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Short communication

Expression of Hemagglutinin-Neuraminidase glycoprotein of Newcastle Disease Virus in agroinfiltrated *Nicotiana benthamiana* plants

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ABSTRACT

The worldwide need for producing safer and less expensive vaccines with minor manufacture and processing requirements, together with the advances made through biotechnology, has promoted the development of efficient alternative tools to conventional vaccines. One of these is the use of plants or plant cell culture as production platforms of vaccine antigens with potential use as immunogens. We have already described the use of transgenic potato plants as immunogens against Newcastle Disease Virus (NDV), although the amount of the recombinant antigen recovered was low. The main objective of the work presented here was to enhance the expression of the HN glycoprotein of NDV through a protein targeting strategy and a promoter change. We have cloned the HN coding region under the regulation of the rubisco small subunit promoter in 5 different versions in a subcellular localization strategy, and we have established that the construct harboring the complete HN gene with its own signal peptide, fused to KDEL retention peptide, rendered the best expressed/accumulated HN protein level whether a transient or a stable transformation assay was performed. We conclude that agroinfiltration results in a simple and useful tool for selecting suitable genetic constructions to be used in stable plant transformation and, moreover, it could be used as a method to produce immunogens for vaccine developments.

Plant-based expression systems have been extensively demonstrated to be efficient in generating large quantities of heterologous proteins and inducing both, parenteral and mucosal immune responses when they are used as immunogens (Moravec et al., 2007; Wigdorovitz et al., 1999). In particular, the Agrobacterium-mediated transient expression assay has been shown to be a powerful tool to evaluate, within a very short period of time, gene functionality and different features of the foreign protein as conformation and folding (virus like particles formation) (Huang and Mason, 2004), glycosylation (van der Hoorn et al., 2005) and protein–protein interaction (Citovsky et al., 2006), among others. This expression system was also used to generate plant-derived antigens to be used as immunogens (Mett et al., 2008) and for the production of molecules of interest to the pharmacologic industry as monoclonal antibodies (reviewed by Ma et al., 2005).

We have already described the use of transgenic potato plants as immunogen against Newcastle Disease (Berinstein et al., 2005). The pathogen responsible for this disease is Newcastle Disease Virus (NDV), a paramixovirus which major antigens are Hemagglutinin-Neuraminidase (HN) and Fusion (F) surface glycoproteins. HN-transgenic potato plants obtained in our laboratory expressed the glycoprotein under the regulation of the CaMV-35S promoter with low yield (0.3–0.6 μg of HN protein per mg of total leaf protein). Nevertheless, they were capable to induce a specific parenteral and mucosal humoral immune response in a murine model (Berinstein et al., 2005).

The main objective of the present work was to determine differences in expression levels of the HN glycoprotein of NDV through a subcellular localization strategy using an agroinfiltration transient assay. The expected enhanced expression would allow obtaining enough material to produce a plant-based vaccine for susceptible hosts.

Five constructs were generated depending on the desired protein destination (Fig. 1): $pSP_{HN}HN$ and $pSP_E/SP_{HN}HN$ as membrane versions; $pSP_E/SP_{HN}HN/KDEL$, $pSP_{HN}HN/KDEL$ and $pSP_E/HN/KDEL$ as retained version. The coding region for HN was cloned with the fragment corresponding to the transmembrane anchor domain in which the natural signal peptide is contained. In the last construct ($pSP_E/HN/KDEL$), this fragment was removed. SP_E stands for the signal peptide of sea anemone equistatin, provided by the

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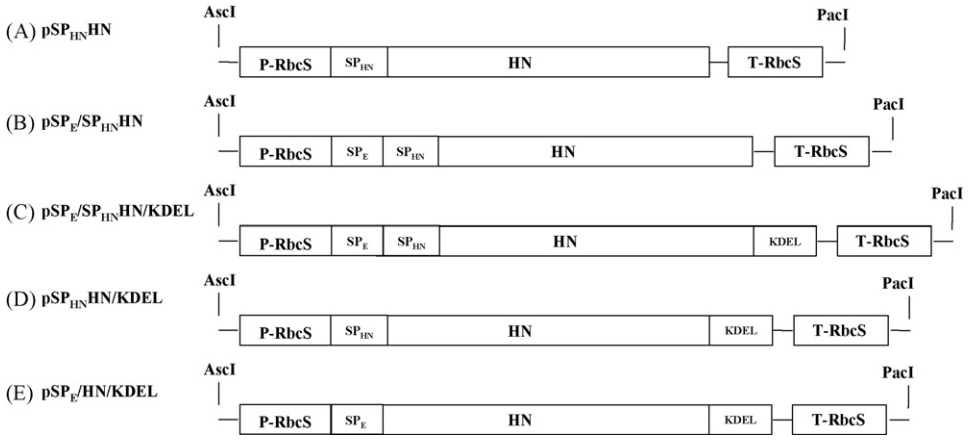

Fig. 1. Schematic representation of the constructs used in this study. P-RbcS, Rubisco small subunit promoter; SP_E, sea anemone equistatin signal peptide; SP_{HN}, HN protein signal peptide; KDEL, ER retention sequence; T-RbcS, transcription termination signal (ImpactVector^{TM}).

commercial plasmid vector used (Outchkourov et al., 2003a,b), and it was maintained in 3 constructs to study if its performance was superior to the viral HN signal peptide. It is known that the ER has a crucial role in guaranteeing the proper folding and assembly of proteins by means of its chaperon machinery and a quality control mechanism. The addition of the KDEL sequence to the C-terminal end of a protein allows its retention within the ER or at least, a delay in the export and degradation downstream the cellular secretory pathway. This is accomplished due to interactions with the ER resident's proteins (Munro and Pelham, 1987). Consequently, we would expect to increase the stability and yield of the HN protein by fusing the KDEL retention sequence to it.

In all constructs, the protein was also designed to be fused to a c-Myc tag for further immunodetection employing an anti-c-Myc monoclonal antibody (Sigma–Aldrich, St. Louis, MO).

The agroinfiltration was conducted as described by Huang and Mason (2004) with minor modifications. Briefly, the resulted binary vectors were introduced into *Agrobacterium tumefaciens* GV3101 by electroporation. The recombinant bacteria were grown for 48 h at 28 °C in Luria-Bertani medium containing 0.1 mg ml^{-1} rifampycin, 0.04 mg ml^{-1} gentamycin and 0.1 mg ml^{-1} kanamycin. Agrobacteria were then pelleted by centrifugation and resuspended in infiltration buffer (10 mM (N-morpholino) ethanesulfonic acid (MES), 10 mM $MgCl_2$) containing 100 μM acetosyringone, to reach an $OD_{600\,nm}$ = 1. Once virulent genes were induced for 5 h at room temperature on the bench, full expanded *Nicotiana benthamiana* leaves were agroinfiltrated and 48 h later total proteins were extracted with 3 volumes of chilled buffer (Isogai et al., 1998).

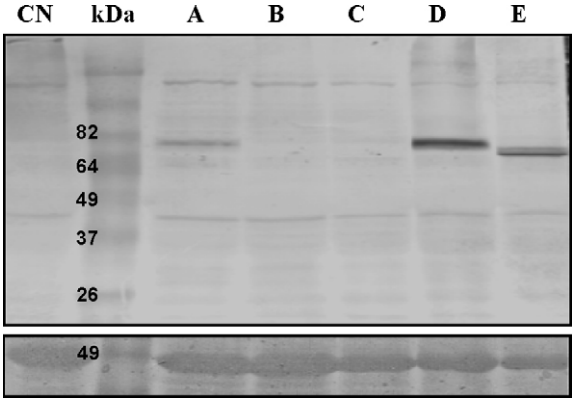

Fig. 2. Expression of NDV HN glycoprotein in transiently transformed plants. Upper panel: Immunoblot analysis using the anti-c-Myc monoclonal antibody. (A) pSP$_{HN}$HN, (B) pSP$_E$/SP$_{HN}$HN, (C) pSP$_E$/SP$_{HN}$HN/KDEL, (D) pSP$_{HN}$HN/KDEL, and (E) pSP$_E$/HN/KDEL. CN: Plant transformed with no related construct. Bottom panel: Red ponceau staining of area, where the rubisco large subunit is usually found, showing similar quantities loaded.

Western blot assays showed the specific band of the HN protein in the extracts of agroinfiltrated leaves (Fig. 2). This result was obtained for the products of constructs pSP$_{HN}$HN, pSP$_{HN}$HN/KDEL and pSP$_E$/HN/KDEL (lanes A, D and E respectively) which contained just one signal peptide (Fig. 2). Notably, very low amount of recombinant protein was detected when both signal peptides were present in tandem in the constructs pSP$_E$/SP$_{HN}$HN and pSP$_E$/SP$_{HN}$HN/KDEL by Western blot assay (Fig. 2). This could be due to a signaling conflict between both signal peptides, that impairs the insertion of the protein in the ER, since not even the inclusion of KDEL is able to allow the detection of significant recombinant protein in the agroinfiltrated plants (lane C, Fig. 2). Similar results were also reported by Huang and co-workers when expressing the H protein from another paramyxovirus, the measles virus, in stably transformed tobacco plants. Despite the presence of mRNA, observed by Northern blot, the authors could not detect the recombinant protein by ELISA in those plants harboring the coding region of the H protein where a plant signal peptide was cloned upstream of its own (Huang et al., 2001). Interestingly, transient transformation rendered similar amounts of HN protein when evaluated by Western blot analysis, using either homologous (pSP$_{HN}$HN/KDEL) or heterologous SP (pSP$_E$/HN/KDEL), indicating that the use of the SP provided by the system did not give any advantage over the use of viral one.

Also, it could be observed that the molecular size of HN protein from pSP$_E$/HN/KDEL was lower than 74 kDa, the apparent molecular size approximately predicted for HN, because of the removal of the 50 N-terminal aminoacids (transmembrane anchor domain) (Fig. 2, lane E).

Panda et al. (2004) demonstrated that the deglycosylation of HN drastically influenced the virulence and antigenicity of NDV, highlighting the importance of carbohydrates in the stability and functionality of HN in the pathogenesis. Moreover, Long et al. (1986) have demonstrated that conformational and glycosides dependent epitopes exist in HN protein. In this regard, a PNGase F treatment was performed to determine the glycosylation status of the expressed protein following the manufacturer's indications (New England, BioLabs). PNGase F is an endoglycosidase that cleaves oligosaccharides from N-linked glycoproteins. Fig. 3 shows a reduction in the apparent molecular weight of the recombinant protein, indicating that the HN protein transiently expressed in *N. benthamiana* leaves was post-translationally modified by the addition of N-linked glycosides.