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Analysis of the interaction between single-chain variable fragments and their antigen in a reducing intracellular environment using the two-hybrid system

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Abstract The coding sequences of three single-chain variable (scFv) fragments (A4, G4 and H3), which bind to dihydroflavonol-4-reductase (DFR) of *Petunia hybrida*, and the DFR-encoding sequence were cloned in two-hybrid vectors. The vectors were transformed in the yeast strain HF7c (*his3-200*, *trp1-901*, *leu2-3*) and the scFv-DFR interaction was analyzed by measuring yeast growth on medium without histidine. ScFv-G4 and, to a lesser extent, scFv-A4 could interact with DFR in the yeast nucleus. On the contrary, scFv-H3 showed no interaction with its antigen in yeast. The results of a previous expression analysis of the same scFv fragments in the plant cytosol correlate with those of the two-hybrid test. This suggests that it is possible to evaluate the antigen-scFv interaction in a reducing subcellular environment with the two-hybrid test. Therefore, the yeast two-hybrid system can be useful to identify candidate scFv fragments for intracellular antibody applications.

Key words: Dihydroflavonol-4-reductase; Two-hybrid; Immunomodulation; Reducing subcellular environment; Single-chain variable fragment

1. Introduction

Various reports have shown that the accumulation capacity and antigen-binding properties of single-chain variable (scFv) fragments in the cytosol of the plant cell are highly dependent on the intrinsic characteristics of the expressed scFv fragment, making the scFv's intracellular performance as yet unpredictable (for a review, see [1,2]). So far, only few scFv fragments were found to accumulate to high levels in the cytosol [1,3,4]. Several scFv fragments do not accumulate to detectable levels in the cytosol, even when the corresponding mRNA levels are high [5–7], showing that the low protein accumulation levels result from instability, inefficient synthesis or bad folding of the proteins. Moreover, some scFv fragments with a high accumulation capacity in the cytosol can have a low antigen-binding affinity [1]. The absence of proper disulfide bond formation in the cytosol might destabilize the scFv fragments or induce conformational changes that inactivate the scFv protein or lower its affinity.

When plant-made scFv fragments are intended for immunomodulation of antigens present in the cytosol, it is a prerequisite that they are able to accumulate and to bind their antigen in the cytosol. Consequently, a quick assay is needed to analyze the isolated scFv fragments for their accumulation capacity and antigen-binding properties in the cytosol. For this purpose, we tested an in vivo assay based on the two-hybrid system because in such a system, the protein interactions occur in the reducing environment of the cytosol and the nucleus. The murine scFv fragments A4, G4 and H3 binding to dihydroflavonol-4-reductase (DFR) from *Petunia hybrida* [8] were chosen as model proteins. After extraction from tobacco leaves that transiently express these scFv fragments and from stably transformed petunia plants, the accumulation capacity and antigen-binding activity of these three scFv fragments were shown to differ upon expression in the plant cytosol [1]. Both scFv-A4 and scFv-G4 can accumulate to high levels in the cytosol, reaching 0.3% and 1% of total soluble protein (TSP) in transgenic petals, respectively. However, when their antigen-binding activity is compared after extraction from the plant cytosol (in absence of disulfide bonds) and after extraction from the periplasm of *Escherichia coli* (in presence of disulfide bonds), both scFv fragments clearly differ. ScFv-G4 has a similar antigen-binding affinity in the absence as well as in the presence of disulfide bonds, whereas scFv-A4 has a much lower antigen-binding affinity in the absence than in presence of disulfide bonds. ScFv-H3 accumulation is approximately 10-fold lower, reaching 0.05% of TSP in petals. The cytosolic accumulation of H3 was too low both transiently and in stably transformed plants [1] to properly check its antigen-binding activity after extraction. Here, the interaction between these scFv fragments and their antigen is analyzed in a reducing intracellular environment by using the two-hybrid system in yeast.

2. Materials and methods

2.1. Cloning of the scFv- and DFR-encoding sequences in the two-hybrid vectors pGAD424 and pGBT9

The two-hybrid vector pGAD424 (Clontech, Palo Alto, CA, USA) was adapted for cloning scFv-encoding sequences. Therefore, an oligonucleotide was designed containing the restriction sites *Sfi*I and *Not*I separated by an *Eco*RV site and flanked by an *Eco*RI site and a *Bam*HI site, respectively. The oligonucleotide was cloned between the restriction sites *Eco*RI and *Bam*HI from pGAD424. The coding sequences of the scFv fragments A4, G4 and H3 were cut from the phage display vectors pHENI-A4, pHENI-G4 and pHENI-H3 [8] by using the restriction sites *Sfi*I and *Not*I and cloned in the adapted pGAD424 vector. The resulting plasmids pGAD-A4, pGAD-G4

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Abbreviations: DFR, dihydroflavonol-4-reductase; OD, optical density; scFv, single-chain variable fragment; TSP, total soluble protein

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and pGAD-H3 contain the scFv-encoding sequences fused in frame with the coding sequence of the activating domain from the yeast transcription factor GAL4. The DFR-encoding sequence was cut from the construct pMAL-DFR with the restriction sites *Eco*RI and *Pst*I. The pMAL-DFR construct consists of the pMAL-c2 expression vector (New England Biolabs, Beverly, MA, USA) containing the DFR-encoding sequence cloned between the restriction sites *Eco*RI and *Xba*I of the pMAL polylinker. The DFR-encoding DNA fragment was cloned in the two-hybrid vector pGBT9 (Clontech) with the restriction sites *Eco*RI and *Pst*I. The resulting plasmid pGBT-DFR contains the DFR-encoding sequence fused in frame with the coding sequence of the DNA-binding domain of GAL4.

2.2. Transformation of Saccharomyces cerevisiae HF7c

Competent cells of the *S. cerevisiae* strain HF7c were prepared using the lithium acetate method [9]. Transformation was performed by heat shock as described in the Matchmaker Library Protocol (Clontech). Transformants were selected on SD agar medium without leucine and tryptophan (SD/−Leu/−Trp). Plates were incubated at 30°C for 3 days.

2.3. Two-hybrid analysis

The interaction between the scFv fragments and the DFR antigen was studied by three different assays. (i) By streaking on selective medium: single transformed colonies were picked from the SD/−Leu/−Trp agar plates and streaked on a quadrant of a SD/−Leu/−Trp agar plate and of a plate containing SD agar medium lacking leucine, tryptophan and histidine (SD/−Leu/−Trp/−His). Plates were incubated for 2 days at 30°C. (ii) By plating on selective medium: single transformed colonies were picked from the SD/−Leu/−Trp agar plates, resuspended in 1 ml SD/−Leu/−Trp/−His medium, of which 100 μl was plated on SD/−Leu/−Trp and SD/−Leu/−Trp/−His agar plates. Plates were incubated at 30°C for 3 days. (iii) By growing in selective medium: single transformed colonies were resuspended in SD/−Leu/−Trp and SD/−Leu/−Trp/−His liquid medium and incubated by shaking at 30°C. Yeast growth was followed spectrophotometrically by measuring the optical density at 600 nm (OD_{600}) at several time points after inoculation.

3. Results

We analyzed the scFv fragments A4, G4 and H3 for binding their antigen DFR in vivo by using the yeast two-hybrid system. The coding sequences of the three scFv fragments were cloned in the two-hybrid vector pGAD424 resulting in the vectors pGAD-A4, pGAD-G4 and pGAD-H3 expressing the GAL4 activation domain-scFv fusion. The coding sequence of the antigen DFR was cloned in the two-hybrid vector pGBT9 resulting in the vector pGBT-DFR expressing the GAL4 DNA-binding domain-DFR fusion. The yeast strain HF7c was cotransformed with pGBT-DFR and the vectors pGAD-A4, pGAD-G4 and pGAD-H3 and transformants were selected on −Leu/−Trp medium. The auxotrophy for leucine and tryptophan of yeast HF7c could be complemented by introduction of the vectors pGAD424 and pGBT9 which contain a *LEU2* and *TRP1* gene, respectively. As negative controls, we cotransformed each of the three recombinant pGAD vectors with the empty pGBT9 vector and the pGBT-DFR vector together with the empty pGAD424 vector.

Using these transformants, three different two-hybrid assays were performed. In the first two assays, we analyzed yeast growth on agar plates containing −Leu/−Trp/−His selective medium and in the third assay in liquid −Leu/−Trp/−His selective medium. The auxotrophy for histidine of HF7c could be complemented by expression of the genomic *HIS3* reporter gene under control of a GAL4-responsive promoter. Expression of the *HIS3* reporter gene could be achieved solely by reconstitution of a functional GAL4 transcriptional activator, thus by interaction of the scFv fragment with its antigen.

In a first assay, transformants were streaked on agar plates containing −Leu/−Trp medium and −Leu/−Trp/−His medium. None of the transformants lacking the DFR-encoding sequence (pGAD-A4+pGBT9, pGAD-G4+pGBT9 and pGAD-H3+pGBT9) grew on −Leu/−Trp/−His medium (Fig. 1). However, when the antigen DFR-encoding sequence was present, the transformant containing pGAD-G4+pGBT-DFR and, to a lesser extent, also the transformant containing pGAD-A4+pGBT-DFR grew on −Leu/−Trp/−His medium. No significant growth could be observed on −Leu/−Trp/−His medium for the pGAD-H3+pGBT-DFR (Fig. 1) nor for the pGBT-DFR+pGAD424 transformants (result not shown).

In a second assay, single transformed colonies were picked from −Leu/−Trp medium, resuspended and plated on −Leu/−Trp and −Leu/−Trp/−His media. After incubation, colonies were detected on −Leu/−Trp/−His medium for the transformants containing pGAD-A4+pGBT-DFR and pGAD-G4+pGBT-DFR, but not for those with pGAD-H3+pGBT-DFR or for control strain pGAD424+pGBT-DFR (Table 1). Besides, for the transformant containing pGAD-G4+pGBT-DFR, similar amounts of colonies could be counted on −Leu/−Trp and −Leu/−Trp/−His media, whereas for the pGAD-A4+pGBT-DFR transformant only approximately 10% of the colonies on −Leu/−Trp could be counted on −Leu/−Trp/−His medium after 3 days of incubation (Table 1). When the pGAD-A4+pGBT-DFR transformants were further incubated, finally similar amounts of colonies could be counted on −Leu/−Trp and −Leu/−Trp/−His media. At that time, still no colonies could be counted for the pGAD-H3+pGBT-DFR transformants. Hence, most yeast transformants containing pGAD-A4+pGBT-DFR just grew more slowly on −Leu/−Trp/−His than on −Leu/−Trp medium. However, we have no explanation for the observation that part of the yeast cells from a single clone, trans-

Table 1
Two-hybrid analysis by plating yeast transformants on selective medium

Yeast strain	Colonies on +His	Colonies on −His
pGAD-GA+pGBT-DFR	820	1002
pGAD-A4+pGBT-DFR	998	112
pGAD-H3+pGBT-DFR	1000	0
pGAD424+pGBT-DFR	1000	0

Yeast HF7c cells were cotransformed with the plasmid pGBT-DFR, which encodes the GAL4 DNA-binding domain-DFR fusion, and with a pGAD plasmid expressing a GAL4 activation domain-scFv (A4, G4 or H3) fusion or with the pGAD424 plasmid as a control. Transformed colonies were resuspended and plated on medium with (+His) or without (−His) histidine. After incubation, colonies were counted.

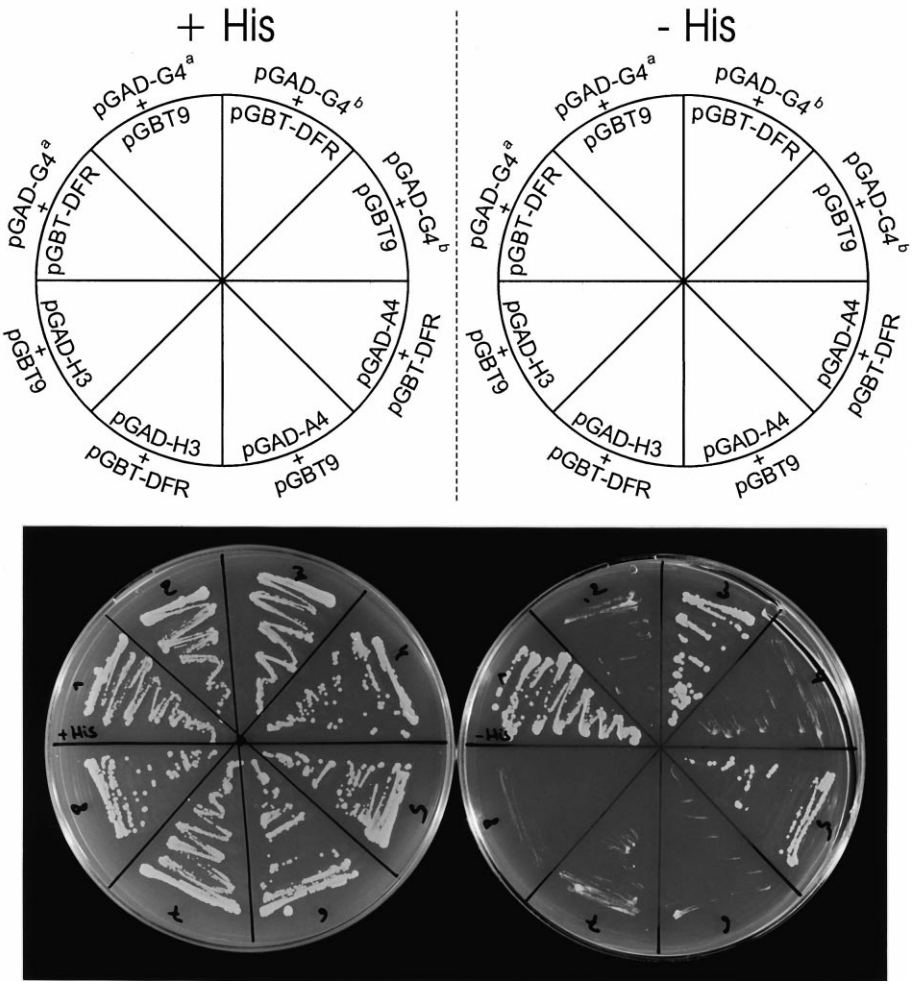

Fig. 1. Two-hybrid analysis by streaking yeast transformants on selective medium. Yeast HF7c cells were cotransformed with a pGAD plasmid expressing a GAL4 activation domain-scFv (A4, G4 or H3) fusion and with the pGBT-DFR plasmid, which encodes the GAL4 DNA-binding domain-DFR fusion, or with the pGBT9 plasmid as a control. Transformants were streaked on plates with (+His) or without (−His) histidine. Reconstitution of GAL4 activity restores the ability to grow on −His medium. The plasmid pGAD-G4[a] was constructed as described in Section 2.1. The plasmid pGAD-G4[b] is a variant of pGAD-G4[a] containing the same scFv fragment G4, but with an additional C-terminal c-*myc* tag.

formed with pGAD-A4+pGBT-DFR, grew faster on −Leu/−Trp/−His medium compared to the remaining cells of the clone.

In a third assay, single transformed colonies were inoculated in −Leu/−Trp and −Leu/−Trp/−His media and yeast growth was followed spectrophotometrically (Fig. 2). Only the transformant containing pGAD-G4+pGBT-DFR grew equally fast in both media, while the transformant with pGAD-A4+pGBT-DFR grew much slower in −Leu/−Trp/−His than in −Leu/−Trp medium. For the transformant containing pGAD-H3+pGBT-DFR, no growth could be measured in −Leu/−Trp/−His medium (Fig. 2A,B). As expected, the other control strains grew in −Leu/−Trp, but not in −Leu/−Trp/−His medium (Fig. 2C,D).

Together, these results show that scFv-G4 can interact with its antigen in the yeast nucleus. The same observation is true for scFv-A4, but the interaction is less pronounced. ScFv-H3, on the contrary, does not interact with DFR in two-hybrid vectors.

4. Discussion

In general, we can conclude that the results from the two-hybrid tests correlate with those from a previous expression analysis in the plant cytosol [1]. In the plant cytosol, only scFv-G4 can accumulate to high levels and keep its antigen-binding activity, whereas in two-hybrid scFv-G4 performs best. In the plant cytosol, scFv-A4 exhibits an accumulation capacity similar to that of scFv-G4, but has a low antigen-binding activity; in two-hybrid, scFv-A4 interacts with the DFR antigen, but less than scFv-G4. In the plant cytosol, scFv-H3 accumulates to low levels, whereas in two-hybrid no interaction can be demonstrated. The difference in intracellular performance of the three scFv fragments could be best observed and quantified in the plating (Table 1) and the liquid medium assays (Fig. 2).

The results presented here show that the use of two-hybrid can demonstrate the in vivo interaction between antibody fragments and their antigen. In this way, the identification

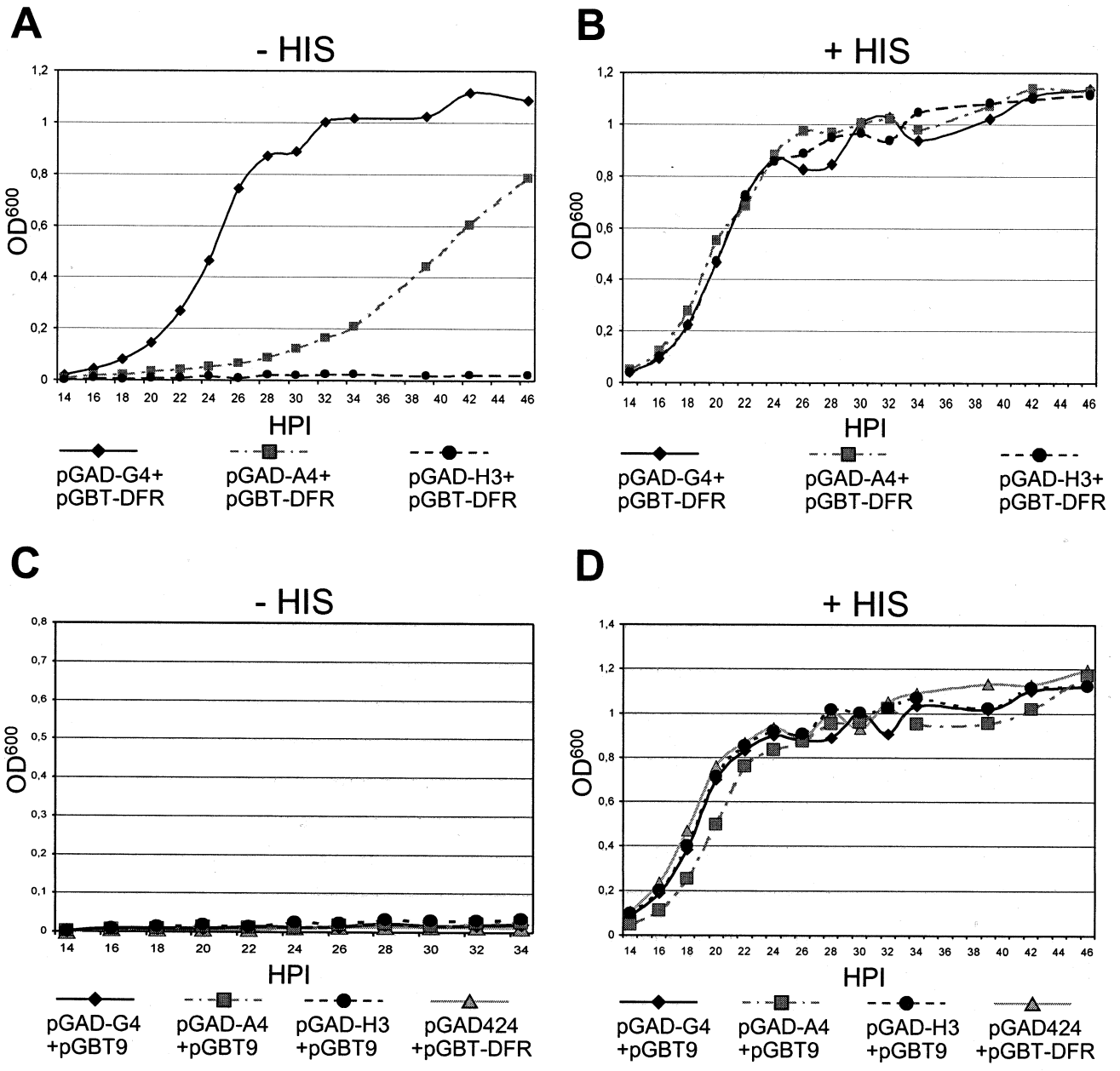

Fig. 2. Two-hybrid analysis by growing yeast transformants in liquid medium. Yeast HF7c cells were cotransformed with a pGAD plasmid expressing a GAL4 activation domain-scFv (A4, G4 or H3) fusion and with the pGBT-DFR plasmid, expressing the GAL4 DNA-binding domain-DFR fusion, or with the pGBT9 plasmid as a control. Transformants were grown in liquid medium with (+His) or without (−His) histidine. OD_{600} was measured spectrophotometrically at several time points after inoculation (HPI) and a growth curve was set up.

of candidate antibodies for intracellular applications can be facilitated, as also recently shown by Visintin et al. [10]. This is an important advance because scFv stability and antigen-binding activity in a reducing environment are the main bottle necks for the general application of immunomodulation. In the future, a combination of phage display technology and two-hybrid could be used to immediately isolate scFv fragments that are stable in the cytosol. A first selection on a large scFv library could be performed by phage display based on specificity for the antigen and a second selection could be performed by two-hybrid analysis.

The observation that G4 can interact with its antigen in the yeast nucleus confirms that this scFv fragment keeps its antigen-binding affinity in a reducing intracellular environment [1]. Hence, G4 could be applied as a scFv scaffold for the grafting of the complementarity-determining regions from scFv fragments that are unstable after expression in the cytosol or the nucleus. As such, the stability of G4 could be combined with the specificity of the unstable scFv.

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