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Simultaneous equimolar expression of multiple proteins in plants from a disarmed potyvirus vector

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ABSTRACT

The use of viral vectors for expression of heterologous proteins in plants is hampered by some limitations including the amount of exogenous genetic information that can be incorporated, difficulties with coexpression of stoichiometric amounts of multiple polypeptides and the risk that infectious clones could escape to environment. Here, a new plant viral vector is described which overcomes these limitations. The technology is based on the replacement of the viral RNA polymerase (NIb) cistron of a potyvirus by a cassette for the coexpression of multiple heterologous proteins. The heterologous proteins are flanked by specific cleavage motifs of a viral protease that mediate their efficient release from the viral polyprotein. The vector only replicates and moves systemically in plants where the viral NIb activity is supplied in *trans* by a transgene or another viral vector. The vector allowed for simultaneous expression of three fluorescent reporter proteins in the same subcellular location or two interacting transcription factors inducing anthocyanin accumulation. The vector had sufficient stability throughout the infectious cycle and the NIb deletion prevented infection of wild-type plants which improves biosecurity.
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1. Introduction

Plants and their viruses form an attractive combination from a biotechnological perspective: plants are ideal biofactories whose expression pattern can be programmed through engineered viral vectors. Through the simple and ingenious manipulation of infectious clones of multiple plant viruses, heterologous gene products of interest have been rapidly and efficiently expressed (Pogue et al., 2002; Cañizares et al., 2005; Gleba et al., 2007; Lico et al., 2008; Shih and Doran, 2009; Sainsbury et al., 2010). One virus group that has traditionally attracted interest in plant biotechnology is the potyviruses (genus *Potyvirus*, family *Potyviridae*). These plant viruses consist of a (+) polarity RNA of circa 10 kb, with a 5′ genome-linked viral protein (VPg) and a 3′ poly(A) tail, encapsidated in flexuous and elongated virions. The genome encodes a polyprotein which is processed in a regulated way by three viral proteases into about ten mature gene products (Urcuqui-Inchima et al., 2001), plus P3N-PIPO resulting from a translational frame shift (Chung et al., 2008; Wei et al., 2010). The proteases P1 and helper-component (HC-Pro), in the amino terminal end of the polyprotein, catalyze their own processing, while nuclear inclusion "a" protease (NIaPro), or its non-completely processed form (NIa), in which the NIaPro domain is still joined to VPg, catalyzes the remaining cleavages in *cis* and in *trans*, recognizing a conserved processing motif of seven amino acids (−6/+1) surrounding the cleavage site (Adams et al., 2005).

Several features make potyviruses appealing as expression vectors. Their expression via a polyprotein which is processed into a series of mature gene products allows for production of heterologous proteins in an amount equimolar to the rest of viral proteins (Dolja et al., 1992; Carrington et al., 1993). If the heterologous proteins are flanked by the specific processing sites of a viral protease, they are efficiently released from the polyprotein (Carrington et al., 1993). The use of different insertion sites allows for coexpression of several heterologous proteins from a single vector (Masuta et al., 2000; Beauchemin et al., 2005; Kelloniemi et al., 2008). The elongated nature of the virion allows it to accommodate large amounts of extraneous genetic material (Kelloniemi et al., 2008). Moreover, potyviruses constitute an extraordinarily diverse group infecting a large number of host plant species (Fauquet et al., 2005), making heterologous technologies broadly applicable.

The use of viral vectors to express heterologous gene products in plants has several limitations. One is the amount of heterologous genetic information that can be expressed from a single vector. Another is difficulties with coexpression of stoichiometric amounts of multiple polypeptides. Finally, there is the risk that recombinant viral vectors could escape to environment. Here, we attempt to overcome these limitations by developing a new vector based

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on the potyvirus *Tobacco etch virus* (TEV). The vector is based on two early findings in TEV molecular biology. First, the viral protease NIaPro (or its unprocessed form NIa) efficiently cleaves an artificial proteolytic site introduced in between a β-glucuronidase reporter gene and viral HC-Pro (Carrington et al., 1993). Second, a TEV mutant with a deletion of the whole viral RNA polymerase (nuclear inclusion "b" protein, NIb) cistron (TEVΔNIb) can be complemented in *trans* in a transgenic plant expressing this protein (Li and Carrington, 1995). Furthermore, recent construction of a stable binary plasmid (pGTEV), containing a TEV infectious clone under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter and terminator allows efficient infection of host plants by agroinoculation (Bedoya and Daròs, 2010). In the work described here, the NIb cistron of a TEV infectious clone was replaced by cassettes to coexpress proteins that were flanked by natural and artificial cleavage sites of the viral protease NIaPro. The resulting vectors were stable and exclusively replicated and moved in plants where viral NIb was supplied in *trans* through a transgene or compatible viral vector. Using this vector we show the coexpressions of up to three different fluorescent proteins in the same subcellular location. We also show the potential use of this vector in metabolic engineering by coexpressing two interacting transcription factors that very efficiently induced accumulation of anthocyanins in infected tissue.

2. Materials and methods

2.1. Plasmid construction

The pGTEV plasmid contains a TEV infectious cDNA (Genbank accession number DQ986288) flanked by CaMV 35S promoter and terminator (Bedoya and Daròs, 2010). Plasmid pTEV89 contains a cDNA fragment of TEV-DQ986288 (positions 6506–9059) inserted in the SmaI site of pBluescript II KS + (Genbank accession number X52327). This cDNA fragment includes the complete NIb cistron flanked by restriction sites Eco81I and BlnI that are unique in pGTEV. Plasmid pgR107 (Genbank accession number AY297842) contains a *Potato virus X* (PVX) infectious clone including a duplicated coat protein (CP) promoter ahead of a small polylinker (Lu et al., 2003). Plasmid pmCherry contains the cDNA of the red fluorescent protein mCherry (Genbank accession number AY678264) (Shaner et al., 2004). Plasmid pDH51-GW-YFP (Genbank accession number AM773752) (Zhong et al., 2008) contains the cDNA of the yellow fluorescent protein Venus (Nagai et al., 2002). Plasmid pmTagBFP contains the cDNA of the blue fluorescent protein mTagBFP (Subach et al., 2008) as in plasmid pTagBFP-N (Evrogen) but including the silent mutation G258A in the mTagBFP cDNA to remove an Eco81I site.

PCR amplification for plasmid construction was performed in 20 μl with 0.4 U of the high fidelity Phusion DNA polymerase (Finnzymes) in HF buffer (Finnzymes), 3% dimethyl sulfoxide, 0.2 mM dNTPs, 0.5 μM each primer and 10 ng of template DNA. The reactions were incubated 30 s at 98 °C followed by 30 cycles of 10 s at 98 °C, 30 s at 55 °C and a variable extension time depending on the length of the expected product (15 s/kb) at 72 °C, and a final extension of 10 min at 72 °C. The constructs were transformed by electroporation into *Escherichia coli* strain DH5α. mCherry cDNA including an amino terminal FLAG tag was amplified from pmCherry with primers PI and PII (the sequences and features of all primers are detailed in supplementary Table S1). Venus cDNA including an amino terminal HA tag was amplified from pDH51-GW-YFP with primers PIII and PIV. mTagBFP cDNA including an amino terminal c-Myc tag was amplified from pmTagBFP with primers PV and PVI. These three cDNAs digested with Eco31I (Fermentas) were ligated to the PCR product resulting from amplification of pTEV89 with primers PVII and PVIII also digested with Eco31I. In the resulting plasmids pTEV89ΔNIb-Cherry, pTEV89ΔNIb-Venus and pTEV89ΔNIb-Blue, each one of the tagged fluorescent protein cDNAs FLAG-mCherry, HA-Venus and c-Myc-mTagBFP, substitute most of NIb cistron and are flanked by the natural NIaPro proteolysis sites NIaPro/NIb and NIb/CP.

The three fluorescent protein cDNAs including the corresponding tags were amplified again to construct the cassette to coexpress the three proteins simultaneously. FLAG-mCherry cDNA was amplified from pTEV89ΔNIb-Cherry with primers PI and PIX. HA-Venus cDNA was amplified from pTEV89ΔNIb-Venus with primers PX and PXI. c-Myc-mTagBFP cDNA was amplified from pTEV89ΔNIb-Blue with primers PXII and PVI. The three cDNAs were digested with Eco31I and ligated to the product of amplification of pTEV89 with primers PVII and PVIII also digested with Eco31I, as described above. The plasmid resulting from assembly of the four DNA fragments was named pTEV89ΔNIb-CVB and contains the FLAG-mCherry, HA-Venus and c-Myc-mTagBFP cDNAs separated by two artificial NIaPro proteolysis sites, substituting most of the NIb cistron and flanked by the natural NIaPro proteolysis sites NIaPro/NIb and NIb/CP.

Similarly, the cDNAs of snapdragon (*Antirrhinum majus* L.) transcription factors Delila and Rosea1 were amplified from a DNA preparation obtained from a transgenic tomato expressing these genes (Butelli et al., 2008) with primers PXIII-PXIV and PXV-PXVI. Both cDNAs were digested with Eco31I and the products ligated, as described above, to the product of amplification of pTEV89 with primers PVII and PVIII. The resulting plasmid pTEV89ΔNIb-DelRos contains the Delila and Rosea1 cDNAs separated by an artificial NIaPro proteolysis site, replacing most of NIb cistron and flanked by natural proteolysis sites NIaPro/NIb and NIb/CP.

Finally, the Eco81I-BlnI cDNA fragment from pGTEV was exchanged with each of the Eco81I-BlnI restriction fragments from plasmids pTEV89ΔNIb-Cherry, pTEV89ΔNIb-Venus, pTEV89ΔNIb-Blue, pTEV89ΔNIb-CVB and pTEV89ΔNIb-DelRos containing the corresponding expression cassettes, to construct the expression vectors pGTEVΔNIb-Cherry, pGTEVΔNIb-Venus, pGTEVΔNIb-Blue, pGTEVΔNIb-CVB and pGTEVΔNIb-DelRos, respectively.

Plasmid pgR107-NIb was constructed by inserting the complete TEV NIb cDNA, including an amino terminal ATG and a carboxy terminal UGA stop codon, amplified from pGTEV with primers PXVII and PXVIII and digested with Eco31I into plasmid pgR107 opened with SmaI-SalI. The resulting plasmid was named pgR107-NIb and contains the TEV NIb cDNA under the control of the duplicated PVX CP promoter.

2.2. Plant inoculation

Wild-type tobacco plants (*Nicotiana tabacum* L. cv. Xanthi nc), transgenic plants from this same cultivar expressing TEV NIb plus an additional amino terminal Met (Li and Carrington, 1995) and wild-type *Nicotiana benthamiana* Domin plants about 6 weeks old were infiltrated in the two youngest expanded leaves (two infiltrations per leaf) and cultivated in a growth chamber under a photoperiod of 16 h day at 25 °C and 8 h night at 23 °C. The agroinoculation was done with cultures of *Agrobacterium tumefaciens* C58C1 containing the helper plasmid pCLEAN-S48 (Thole et al., 2007) and the plasmids (singly or in various combinations) pGTEV, pGTEVΔNIb-Cherry, pGTEVΔNIb-Venus, pGTEVΔNIb-Blue, pGTEVΔNIb-CVB, pGTEVΔNIb-DelRos or pgR107-NIb, adjusted to an OD (600 nm) of approximately 0.5 and induced for 2 h at 28 °C with 150 μM acetosyringone in 10 mM MES-NaOH, pH 5.6, 10 mM $MgCl_2$ (Bedoya and Daròs, 2010).

The plants were also inoculated mechanically with homogenates of infected tissues. Crude homogenates were obtained from 100 mg of frozen tissue ground in a 2-ml Eppendorf

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