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The nucleoprotein of *Tomato spotted wilt virus* as protein tag for easy purification and enhanced production of recombinant proteins in plants

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Abstract

Upon infection, Tomato spotted wilt virus (TSWV) forms ribonucleoprotein particles (RNPs) that consist of nucleoprotein (N) and viral RNA. These aggregates result from the homopolymerization of the N protein, and are highly stable in plant cells. These properties feature the N protein as a potentially useful protein fusion partner. To evaluate this potential, the N protein was fused to the *Aequorea victoria* green fluorescent protein (GFP), either at the amino or carboxy terminus, and expressed in plants from binary vectors in *Nicotiana benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* and evaluated after 4 days, revealing an intense GFP fluorescence under UV light. Microscopic analysis revealed that upon expression of the GFP:N fusion a small number of large aggregates were formed, whereas N:GFP expression led to a large number of smaller aggregates scattered throughout the cytoplasm. A simple purification method was tested, based on centrifugation and filtration, yielding a gross extract that contained large amounts of N:GFP aggregates, as confirmed by GFP fluorescence and Western blot analysis. These results show that the homopolymerization properties of the N protein can be used as a fast and simple way to purify large amounts of proteins from plants.

Keywords: Nucleoprotein; Fusion proteins; Transient expression; Green fluorescent protein; Plant

Molecular farming is arising as an important expression system for the production of high-valued recombinant proteins in plants. Over the last 10–15 years a large number of heterologous proteins have been expressed in plants, including antibodies, antigens, and other proteins of therapeutic and industrial application [1,2]. The low costs, easy scale-up production and safety are some main advantages of this expression system as compared to other production systems based on microorganisms or mammalian cell culture [3,4]. A cost analysis for producing β-glucuronidase (GUS) in corn seeds estimated the cost per gram to be approximately 10 times lower than if production was carried out in *Escherichia coli* [3]. It was also estimated that the purification cost accounted for 94% of the total cost, being directly dependent on the expression level of the target protein [5]. Considering that heterologous protein expression in plants is usually low, ranging from <0.1 to 1% of the total soluble protein, cost of purification can determine the commercial viability of a plant based production platform for recombinant proteins [6].

Much of the research on molecular farming has focused on protein expression level and post-translational modification of glycoproteins, which are the major technical barriers [2,7,8]. Increasing recombinant protein expression levels has been tackled by different approaches, including the choice of efficient promoter and enhancers sequences, by using viral vectors or by optimizing codon usage and RNA stability [1,9]. At the protein level, the subcellular targeting of the recombinant protein can have a major effect on protein accumulation [3,10]. The use of gene fusions has also been shown to increase protein stability and yield.

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In plants, several proteins have been tested as fusion tags including GUS, ubiquitin, heat-labile enterotoxin, oleosin and green fluorescent protein (GFP)[1] [11–14]. Proteins to be used as tags typically show high stability and must accommodate N or C-terminal fusions [15]. In the case of GFP and GUS, these tags also function as a reporter gene, which also facilitates the detection and purification of the fusion product [15].

The ribonucleoprotein particles (RNPs) or nucleocapsids of Tomato spotted wilt virus (TSWV) are composed of the 28 kDa viral encoded nucleoprotein (N) and RNA [16]. Nucleocapsids result from homopolymerization of the N protein, are highly stable in plant cells, and can be easily purified from TSWV infected plants by ultracentrifugation [17]. These properties feature the N protein as a potentially useful protein fusion partner for plant-based expression and purification. The aim of this study was to test the potential application of the N gene as a fusion tag, by evaluating gene fusion constructs with the GFP as a reporter gene.

Materials and methods

Agrobacterium culture conditions and plant inoculation

Agrobacterium tumefaciens strain GV3101 was grown in LB medium. Binary vectors were transformed into electrocompetent cells using a Bio-Rad electroporator according to the manufacturer's instructions. For plant inoculation, 2 ml of an overnight culture was centrifuged at 1200*g* for 10 min and resuspended in 5 ml of MS medium (pH 5.5) containing 150 μM of acetosyringone. The final volume was adjusted to an A_{600} of 0.5. To evaluate the effect of suppressors of RNA silencing on expression, an *Agrobacterium* strain carrying the HcPro gene from Cowpea aphid-born mosaic virus (CABMV) [18] was co-inoculated with the *Agrobacterium* strains carrying the N fusions by mixing the bacteria suspensions (2:1 ratio). As a control, the avirulent *Agrobacterium* strain A136 was used. *Nicotiana benthamiana* plants (4- to 5-week-old) were infiltrated with the *Agrobacterium* suspensions using a needle-less syringe. Plants were kept in a growth chamber at 25 °C with a 12 h photoperiod.

DNA constructs

The TSWV N gene and the GFP gene [19] were amplified by PCR with a proof reading DNA polymerase (*Pfu*I, Promega). Forward and reverse primers for the N gene contained extensions for the restriction sites *Nco*I/*Bam*HI and *Nco*I/*Not*I, respectively, whereas GFP primers contained *Nco*I and *Spe*I sites. The amplified fragments were purified, ligated to pGemT-easy (Promega) and electroporated into *E. coli* strain XL1-Blue. Selected clones containing the N gene were digested with *Bam*HI and *Not*I and the purified fragment cloned into the Gateway™ vector, pEntr11 (Invitrogen), generating the recombinant plasmid designated pEntr-N. The GFP gene was cloned in pEntr11 using the *Nco*I and the *Xba*I compatible site *Spe*I, generating plasmid pEntr-GFP. Another N gene fragment obtained by digestion with *Nco*I was cloned into the *Nco*I site of pEntr-GFP, generating an in frame N:GFP fusion. Vectors pEntr-N, pEntr-GFP and pEntr-N:GFP were recombined with the binary vector pK2GW7 [20] (Fig. 1A–C) using the LR recombinase (Invitrogen). The plasmid pEntr-N was also recombined with the binary vectors pK2GW7, generating a GFP:N fusion (Fig. 1C). The non-fused GFP gene was also cloned into the bacterial expression vector, pQE30 (Qiagen), and transformed to *E. coli* (strain M15).

Protein extraction and Western blot

To purify the N-GFP fusion proteins, 0.3 g of leaves were macerated, with 10 ml of cold 0.1 M potassium phosphate buffer (pH 7.0) using pestle and mortar. The macerate was strained through miracloth and centrifuged at 12,000*g* for 10 min. The supernatant was discarded and the pellet resuspended in 5 ml of potassium phosphate buffer. The extract was transferred to a beaker and stirred for 1 h at 4 °C, in the presence of 1% Nonidet® 40 (Applichem). Next, the extracts were strained through an 11 μm nylon filter (Millipore), and collected in a 15 ml tube. Using a Pasteur pipette, a cushion of sucrose 20% was added to the bottom of the tube, and centrifuged for 10 min at 140*g*. The pellet and the sucrose fractions were collected, transferred to 1.5 ml centrifuge tubes, and centrifuged at 20,000*g* for 5 min. The supernatant was discarded and the pellet was resuspended in 1 ml of potassium phosphate

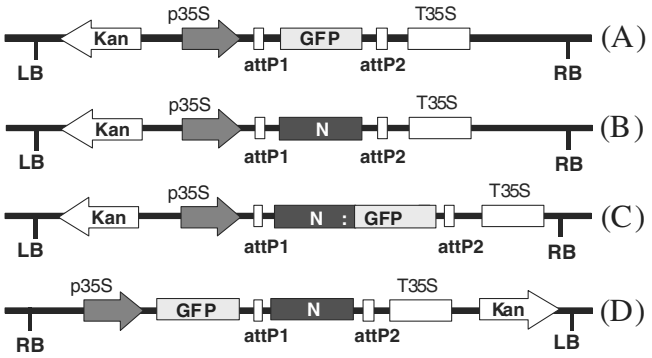

Fig. 1. Schematic representation of the T-DNA region of the utilized binary vectors. Constructs (A), (B) and (C) carrying the GFP gene, the N gene and the N:GFP fusion, respectively, were obtained through Gateway recombination with the destination vector pK2GW7. (D) Construct carrying the gene fusion GFP:N was obtained upon recombination with the destination vector pK7WGF2.0. attP1 and attP2: Gateway recombination sites. p35S: CaMV35S promoter. T35S: CaMV35S terminator. The T-DNA also harbours a kanamycin resistance gene (Kan) under control a *nos* promoter and terminator.

[1] *Abbreviations used:* GFP, green fluorescent protein; RNPs, ribonucleoprotein particles; TSWV, Tomato spotted wilt virus; CAbMV, Cowpea aphid-born mosaic virus; PBS-T, PBS-Tween.

buffer. Samples from these extracts were maintained at 4 °C and used for SDS–PAGE and Western blot analyses and for microscopic observation. Total protein extraction was done by macerating two leaf discs (30 mg) in a tube with 100 μl of PBS, using a plastic pestle. Protein loading buffer was added to these extracts and incubated for 3 min at 95 °C. After protein separation by SDS–PAGE, proteins were transferred to a PVDF membrane (Millipore) using a semi-dry transfer apparatus (Bio-Rad). The membranes were blocked in 10% BSA/PBS for 1 h, and incubated for 1 h with anti-GFP polyclonal antibody (1:2000 in 5% BSA/PBS; Invitrogen). After washing 3 times in 0.1% PBS-Tween (PBS-T), the blots were incubated for 1 h with an anti-rabbit secondary antibody conjugated to alkaline-phosphatase (Sigma), and developed with BCIP/NBT. Protein concentration was estimated using a Fujifilm FLA 3000 scanner with a green laser (532 nm) and an O580 filter. A standard curve with purified GFP was used to estimate the amount of N:GFP and GFP:N present in the purified samples by using the Multigauge software. Hexahistidine tagged GFP was purified from *E. coli* culture using the TALON® CellThru Resin (Clontech), according to the manufacturer's instructions. Concentration of bacteria-purified GFP was determined using the Bio-Rad kit, based on the Bradford assay [21].

Detection of GFP fluorescence and imaging

Close-up UV images were obtained with a digital camera (CoolSnap) mounted on a stereo microscope (M3Z, Leica) with UV light and a blue filter set (465 nm). To facilitate microscopic imaging, protoplasts from infiltrated leaves were prepared, essentially as described by Kikkert et al. [22]. Samples were analyzed under a Zeiss LSM510 laser scanning microscope, using a blue laser light at 488 nm and emission through a 505–530 bandpass filter.

Results and discussion

A gene fusion approach based on the N protein from TSWV was explored in view of its potential to increase the stability of foreign proteins produced in plants. The rationale behind this choice was the known high stability of this viral protein in plant cells, and on the fact that it can form homopolymers, thereby offering a possible purification alternative for a target protein as a gene fusion. The GFP protein was used as a model, for its convenience as an easy and efficient fluorescent reporter protein and its relatively small size (27 kDa).

The N gene was fused either to the N-terminus or to the C-terminus of GFP, generating the binary vectors pK7GW-N:GFP and pK2WG-GFP:N (Fig. 1C and D). The expression of these gene fusions was evaluated by transient gene expression after *Agrobacterium tumefaciens* infiltration in *N. benthamiana* leaves [23]. As *Agrobacterium* infiltration induces RNA silencing of the expressed product, co-expression with a suppressor of RNA silencing was shown to boost expression levels [23], hence this control was included in all experiments to further increase protein expression levels. Four days after *Agrobacterium* inoculation, leaves were analyzed under UV light using a stereo microscope. Intense GFP fluorescence was observed in infiltrated leaves expressing both GFP:N and N:GFP fusions (data not shown).

Protoplasts from leaves expressing the N fusions was analyzed under a laser scanning microscope, revealing marked differences between the N:GFP and GFP:N fusions. Although the fusion proteins are relatively small (55 kDa), large cytoplasmic aggregates of intense fluorescence were seen in cells expressing GFP:N (Fig. 2A), N:GFP fusion formed small aggregates scattered throughout the cytoplasm, resembling aggregates formed by non-fused N (Fig. 2B and D). Cells expressing the non-fused GFP gene showed typical intense fluorescence from the cytoplasm and nucleus (Fig. 2C). In contrast to the other constructs, where fluorescence diminished after about 10 days, bright fluorescence was still visible as late as 30 days post infiltration in leaves expressing the GFP:N fusion. This indicates a greatly increased stability of this particular fusion protein *in planta*.

The formation of N polymers has been studied by two-hybrid screening and *in vitro* experiments leading to the mapping of N- and C-terminal regions that are essential for the interaction between N monomers [24,25]. Based on these studies, a "tail-to-tail" and "head-to head" organization of the N monomers has been proposed [26]. The homotypic interaction of N and its interactions with other TSWV viral proteins have been studied through the expression of GFP fusions of the N gene in mammalian BHK cells. Interestingly, in this cell system no difference was observed between N- or C-terminal N:GFP fusions [27].

In plant cells, as shown here, the N protein fused to the GFP C-terminus showed increased stability, besides providing a simple method for extraction. This extraction was based on the formation of the large aggregates of GFP:N and N:GFP fusions, and took advantage of the GFP fluorescence. The presence of the aggregates after each centrifugation or filtration step was easily checked using a fluorescence microscope. In that way, it was verified that most aggregates were present in the pellet fraction after centrifugation of the leaf extracts, purified N fusions (GFP:N and N:GFP) retained GFP fluorescence (Fig. 2F). Though nearly lacking any contaminating protein (Fig. 3) the resulting extract contained some starch grains, as identified by iodine stain (data not shown), and other cell debris. To separate the protein aggregates from the starch, centrifugation over a 20% sucrose cushion was added to the procedure. Western blot analysis of these purified extracts confirmed the presence of the fusion proteins (Fig. 4, lanes 1–3). Quantification of the purified N fusions evaluated by scanning of the immunodetected signal showed that the yield was approximately 6.5 μg/g of fresh weight (FW). Total leaf extract of infiltrated leaves expressing GFP:N and non-fused GFP (Fig. 4, lanes 3

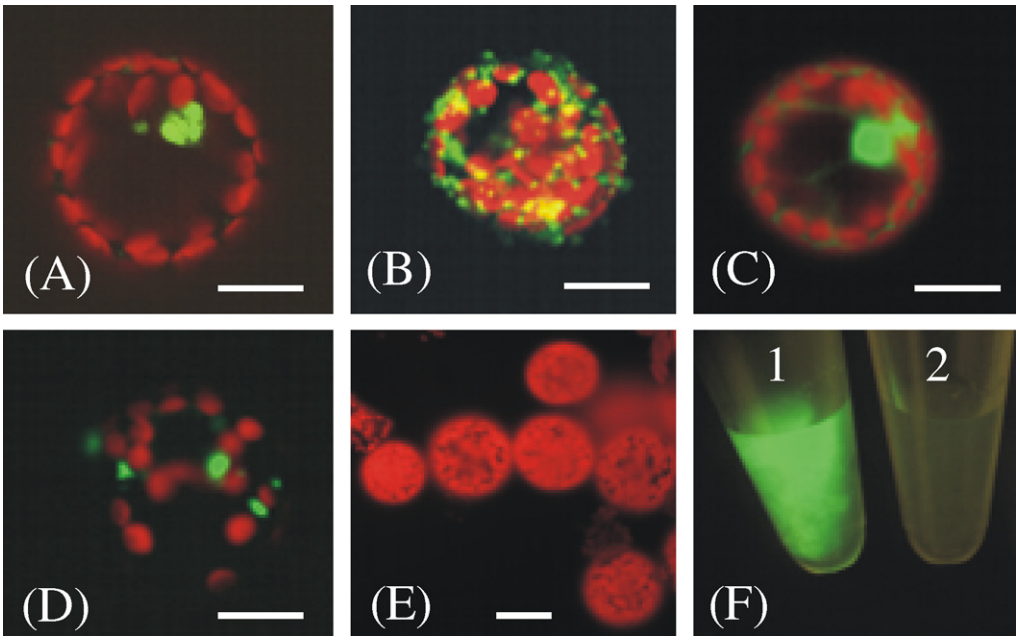

Fig. 2. Laser scanning microscope imaging of protoplasts of *N. benthamiana*. (A–C) Protoplasts expressing GFP:N (A), N:GFP (B) and free GFP. (C and D) Immunolocalization of N in protoplasts using anti-rabbit conjugated alexaR 504 fluorochrome. (E) Non transformed protoplasts as negative fluorescence controls. (F) Microcentrifuge tubes containing samples purified from leaves expressing the GFP:N fusion (1) and the N gene (2), observed under UV light. Bars represent 5 μm.

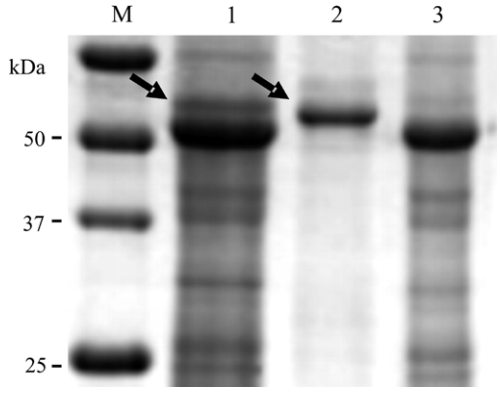

Fig. 3. Analysis of purified GFP:N sample by SDS–PAGE and revealed by Coomassie blue staining. Lane 1, total soluble protein extract from leaves expressing GFP:N. Lane 2, purified GFP:N. Lane 3, total soluble protein extract from non inoculated leaf. M: Precision Plus Protein™ standard (Bio-Rad). The GFP:N fusion (55 kDa) on lanes 1 and 2 are indicated (arrows).

and 4) contained approximately the same amount (7.0 μg/g FW), indicating that the fusion to the N gene does not lead to a large increase in GFP accumulation, but rather that purification is highly efficient. This simple method for extraction of the large GFP:N and N:GFP aggregates can greatly facilitate further purification processing. A large volume of plant extract can be easily reduced to a concentrated precipitate, allowing the separation of this protein fusion complex from almost all cellular proteins, alkaloids, phenolic compounds and other undesirable substances [6,8].

Protein purification methods for large scale production have been developed for some species, mainly corn and canola seeds [6,28]. Large scale purification of apoplast proteins from tobacco leaves has also been used. Other methods for purification involve the rhizosecretion of the recombinant protein to the culture medium or the secretion of recombinant proteins from the leaf gutation fluid [29–31]. To further increase yield and facilitate extraction, strategies based on gene fusion were developed. These include the fusion to oleosin, allowing the fusion protein to be recovered from oil bodies, and further treated with an endoprotease to liberate the recombinant target protein [12,13]. Another approach is based on a fusion construct that contains an integral membrane-spanning domain leading the fusion protein to accumulate at the plasma membrane, thereby facilitating the extraction [32].

We have shown that the N protein as a fusion to GFP presents several advantages and can be further explored. Testing the N fusion tag with different proteins is essential, as every protein is different. As a consequence, fusion proteins often do not function equally well with all partner proteins, and separating the fusion protein from the passenger may also be a challenge as the cleavage site may not be accessible [33]. The GFP:N or N:GFP fusions can also be useful for the production of small peptides, which are often unstable and do not accumulate within the cell [34,35]. A fusion with GFP and N and the target peptide could result in stable fusion aggregates that are easily extracted. This extract, free of proteases and phenolic compounds, would subsequently be subjected to endoprotease cleavage, yielding the target peptide to be further purified by chromatography or affinity columns [6]. The aggregated nature of the N protein fusion, however, may hamper the cleavage of the passenger peptide and further solubilising

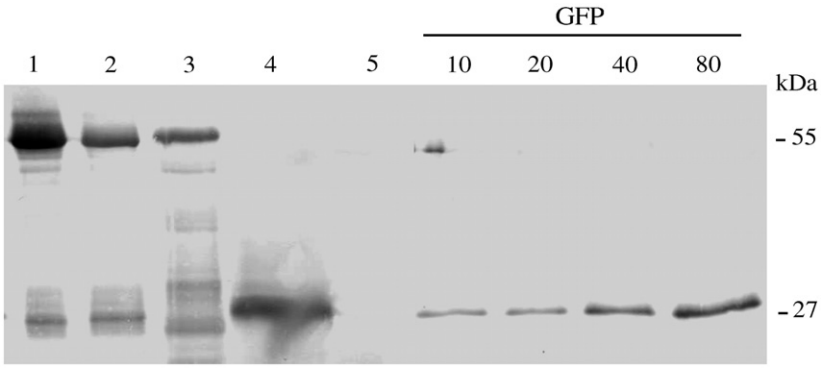

Fig. 4. Western blot analysis of N-GFP fusions. Lane 1, GFP:N fusion protein purified from infiltrated leaves. Lane 2, purified N:GFP protein fusion. Lane 3, total soluble protein extracted from leaves expressing the GFP:N fusion. Lane 4, total soluble protein extract of leaves expressing non-fused GFP. Lane 5, total soluble protein extract from non-infiltrated leaves. GFP standards purified from *E. coli* (10–80 ng per well). Precision Plus Protein™ standard (Bio-Rad) was used as size marker.

may be required [33]. Another potential application of this fusion system is to induce immune response, considering the large size and the stability of the aggregates, functioning as an epitope presentation system, this system may not be subjected to the same size and steric constrains as virus particle fusions [4,36]. In all, this investigation has demonstrated that the N protein as a fusion tag has a large potential for recombinant protein production and purification from plants.

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