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Research Article

Cloning and Expression of Fusion (F) and Haemagglutinin-neuraminidase (HN) Epitopes in Hairy Roots of Tobacco (*Nicotiana tabaccum*) as a Step Toward Developing a Candidate Recombinant Vaccine Against Newcastle Disease

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Abstract

Newcastle is a significant avian disease continuing to cause considerable loss. Developments in genetic engineering have led to plant-based platforms for human and animal vaccine production. Recombinant vaccine production in hairy root systems have several advantages over stable expression in whole plants, including high growth rates, ready genetic manipulations, high levels of recombinant protein production, and the potential for bioreactor culture. In an attempt to develop a recombinant vaccine in hairy roots, the sequences encoding fusion (F) and haemagglutinin-neuraminidase (HN) epitopes of Newcastle disease virus were cloned in pBI121 expression vector which was then transferred into leaf disks of tobacco (*Nicotiana tabaccum*) 'Turkish' cultivar by means of *Agrobacterium rhizogenes*. Hairy roots developed on MS medium containing 50 mg/L kanamycin and 30 mg/L meropenem. Incorporation of the heterologous gene in the genome of hairy roots was confirmed by PCR. Expression analyses were performed by real-time PCR at transcription level and by dot-blot and ELISA assays at translation level, all confirming the expression of the heterologous gene and production of the recombinant protein.

***Keywords*:** Recombinant Vaccine, Antigen Expression, Newcastle Disease, Hairy Roots, *Agrobacterium Rhizogenes*

Introduction

Recombinant vaccines based on viral coat protein subunits represent an efficient tool as a substitute for conventional, attenuated virus based vaccines (Makela, 2000). They are called edible recombinant vaccines as they are administered orally. A recombinant (or subunit) vaccine is an antigen produced with the help of genetic engineering methods in a suitable expression system. Theoretically, the genes encoding every protein can be cloned and expressed in bacteria, yeasts or mammalian cells. A number of genes encoding surface antigens from viruses, bacteria and single-celled pathogens have been cloned in expression systems and the expressed antigens have been used as vaccines (Arntzen and Mason, 1995). Plant-based platforms are one of the highly preferred expression systems employed for recombinant vaccine production (Sala et al., 2003). Transgenic plants expressing industrially or pharmaceutically valuable heterologous proteins can well be a substitute for fermented systems. Several vaccines have been produced on a transient or stable basis in plants and proved to conserve the necessary conformation for evoking the immune response in human or animals.

Transgenic plants are highly interesting candidates for recombinant vaccine production. The main benefits of plant-based edible vaccines include ease of production and administration, as well as a very high level of immunogenicity because they elicit the mucosal immune system, which is the first and most important barrier against different pathogens. The majority of animal pathogens enter the host's body through respiratory or digestive tracts, where the mucosal immune system operates; and the mucosal immune response elicited by edible vaccines will have a very high potency against pathogens (Arntzen and Mason, 1995). Production of recombinant vaccines against veterinary diseases such as Norwalk virus and foot and mouth disease has been reported by several researchers (Mason et

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al., 1996; Lai et al., 2012). In each case, epitopes evoking the immune system have been used in bacteria, yeast or plant-based expression platforms (Mason et al., 1996; Lai et al., 2012), among which the plant-based systems possess a number of advantages as follows: 1- the potential to elicit humoral, cell-mediated, and mucosal immune systems; 2- plant cell walls preserve the antigen from digestion, and it will be released gradually into lymph and later into blood; 3- packaging of viral antigen in plants is exactly similar to that observed in diseased individuals, assembling into subviral particles (Sala et al., 2003); 4- the costs of vaccine production in transgenic plants are considerably lower than conventional methods, with the costs being limited to those of typical plant cultivation and harvest; 5- plant parts or extracts can be kept at room temperature without any special equipments; 6- plants are not hosts of animal viruses, therefore eliminating the risk of mammalian virus contamination; 7- vaccines produced in plants can be consumed as a dietary supplement (Kim and Yang, 2010).

There are two methods for incorporating antigens into plant genomes and producing recombinant vaccines (Fieler et al., 1997). The first method includes insertion of the antigen coding gene into nuclear or organellar genome, resulting in a stable transgenic plant. In the second method, the gene is transferred to the plant by means of *Agrobacterium* or a plant virus, and eliminating the need for *in vitro* tissue culture and plant regeneration (Waugh, 2005).

Expression of recombinant vaccines in hairy roots mediated by *A. rhizogenes* has several advantages over stable expression in whole plants. Among these advantages are high growth rates, low duplication times, easy genetic manipulation, high capacity for recombinant protein production, the potential for growth in bioreactors, the possibility of evaluating expression courses and levels, and verification of the constructs without the need for development of stable transgenic plants (Giri and Narasu, 2000; Fischer and Schilberg, 2004).

Hairy root production in tobacco is preferred over other plants due to several factors such as ease of transformation, availability of optimized tissue culture protocols, and relatively high levels of transgene expression (Tripurani et al., 2003; Arntzen and Mason, 1995).

Newcastle virus is a prominent avian pathogen, both epidemiologically and economically. Killed or attenuated virus is currently used for Newcastle vaccination (Zhao and Hammond, 2005), often forming an effective solution. High costs and the risk of improper attenuation however necessitate a new means of vaccination against Newcastle disease (Zhao and Hammond, 2005).

Newcastle disease virus (NDV), a member of the *Rubulavirus* genus in the *Paramyxoviridae* family, is an enveloped virus with a single-stranded, monopartite anti-sense RNA genome of 15 kb length. Its genome encodes 6 principal structural and non-structural proteins including nucleocapsid (NP), phosphoprotein (P), matrix (M), fusion (F), haemagglutinin-neuraminidase (HN) and RNA-dependent RNA polymerase (L) (Berinstein et al., 2005).

F and HN are trans-membrane glycoproteins necessary for infectivity and pathogenicity and both of them can evoke immune response (Berinstein et al., 2005). Amino acid residues 65 to 81 of F and 346 to 353 of HN are known to constitute the most influential immunogenic sites for antibody induction (Zhao and Hammond, 2005).

The objective of this work was to transform and express the F and HN epitopes in tobacco hairy roots. Tobacco is a model organism in transgene expression studies, and successful expression of antigens in its hairy roots could be a step toward recombinant vaccine production in other plants used as avian feed.

Materials and Methods

Design and preparation of the construct

The construct used in this work included 4 tandem repeats of the HN epitope (encoding residues 346-353 of the HN protein) followed by 3 tandem repeats of the F epitope (encoding residues 65-81 of the F protein). The sequences were retrieved from the NCBI database, and optimized according to the codon bias of the host plant in order to guarantee a high level of expression in its hairy roots.

After optimization, the sequence was examined with the Lasergene software (DNASTAR) to ensure the absence of any transcription or translation inhibitor sequences. A histidine tag (18 bp) was then ligated upstream the construct, followed by the omega sequence (67 bp) as the ribosome attachment site. A 18 bp sequence encoding the endoplasmic reticulum retention signal peptide (SEKDEL) was also fused to the 3' terminus. The omega sequence (Berinstein et al., 2005) and signal peptide (Kang et al., 2004) serve an important role in enhancing the expression level of epitopes.

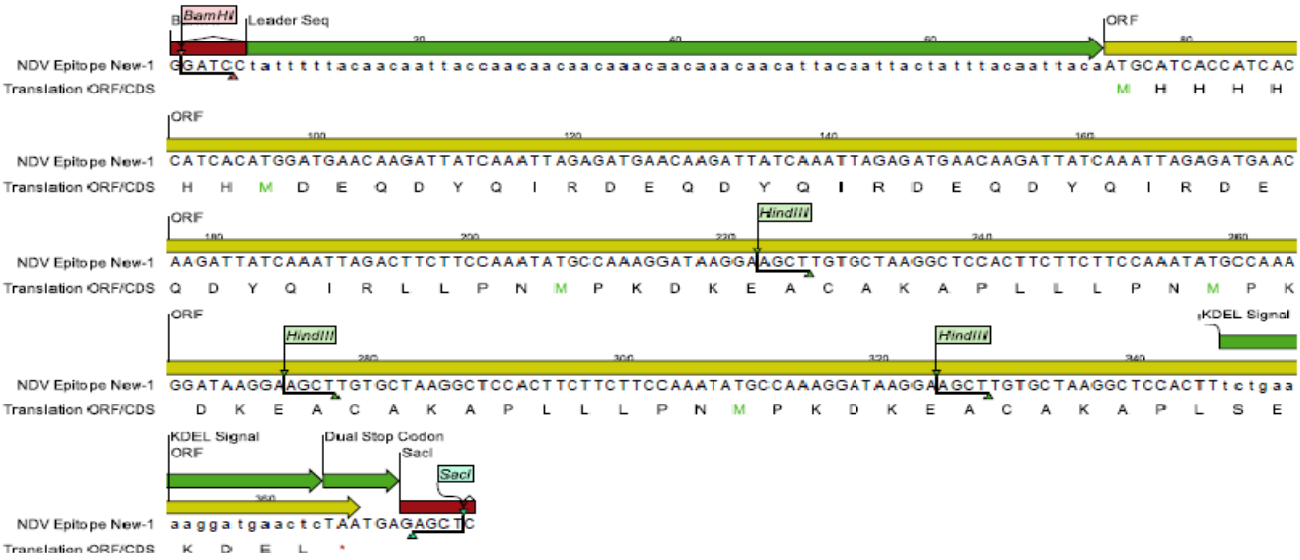

Figure 1. Expression cassette incorporating the F and HN epitopes together with the other sequences necessary for expression in eukaryotic systems

Finally, two restriction sites, *Bam*HI and *Sac*I, were incorporated at 5' and 3' termini respectively to facilitate the cloning process. These elements increased the size of the cassette to 376 bp. The resulting F-HN cassette (fig. 1) was designed with CLC software package, synthesized by Gene Ray, and cloned into pGH vector.

Preparation of pBI121 vector carrying F and HN epitopes for transformation of the nuclear genome

The F-HN cassette was extracted from pGH plasmid by enzymatic digestion with *Bam*HI and *Sac*I. pBI121 binary vector harbouring CaMV 35S promoter and NOS terminator was also digested at the same sites. After elimination of the GUS sequence, the F-HN cassette was inserted between promoter and terminator (fig. 2).

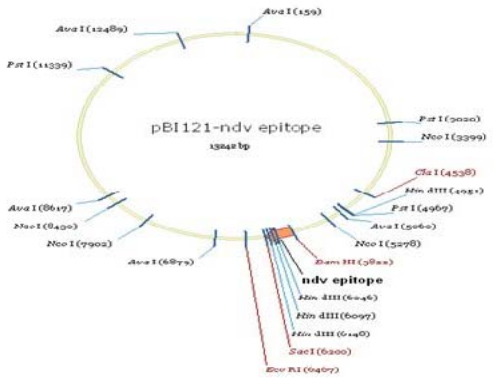

Figure 2. Schematic structure of the recombinant plasmid pBI121- NDV epitope for Agrobacterium-mediated transformation of tobacco hairy root.The DNA sequence encoding NDV epitope was cloned the downstream of the CaMV 35S promoter of a binary plasmid pBI 121

The resulting construct (named pBI121-NDV epitope) was introduced into *A. rhizogenes* by electroporation, and its proper placement downstream the promoter was confirmed by plasmid extraction and digestion with *Bam*HI and *Sac*I, which gave a 376 bp band on the electrophoresis gel (fig. 3).

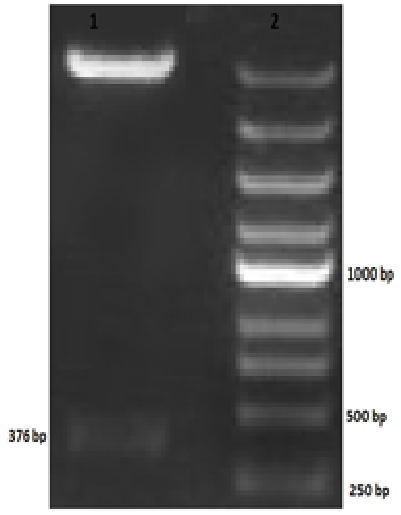

Figure 3. pBI121-NDV epitope extracted from *A. rhizogenes*, digested with *Bam*HI and *Sac*I: digested plasmid (left) and size marker (right)

Generation of transformed hairy roots

A. rhizogenes strain Ar15834 carrying the construct (pBI121-NDV epitope) was cultured in Luria-Bertani (LB) medium for 18 hours, to an optical density (OD) at 600 nm of approximately 0.6%. Suspensions were centrifuged at 3500 rpm for 20 minutes at 4°C. The pellets were suspended in MS medium (pH 5.2) with 0.05 mMacetosyringone (3′, 5′-dimethoxy-4′-hydr -

oxyacetophenone) and injected into tobacco leaves with insulin syringe.
Transformed tobacco leaves were cultured on MS medium (Murashige and Skoog, 1962) with no plant growth regulators (co-culture medium), and subcultured on MS medium containing 50 mg/l kanamycin and 30 mg/l meropenem (selection medium) upon the emergence of bacterial colonies around them. Hairy roots were generated in ample quantities within 35 days (fig. 4), and subcultured in liquid, hormone-free MS medium.

Figure 4. Generation of transformed hairy roots on MS medium containing 50 mg/l kanamycin and 30 mg/l meropenem

Molecular verification of hairy roots

Molecular verification of hairy roots was achieved by PCR amplification of a 194 bp fragment of the rolB gene. Genomic DNA was extracted from hairy roots following Dellaporta et al. (1983), and PCR reaction was performed with the following primers:

Forward:
5'AAGTGCTGAAGGAACAATC 3'
Reverse:
5' CAAGTGAATGAACAAGGAAC 3'

PCR cycles comprised denaturation at 94°C for 1 minute, annealing at 51.5°C for 30 seconds, and extension at 72°C for 20 seconds.

Examination of transformed hairy roots for the presence of F and HN epitopes

Transformation of hairy roots was confirmed by PCR. Genomic DNA was extracted following Dellaporta et al. (1983). A 741 bp fragment was amplified with the following specific primers:

Forward:
5' ATGAACAAGATTATCAAATTAGAC 3'
Reverse:
5' CCGTAAAGCACTAAATCG 3'

The 35 PCR cycles comprised denaturation at 94°C for 1 minute, annealing at 46°C for 1 minute and extension at 72°C for 1 minute. Genomic DNA from untransformed hairy roots was used as the negative control.

Expression analysis at transcription level with real-time PCR

Quantitative Real-Time PCR experiments were performed in duplicate for each sample to quantify the expression level of the transgene. Total RNA was extracted from the transformed hairy roots. cDNA was constructed by reverse transcription using oligo-dT primers and used as the real-time PCR template. Real-time PCR reaction volumes of 20 µl included 0.5 µl of each primer and 10 µl SYBR Green Real time PCR master mix., and the following specific primers:

Forward:
5' GACTTCTTCCAAATATGCCAAAG 3'
Reverse:
5' TGGAGCCTTAGCACAAGC 3'

Expression analysis at translation level with dot-blotting

Production of the recombinant protein in tobacco hairy roots was verified by dot-blotting. A protein sample of 3 µl volume was spotted onto the membrane and allowed to dry at 37°C. The membrane was incubated for 1 hour with blocking solution (BSA) which prevents non-specific reactions, then incubated for 1 hour with anti-His tag, rinsed 3 times with PBST/PBS, incubated for 1 hour with the secondary antibody (at 37°C), rinsed 3 times with PBST/PBS, and finally incubated with OPD substrate. A 3 µl sample of commercial NDV vaccine and the same volume of the protein from untransformed plants were used as positive and negative controls, respectively.

Quantification of protein expression with ELISA assay

Expression of the heterologous protein was evaluated by ELISA assay. ELISA plate was coated with total soluble proteins from transformed and untransformed hairy roots and the NDV antigen at 37°C for one hour and then incubated with 1% BSA in PBS at 37°C for 2 hours in order to prevent non-specific binding. The wells were washed by PBST/PBS, incubated with anti-His tag (1:1000 dilutions) and then with alkaline phosphatase conjugated with anti rabbit IgG (1:1500 dilution), and finally developed with TMB substrate. Colour reaction was stopped by adding 2N H_2SO_4 and read at a wavelength of 405 nm.

Results

In this research transformed hairy roots were obtained by co-culture of tobacco leaf disks with *A. rhizogenes* carrying the binary vector pBI121-NDV epitope. PCR amplification of a 194 bp fragment of the rolB gene confirmed that the generated hairy roots are a result of A. rhizogenes infection (fig. 5).

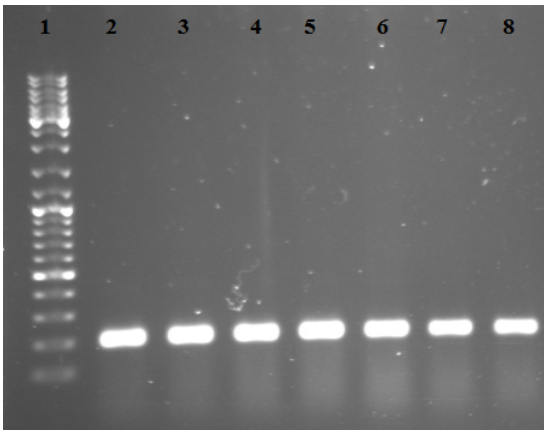

Figure 5. Molecular verification of hairy roots by amplification of a 194 bp fragment of the rolB gene. Lane 1 is 100bp size marker; lanes 2-8 are the amplified 194 bp rolB fragment

A second PCR confirmed the presence of the construct carrying F and HN epitopes in the transformed hairy roots (fig. 6). As shown in fig. 6, the 741 bp band is only observed in transformed hairy roots.

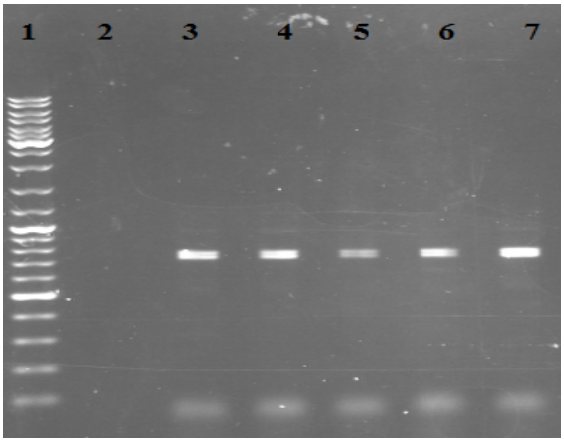

Figure 6. Verification of the presence of the construct harbouring F and HN epitopes in hairy roots by PCR amplification of a 741 bp fragment of the construct: 100bp size marker (1), untransformed hairy root as negative control (2), plasmid pBI121-NDV as positive control (3) and transformed hairy root (4-7)

Expression of the construct harbouring F and HN epitopes was quantified at transcription level with real-time PCR. The results indicated that the transgene is expressed in transformed hairy roots (chart 1).

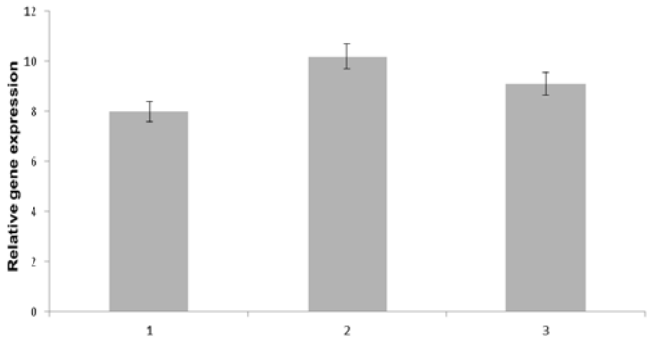

Chart 1. Expression analysis of the construct carrying F and HN epitopes at transcription level in transformed hairy roots by means of real-time PCR (1,2 and 3 are transformed hairy root samples)

Recombinant protein production was verified by dot-blot assay, confirming that the recombinant protein is produced in transformed, but not in untransformed hairy roots (fig. 7).

Figure 7. Verification of recombinant protein production in transformed hairy roots with dot-blotting: positive control (1); protein from transformed hairy roots (2); wild-type protein as negative control (3)

Recombinant protein production was also quantified by ELISA, with the results indicating production of very high levels of recombinant protein in transformed hairy roots, whereas untransformed roots were lacking a cogent evidence of recombinant protein production (chart 2).

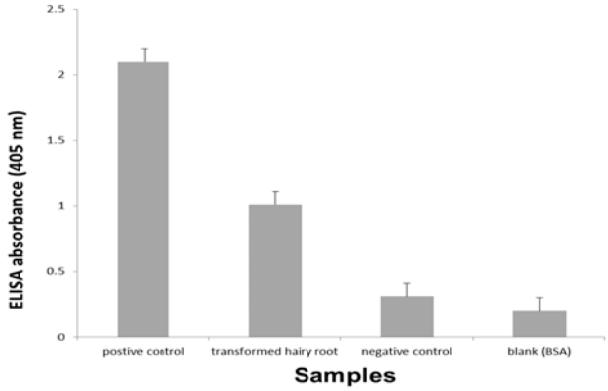

Chart 2. ELISA results showing production of F and HN epitopes in hairy roots. From left to right: commercial vaccine as positive control; transformed hairy root; untransformed hairy root as negative control; BSA as blank sample

Discussion

Green plants represent appropriate systems for expression of recombinant vaccines and other pharmaceutical proteins; but several factors such as the considerably long time required for development of transformed lines, relatively high costs, and low expression levels remain major obstacles to commercialize recombinant vaccine production using *A. tumefaciens*-mediated plant genetic engineering (Bendahmane et al., 2000).

expression of antigens in hairy roots or in whole plants using plant viral vectors has been proposed as a solution to these pitfalls. Although expression in hairy roots is not a suitable method for commercial production of recombinant vaccine in plants, the antigens produced in this procedure can be used for production of specific antibodies required in molecular diagnosis. Moreover, this method makes it possible to evaluate efficacy of a potential recombinant vaccine in a short time.

Expression in hairy roots involves introduction of the antigen coding sequence into the plant tissue by means of *A. rhizogenes*, and induction of transformed hairy root production.

In the present work, F and HN epitopes of Newcastle disease virus were expressed in tobacco hairy roots with the help of *A. rhizogenes*. As confirmed by Real Time PCR assay, expression level of the transgene was fairly high which is in agreement with the results obtained other investigators who had reported that foreign gene expression in transient gene expression assays is much higher than that of stable transformation (Leckie and Stewart, 2011; Wroblewski et al., 2005).

As indicated by dot-blot assay, the protein sample from transformed hairy roots produced an intense signal comparable to that from commercial Newcastle vaccines whereas protein of wild type plant was not detectable.

ELISA assay, which quantitates protein levels, also showed that the recombinant protein is produced at very high levels in transformed hairy roots. The faint absorbance observed for untransformed hairy root sample is probably resulting from the non-specific binding of proteins with the anti-histidine tag.

Plant viruses are also used as vectors for transient antigen expression in plants, where the desired epitope is inserted inside the gene encoding viral coat protein (Sala et al, 2003). Despite being a highly efficient method for recombinant protein production, preparation of viral vectors is a very time consuming and labour intensive task. Another drawback of viral vectors is that insertion of genes larger than a threshold size will diminish the efficiency of the vector (Sala et al., 2003).

On the other hand, the problems associated with recombinant protein production in stably transformed plants such as post-transformation gene silencing, undesirable alterations due to bond formation with complex carbohydrates, the considerable time required for development of a stable transgenic line with appropriate protein production capacity, and most importantly the environmental issues and the possibility of transgene escape (Jianxiang et al., 2007; Julian et al., 2003) have stimulated the application of alternative means such as transformed hairy roots for production of recombinant proteins or other products (Giding et al., 2000).

In the present work, different elements including the CaMV35s promoter, omega and SEKDEL sequences, and codon optimization were used in order to enhance the expression of F and HN epitopes, building on the published literature (Streatfield et al., 2001; Kang et al., 2004; Sala et al., 2003; Streatfield, 2005) reporting them to be important factors which increase expression levels of epitopes in the process of recombinant vaccine production.

It should be noted that the observed increase in expression levels can not be conclusively attributed to the mentioned elements, as the authors did not examine their influence; and this could be the subject of further studies.

Conclusion

Developments in genetic engineering have led to plant-based systems for recombinant vaccine production; but these systems are accompanied by a number of drawbacks which might be overcome with transient expression systems such as hairy roots. Here we report the application of tobacco hairy roots for expression of the F and HN epitopes of Newcastle disease virus. The results showed that hairy roots represent an efficient tool for expression of these viral antigens.

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