C-terminus of scFv which presumably protect it from proteolytic processes (Schouten et al. 1996, 1997). Besides, an efficient 5’-UTR derived from PGIP protein of Phaseolus vulgaris...
was placed upstream of scFv in all constructs to improve mRNA translation efficiency.

Over twenty T0 transgenic events for each construct were self-pollinated and T1 progenies were screened for kanamycin resistance to identify transgenic events with single locus insertions. Two C, five CC, six A and seven M transgenic events showing 3:1 Mendelian ratios for kanamycin resistance trait were selected for further experiments.

Expression of scFv antibody in transgenic plants

Western blot analysis was carried out to monitor the expression levels of the scFv in T1 transgenic plants as shown in Figure 2. A protein band was detected for scFv at approximately 35 kDa in the root extracts which is heavier than the predicted size, 31 kDa. It is possible that posttranslational modifications of protein shifted the position of the scFv as previously reported for other scFvs (Cervera et al. 2010). All examined transgenic plants displayed detectable levels of scFv protein, although variable expression rates were observed.

Comparing viral accumulation in transgenic plants

Seven to ten T1 progenies from each transgenic event were challenged in BNYVV-infested soil. After two months, the virus titers in the roots and leaves were determined by ELISA method. The presence of virus coat protein was not detectable in the leaves of non-transgenic control plants (data not shown) whereas their roots were highly susceptible to virus infection. The means of viral accumulation of all the challenged transgenic events transformed with cytosolic, apoplastic or mitochondrial targeting cassettes were significantly different from the infected wild-type plants, except for M4 and M50 events. While almost all transgenic events carrying M cassette and also event CC50 presented accumulation values somewhat between the infected and non-infected wild-type plants, the other transgenic events produced values that were not significantly higher than the non-infected wild-type plants (Figure 3).

BNYVV inhibition by expressing scFv in different subcellular locations

The efficacy of each cassette was assessed by looking at the percentages of immunized plants against the infection as shown in Figure 4. All the transgenic plants showed a significantly higher number of immunized plants than the infected wild-type one ranging from 45.2% to 58.57%. There were no significant differences among the number of immunized plants carrying either construct.

Discussion

Here, we have shown that putative cytosolic, apoplastic and mitochondrial expressions of the scFv against BNYVV coat protein led to reduced viral accumulation in transgenic N. benthamiana plants. To our knowledge, this is the first successful report on the interference with BNYVV infection that introduce a new possibility for the control of this economically important virus. Fecker et al. (1996) expressed scFv genes originated from hybridoma against BNYVV coat
protein and 25 kDa. Transgenic plants expressing CP-specific scFv in the endoplasmic reticulum displayed a delay of virus infection. However, eventually all scFv-expressing plants became infected within 30 days after inoculation (Fecker et al. 1997). Whereas almost all the examined scFv-expressing events in this study strongly inhibited viral accumulation for the duration of experiment (60 days) while continuously exposed to the BNYVV-transmitting P. betae.

Effective antibody mediated resistance depends on targeting of recombinant antibodies in the appropriate cell compartment where the relevant antigen is present. Expression levels as well as the stability and binding affinity to proper target protein are the other contributing factors. Moreover, the antibody fragments generated via phage display is believed to be more stable in the cytosol than the hybridoma-derived ones as demonstrated before (De Jaeger et al. 2000; Schillberg et al. 2001). Furthermore, the use of 5′-UTR and Kozak sequences could contribute to higher expression of the scFv proteins.

In this work, we could not find clear associations between scFv accumulation levels as shown by western blotting and viral titers (Figures 2 and 3). Generally, events carrying A cassette displayed moderate levels of scFv, while M plants showed the least amounts of the scFv accumulation. In contrast, C and CC plants showed the highest levels of scFv accumulation in the cytosol.

Cytosol is usually considered as an ideal place for neutralizing viral infection where the crucial steps of virus life cycles occur. It is assumed that the reducing environment of cytosol and lack of chaperons discourage stability, accurate folding and assembly of antibodies. Nevertheless, broad range of cytosolic accumulation levels from barely detectable (for a review, see Safarnejad et al. 2011) to high amounts of total soluble proteins have been shown (Nölke et al. 2005; Prins et al. 2005; Villani et al. 2005; Zhang et al. 2008), suggesting that specific properties of each scFv may affect their stability and efficacy.

Some small peptide like the KDEL sequence might protect the proteins from C-terminal degradation by proteinases (Schouten et al. 1996, 1997). In this work, we did not distinguish any differences in the expression levels and resistance rates related to this motif (Figures 2 and 4). Similarly, other researchers have challenged this idea as they did not find any effect of using the same motif (Malembic-Maher et al. 2005).

It has already been shown that remarkable enhancement in antibody accumulation occurs by apoplast targeting (Owen et al. 1992; Conrad and Fiedler 1998; Zimmermann et al. 1998; Malembic-Maher et al. 2005). It is proposed that antibodies assemble and fold much more efficiently in the apoplast than the cytosol. In this research, transgenic plants producing antibodies putatively targeted to apoplast exhibited various rates of viral inhibition, all of which ranked with the non-infected wild-type plants. Indeed, we did not find a remarkable difference between cytosolic and apoplastic...
scFv accumulation levels. It could be hypothesized that A-po- 
plastic scFv would be able to block intercellular movements of 
viral components and, thereby, reduce the spread of infection 
to the neighboring cells. Alternatively, scFv may diffuse from 
apoplastic space to the cytoplasm through disrupted routes or 
lesion caused by virus vector. As shown before, BNYVV par-
ticles confined to the cytoplasmic surfaces of mitochondria at 
the early times of infection and assembly of the virus occurs 
on this organelle. They are later released from mitochondria 
and relocated to the cytoplasm (Erhardt et al. 2001; Valentin 
et al. 2005). Transgenic N. benthamiana plants expressing 
mitochondrion-targeted scFv showed a partial protection 
level against BNYVV possibly due to interference with coat 
protein of virus at primary stages of postinfection.

In conclusion, our data show that expression of scFv anti-
bodies directed against BNYVV P21 targeted to different cel-
lar locations confer virus resistance in the model plant N. 
 benthamiana offering a powerful approach to generate 
 virus-resistant sugar beet varieties.

Acknowledgments
We greatly thank Dr. Mehdi Arbabi for kindly providing BNYVV coat 
protein-specific single chain antibody. This work was financed by the 
National Institute of Genetic Engineering and Biotechnology of I.R. Iran.

Disclosure statement
No potential conflict of interest was reported by the authors.

Funding
This work was supported by National Institute of Genetic Engineering 
and Biotechnology of I.R. Iran.

Compliance with ethical standards
All authors declare no conflict of interest. This article does not 
contain any studies with human participants or animals 
performed by any of the authors.

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