Transgenic plant production of Cyanovirin-N, an HIV microbicide

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ABSTRACT

Cyanovirin-N (CV-N) is a microbicide candidate that inactivates a wide range of HIV strains by binding to gp120. Production of CV-N, or any protein microbicide, needs to be at extremely high levels and low cost to have an impact on global health. Thus, it is unlikely that fermentor-based systems will be suitable, including recombinant E. coli, where CV-N aggregates and dimers have consistently been found. Transgenic plants may provide a suitable expression system for protein microbicides, as production can be easily and economically scaled up. Here, Nicotiana tabacum was transformed with a gene encoding CV-N to explore proof of concept for the production of CV-N in transgenic plants. Plant-derived rCV-N was recoverable at levels of 130 ng/mg of fresh leaf tissue, or at least 0.85% of total soluble plant protein. Western blot analysis demonstrated that virtually all of the rCV-N was expressed in the desired monomeric form. Functionality was demonstrated by specific binding to gp120 and protection of T-cells from in vitro HIV infection. Hydroponic culturing of transgenic plants demonstrated CV-N rhizosecretion at levels of 0.64 μg/ml hydroponic media after 24 days. Therefore, we suggest that transgenic plants have the potential to provide strategies for large-scale protein microbicide production.

Key words: protein expression • rhizosecretion

Topical microbicides are products that can be applied to the vagina or rectum prior to intercourse to prevent the sexual transmission of HIV. They are attracting increasing interest as a prevention strategy for HIV. Among the protein class of microbicides, Cyanovirin-N (CV-N) is a promising candidate (1). This 11 kDa protein can permanently inactivate an extensive range of HIV strains as well as prevent cell-to-cell fusion and transmission of HIV, with effective concentrations (EC50) in the nanomolar range (1). It acts by binding with high-affinity to high-mannose oligosaccharides on the HIV surface glycoprotein gp120, to interfere with viral-target cell receptor interactions essential for viral entry and cell-to-cell fusion (2). Recent studies have shown promising in vivo efficacy of CV-N formulated as a gel and used as a topical vaginal or rectal microbicide in macaque monkeys challenged with SHIV89.6P (3, 4).
As well as its antiviral activity, the remarkable stability and limited toxicity of CV-N offer additional attractive benefits. CV-N is resistant to boiling, multiple freeze-thaw cycles, dissolution in organic solvents such as methanol and DMSO, and treatment with high salt (8 M guanidine hydrochloride), detergent (0.5% SDS), or 0.5% hydrogen peroxide (1). As well as toxicity tests in vitro (1), a 10 mg/ml gel formulation of CV-N applied to the vagina and rectum of macaques demonstrated no irritation or histopathology (3, 4).

CV-N is naturally produced by *Nostoc ellipsosporum*, and it was discovered and isolated by an empirical screen by the National Cancer Institute. It was originally obtained by a series of extraction, filtration, and chromatography steps (1). The feasibility of a recombinant approach to CV-N production was first demonstrated in *E. coli* (1). Optimization of this system has led to yields of up to 140 mg/L. However, this expression system remains problematic, as it consistently produces a significant fraction of dimeric species and aggregates, as well as possible side-chain modifications (5). As an alternative, the production of CV-N in the eukaryotic system *Pichia pastoris* has been assessed by using mutations to remove a potential N-glycosylation site (N30), which, when glycosylated, causes loss of anti-viral activity as well as a mutation in the hinge region (P51G) to help avoid dimerization by proline mediated misfolding (6). The expressed mutants showed indistinguishable anti-viral activity compared with wild-type CV-N, and the results demonstrate the potential for expression of rCV-N in a eukaryotic system. Nevertheless, the potential global requirement for a protein microbicide far outstrips the production capacity using any cell culture system currently available (7). Consequently, in this study we assess the feasibility for rCV-N production in transgenic plants, which have the potential to provide a safe and inexpensive approach for the large-scale production of protein microbicides.

In recent years, considerable interest has emerged in the use of transgenic plants to generate compounds for medical and veterinary use. A variety of molecules have been successfully expressed in plants, including viral and bacterial antigens such as hepatitis B surface antigen (8), measles H protein (9), Norwalk virus capsid protein (10), and anthrax protective antigen (11), as well as many different forms of antibodies (12). Plant systems would offer many advantages for the production of CV-N as a microbicide; transgenic plant production can be scaled up to generate adequate supplies of the microbicide and production would benefit from the low cost of initial investment required to establish the necessary agricultural infrastructure. These factors are particularly important for microbicide production, given that a low-cost, high-output system is required in order to offer worldwide availability extending to developing countries.

**MATERIALS AND METHODS**

**CV-N cloning**

The gene encoding CV-N (CV-N N30Q/P51G) was amplified by PCR using the forward primer 5′-AAACTCGAGCCTTGGTAAATCTCCAG- 3′, and the reverse primer 5′-CGGGAATTCCTTATTCGTAT TTCAGGGT- 3′ designed to incorporate a 5′ XhoI and a 3′ EcoRI restriction site, respectively. The resulting 325 bp fragment was ligated into a derivative of vector pMON530 (13) The binary vector contains a cauliflower mosaic virus 35S promoter, a 60 bp murine IgG leader sequence to direct the recombinant protein to the plant endomembrane...
system, and the Nopaline synthetase 3' terminator, as well genes for resistance to spectinomycin and kanamycin. Recombinant vector was used to transform *E. coli* (DH5α, Invitrogen, Carlsbad, CA) by heat-shock transformation (14) before insertion into *Agrobacterium tumefaciens* (LBA4404, Invitrogen) by electroporation.

Recombinant *A. tumefaciens* was used to transform *Nicotiana tabacum* (var. *xanthii*) (13). Briefly, sterile leaf discs were incubated for 48 h with a log culture of the recombinant *A. tumefaciens*. Infected leaf discs were then transferred to RM3 agar [4.4 g/L Murashige and Skoog basal medium (Sigma, St. Louis, MO), 30 g/L sucrose, 0.1 mg/L α-Naphthaleneacetic acid (Sigma), 1 mg/L 6-Benzylaminopurine (Sigma)], supplemented with kanamycin (200 μg/ml) and carbenicillin (500 μg/ml), to stimulate callus formation and regeneration of shoots. Once shoots had developed, rooting was induced by transplanting supplemented MS agar (4.4 g/L Murashige and Skoog basal medium (Sigma), 30 g/L sucrose, 8 g/L agar) onto kanamycin (200 μg/ml) and carbenicillin (500 μg/ml). Resulting plantlets were transferred to soil and screened for expression of CV-N by Western blot. CV-N positive plants were allowed to flower for self-pollination and the production of seed.

**Transgenic plant leaf extracts**

Leaf extracts of plants were prepared by grinding one leaf disc (approximate fresh weight of 10 mg) in 200 μl of TBS plus 10 μg/ml leupeptin (Sigma) with a plastic pestle in a microfuge tube and centrifuging at 20,000 × g for 10 min at 4°C to remove plant debris.

**Western blot to detect CV-N**

Leaf extract (12.5 μl) was mixed with 7.5 μl SDS-loading buffer [0.3M Tris Hcl pH 6.8, 10% (w/v) SDS, 50% (v/v) glycerol, 0.125% (w/v) bromophenol blue] and loaded onto a 15% polyacrylamide gel. The gels were blotted onto nitrocellulose membrane using a semi-dry transfer system (Hoefer™ TE70, Amersham Biosciences, Piscataway, NJ) and blocked with 5% non-fat dried milk in TBS. Blots were incubated with a 1:1000 dilution of anti-CV-N polyclonal rabbit serum. After stringent washing, an alkaline phosphatase conjugated anti-rabbit IgG antiserum (Sigma) diluted 1:1000 was applied. Antibody binding was detected using AP conjugate substrate kit (BioRad, Hercules, CA).

**HIV-1 gp120 binding ELISA**

The ability of CV-N to bind to HIV-1 gp120 was assessed by a conventional ELISA according to the method of Boyd et al. (1). Briefly, 100 ng of recombinant gp120 (HIV-1IIIB unless otherwise stated; EVA607, MRC Centralised Facility for AIDS Reagents, Potters Bar, UK) was added to each well of a 96-well plate (Nunc Maxisorp Immuno™ plates, Rochester, NY) and incubated at room temperature for 2 h. After washing and blocking of the plate, serial dilutions of plant extracts were added alongside a purified CV-N positive control (recombinantly produced in *E. coli*). Bound CV-N was detected with a 1:1000 dilution of anti-CV-N polyclonal rabbit serum. Alkaline phosphatase conjugated anti-rabbit antibody (Sigma) diluted 1:2500 was used as the secondary antibody. Alkaline phosphatase substrate (pNPP, Sigma) was added, and the absorbance read at 405 nm.
Anti HIV assay

Plant extracts were obtained by grinding 4 leaf discs (~40 mg) in 800 μl RPMI 10% (without phenol red, Sigma) supplemented with 10 μg/ml leupeptin. Extracts were centrifuged at 20,000 × g for 10 min at 4°C, and supernatants were sterilized through a 0.22 μM filter. Serial dilutions of plant extract were added to individual wells of a 96-well plate (Nunc 96 Microwell flat bottom with lid) to give a final volume of 50 μl per well. C8166 cells (4×10^4) in a volume of 50 μl (ARP013, MRC Centralised Facility for AIDS Reagents) and 25 μl of a 1:1000 dilution of HIV-1Ⅲb (equivalent to 115 ng/ml p24, propagated from chronically infected H9 cells) were added to appropriate wells. The plate was incubated at 37°C with 5% CO₂ for 5 days. Cytopathic effects, as indicated by the presence of syncytia, were observed and recorded at days 3 and 5 (Leica DMIL microscope, 100× magnification). At day 5 an XTT assay was performed to assess viable cells. XTT (25 μl of 1 mg/ml; 2,3-bis(2-Methoxy-4-nitro-5-sulfophenyl-2H-tetrazolium-5-carboxanilide, Sigma) mixed with 0.02 mM phenazine methosulphate (Sigma) was added to each well. The plate was incubated at 37°C with 5% CO₂ for 2 h and then agitated on a plate shaker for 30 min before reading the absorbance at 450 nm. The absorbance was also measured at 650 nm to detect cell background, and this reading was subtracted from the 450 nm reading. Absorbance readings obtained from the virus control wells represent cells remaining after what is considered 100% infection. This reading was subtracted from all absorbance readings. Resulting absorbance readings for samples were then used to calculate the relative infectivity of the virus and were expressed as a percentage in relation to cell control absorbance readings.

Hydroponic cultures

Hydroponic cultures of transgenic plants were established in MS media. Sterile seedlings were transferred to 30 ml MS media in a Magenta box supported by a plastic platform to ensure roots were submersed in media while the leaves were kept above the media. Seedlings were allowed to grow to a height of ~7 cm, at which point all media were removed and fresh media added. Media aliquots were then removed at required time points to store at –20°C before analysis. Fresh media were added to replenish the aliquot sampled. Samples were assessed for the presence of CV-N secreted into the media by gp120 binding ELISA and Western blot.

Total soluble protein of leaf extracts

Total soluble protein of leaf extracts was assessed by use of a BCA Protein Assay (Pierce Biotechnology, Rockford, IL) in 96-well plate format. Dilutions of bovine serum albumin (BSA) were used to construct a protein concentration curve to calibrate sample protein concentrations.

RESULTS

CV-N construct

Sixty-two independently transformed tobacco plants were regenerated. Of these, 26 were screened positive by Western blot of leaf extracts using a polyclonal anti-serum to detect CV-N (Fig. 1). As a positive control, the bacterial recombinant CV-N is shown (lane 7). In this sample, in addition to the expected 11 kDa band, the production of dimers and higher aggregates of CV-
N is evident, and these represent a significant proportion of the total protein as described previously (15). Plants expressing CV-N demonstrated the presence of a band at ~11 kDa, the predicted MW of CV-N (Fig. 1, lanes 2–4). Some higher molecular weight nonspecific bands were evident at around 50 kDa and above, but these were also present in the wild-type plant extracts (Fig. 1, lane 8) and thus are likely to be due to cross-reactivity of the polyclonal rabbit serum with plant proteins. No higher weight bands corresponding to oligomers were detected in the plant extracts, which suggests that the plant expressed CV-N is almost all in the monomeric form. When the E. coli-derived CV-N was diluted to give an 11 kDa band of equal intensity to the plant sample, the bands corresponding to the CV-N dimer was still clearly detectable in the E. coli preparation (data not shown).

**gp120 Binding of plant produced CV-N**

Functional activity of plant derived CV-N was assessed by a gp120 binding ELISA. All transgenic plant extracts tested demonstrated binding to soluble gp120IIIb in a concentration-dependent manner. Figure 2 shows the mean and standard deviation of four transgenic plants in comparison with a titration curve for E. coli produced CV-N. No binding of the plant extracts to a control protein, BSA, was detected (data not shown). Wild-type plants did not demonstrate binding to gp120IIIb, confirming that all activity was due to the CV-N produced by the plants and not caused by cross-reactive native plant proteins (Fig. 2).

Binding to gp120 proteins from different HIV-1 strains (SF2 and MN; MRC Centralised Facility for AIDS Reagents, ref # ARP629 and EVA646) was assessed by the same method. As seen with gp120IIIb, all plants tested bound to gp120SF and gp120MN in a similar concentration dependent manner (data not shown).

**Anti-HIV-1 activity of plant produced CV-N**

To assess anti-HIV effects of plant derived CV-N, crude plant extracts were incubated with HIV-1IIIb (from chronically infected H9 cells) and a susceptible T cell line (C8166). Cells incubated with transgenic CV-N plant extracts showed a complete absence of HIV-induced cytopathic effects, as indicated by syncytia formation, comparable with the control E. coli derived CV-N incubated wells and the cell only control (Fig. 3Aa, b, and d). The presence of syncytia in control infection wells and those wells incubated with wild-type tobacco plant extracts demonstrated that there was efficient HIV infection of the T cells (Fig. 3Ac and e).

HIV-induced cytopathic effects result in cell death. Thus, cell viability was assessed by XTT assay at day 5 post-infection, to indicate HIV infection. Results of the XTT assay are expressed as relative infectivity compared with control well readings corrected for uninfected cells. Figure 3B shows that E. coli produced CV-N inhibits HIV infectivity in a concentration-dependent manner. The extracts from three separate CV-N transgenic plants inhibit HIV infection in a similar manner, and no inhibition was observed for wild-type tobacco plants. Leupeptin, a serine/thiol protease inhibitor used in the plant extraction to inhibit plant proteases was also included in the assay to ensure it had no effect on HIV activity (data not shown).

Results from undiluted extracts from both non-transformed and transgenic plants showed an apparent reduction in T cell number (data not shown). However, the absence of syncytia
formation suggests that this effect is unlikely to be caused by HIV infection and is presumably caused by the cytotoxic effects of compounds that are present in the crude tobacco plant extracts.

The results indicate that the CV-N in the crude extract of the transgenic plants demonstrates anti-HIV activity. The biological activity of the CV-N in crude plant extract without purification is encouraging, although the possible cytotoxic effects of the plant extract indicate that purification will be required.

**Plant CV-N concentrations**

We used gp120 binding ELISA results to quantify CV-N concentrations in the transgenic tobacco plants using *E. coli* derived CV-N as a standard. CV-N concentrations were calculated for five of the higher expressing independently transformed plants. In these plants the concentration ranged from 31.0 to 130.3 ng CV-N per mg of leaf tissue. The difference in expression levels is explained by the random insertion of the gene construct into the plant genome by Agrobacteria-mediated transformation (16).

Total soluble protein (TSP) of the plant extracts was analyzed by BCA protein assay (Pierce). TSP data in conjunction with CV-N concentrations were used to calculate CV-N levels expressed as a percentage of total soluble protein (% TSP). Plant 1, the highest CV-N expressing plant out of those tested, yielded an average CV-N production of 0.85% TSP. These observations were confirmed functionally by measuring the comparable HIV inhibitory effects of plant derived CV-N with *E. coli* produced CV-N. Concentrations were calculated in conjunction with the TSP data and found to correspond with % TSP results calculated from the gp120 binding assays (data not shown).

**Rhizosecretion of CV-N from transgenic plant roots**

Hydroponic systems are commonly used for the cultivation of plants in greenhouses. With the demonstration that transgenic plants can rhizosecrete recombinant proteins (17, 18), this system is an attractive method of production as well as potentially simplifying the purification of the protein from the plant material. The plant expression vector contains a murine leader sequence that targets the CV-N to the cellular secretory pathway. In addition, the CaMV 35S promoter drives transcription in all cells of the plant; thus, CV-N should be secreted from the roots of the transgenic plants. Hydroponic cultures of the CV-N transgenic plants were set up, and the hydroponic medium was analyzed for the presence of CV-N by assessing its ability to bind to gp120. Binding data of sequentially collected media samples indicate that CV-N is indeed secreted by the plant roots and shows an accumulation in the media up to day 24 (Fig. 4A). These results were confirmed by Western blots of media samples to detect CV-N (Fig. 4B) where an 11 kDa band indicative of CV-N is viewed for samples harvested at days 14 and 24. The CV-N concentration of the media at day 24 was calculated based on *E. coli*-derived CV-N concentrations and found to be 0.635 μg/ml (±0.298 μg/ml) (data not shown). It is promising that secreted CV-N is readily detectable in the hydroponic media as this could provide a simpler, alternative strategy for CV-N purification from plants.
DISCUSSION

In this study, transgenic tobacco plants expressing the CV-N gene were successfully regenerated. The expressed recombinant protein was detectable by Western blotting and HIV gp120-specific ELISA, and crude plant extracts demonstrated functional anti-viral activity, as demonstrated by protection of C8166 cells from HIV-1llIB infection.

The expression levels in plants were up to 130 ng/mg of fresh leaf material, equivalent to at least 0.85% of TSP. This may represent a significant underestimate of the real levels of expression in plants because of uncertainty regarding the CV-N concentration in the positive control *E. coli* extract that was used for standardization (5) and the fact that the extraction procedure from plants has yet to be optimized. Nevertheless, 0.85% compares favorably with other recombinant proteins that have been expressed in transgenic plants, ranging from 0.01% TSP for hepatitis B surface antigen (19) to 1% TSP for IgG monoclonal antibodies (14). It has been suggested that a protein expression level of 1% TSP is necessary to make plants viable as an expression system (20), as this minimizes handling of bulk material and avoids complications for purification.

CV-N is a relatively simple polypeptide. It is an elongated ellipsoid characterized by an internal twofold pseudo-symmetry, comprising two structural domains, which associate through the formation of two disulphide bonds that are essential for anti-HIV activity. However, attempts at expression in *E. coli* have resulted in a number of technical difficulties, including low levels of expression as well as the formation of unwanted dimers and aggregates (as seen in the *E. coli* control lane of Fig. 1). The latter are thought to be due to the formation of domain swapped dimers, caused by rotation around the hinge region of the molecule (15).

A mutation in the hinge region of the protein has been investigated (P51G), which was postulated to stabilize the monomeric form of CV-N and avoid dimerization by minimizing the possibility of proline mediated misfolding or conformational heterogeneity (15). In addition, a further construct was made containing the P51G mutation as well as a N30Q mutation to remove a putative N-glycosylation site, and this is the construct used in the present study. Early studies of this double mutant in yeast demonstrated enhanced stability, although aberrant processing of the signal sequence was an unexpected hindrance to the development of this expression system (6). This mutation turned out to be of limited value in *E. coli* expression, with dimerization remaining a significant concern. Consequently recent efforts to express rCV-N in *E. coli* have focused on expression and purification from inclusion bodies (5). Yields reaching 140 mg/L can be achieved with a fed-batch system, a 14-fold increase on previous attempts of *E. coli* periplasmic expression. The resulting CV-N was found to be ~95% pure protein and have equivalent anti-HIV activity to its native counterpart. However, although the stability of CV-N allows it to withstand the harsh inclusion of the body purification regime and refolding of the protein, anion exchange chromatography resulted in a significant proportion of CV-N being retained on the column probably due to the presence of CV-N species with side chain modifications as well as higher weight oligomers. It is unclear whether these have been produced as a result of the expression or the purification process. However, further optimization of this system is required to reduce the formation of these aberrant forms and thus increase the yield of monomeric CV-N. In contrast the absence of any apparent dimers or aggregates of CV-N from the transgenic plants in this study suggests that the plant-derived CV-N appears to be expressed primarily, and optimally, in a monomeric form. It will be interesting to determine why this
phenomenon occurs. Further study will be required to explore our hypothesis that, in common with other plant expressed recombinant proteins, CV-N production is facilitated by molecular chaperones in the plant secretory pathway (21).

Targeting of CV-N to the plant secretory endomembrane system using a leader sequence results in rhizosecretion of intact and functional CV-N into hydroponic culture media. Hydroponic culturing has proved a successful method for the secretion of IgG, bacterial xylanase, green fluorescent protein, and human placental secreted alkaline phosphatase (17, 18). In the case of IgG, levels of 0.28 μg/25 ml media/day were achieved (18). In the present study, the mean CV-N concentration was 0.64 μg/ml in hydroponic culture media after 24 days. This equates to 0.67 μg/25 ml/day. The remarkable stability of CV-N in relation to denaturation (1) may make this protein particularly suitable for hydroponic harvesting, as it appears that the CV-N can accumulate in the medium for up to 24 days before harvesting, unlike IgG, which degraded significantly after only 11 days of culturing and also required the presence of gelatin in the media to stabilize the antibody (18). Hydroponic culturing is a potentially advantageous production method for several reasons. The recombinant protein can be continually produced and harvested throughout a plant’s lifetime; in addition, the recombinant protein production can take place in a highly controlled and contained environment that is more consistent with current standards for good manufacturing practice. Furthermore, the recombinant protein needs only to be isolated from simple liquid medium rather than a complex plant extract, thus simplifying and reducing the cost of purification. This is of particular importance when using tobacco, which contains a high content of toxic alkaloids (22). It is likely that these components were responsible for the cytotoxic effects observed on T-cells when incubated with undiluted plant extract in anti-HIV assays, and these would need to be removed before using the plant derived CV-N clinically.

CV-N is a promising candidate for a topical HIV microbicide. However, its usefulness in helping to tackle the global HIV epidemic ultimately depends on the ability to produce enough of this protein to satisfy demand not only in the West, but also in sub-Saharan Africa. A 5 mg dose, which proved to be effective in macaque studies, requires a production capacity of 5000 kg a year to supply just 10 million women twice a week (7). This scale of microbicide production may be achievable only by using plant expression systems, as transgenic plants are uniquely amenable to large-volume scale up and low production costs. This study provides proof of concept that functional CV-N can be produced in plants at expression levels suitable for commercial production. Furthermore, the molecular stability of CV-N may mean that it can be extracted from plants more efficiently than other recombinant proteins because simpler purification steps can be used, thus reducing downstream processing costs. Efficient rhizosecretion of CV-N also offers promising approaches to deal with regulatory issues. We believe these plant expression strategies could represent an important step in the development of protein microbicides for public health.

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REFERENCES


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Fig. 1

Figure 1. Western blot of CV-N transformed plant extracts. Crude plant extracts were run on a 15% polyacrylamide gel under non-reducing conditions and probed with anti-CV-N polyclonal rabbit sera. Lanes 1 and 6: protein molecular weight standards. Lanes 2–5: four different independently transformed transgenic plants (12.5 µl of extract). Lane 7: control purified CV-N (0.22 µg). Lane 8: non-transformed tobacco plant.
Figure 2. gp120 binding of transgenic plant extract measured by ELISA. Results show the mean and SD of four transgenic plants. Purified recombinant CV-N (CV-N) from *E. coli* was used as a positive control at a top concentration of 250 ng/ml. Data for non-transformed plant are mean and standard deviation of three experiments.
Figure 3. A) Prevention of syncytium formation by plant-derived CV-N. C8166 cells were incubated with HIV-1$_{\text{IIIB}}$ and (a) E. coli-derived CV-N (31 nM); (b) crude plant extract from transgenic tobacco expressing CV-N (diluted 1:8); (c) crude plant extract from wild-type tobacco (diluted 1:8). Cell only control and cells plus virus control are shown in (d) and (e), respectively. Cells were observed under 100× magnification on day 5 post-infection. B) Inhibition of HIV infection by plant derived CV-N. XTT assays were performed after incubation of cells and HIV-1$_{\text{IIIB}}$ with plant extracts or E. coli-derived CV-N (top concentration of 2.75 μg/ml) for 5 days. Absorbances were adjusted for uninfected and infected controls to give relative infectivity of HIV. Results are shown for the mean of duplicate wells in a single representative experiment.
Figure 4. Detection of CV-N in hydroponic culture media. A) gp120 binding ELISA of sequential media samples diluted 1:4 for CV-N transformed plants (○). One data point is shown for the medium from a non-transformed plant at day 30 (▲). Results are the mean and standard deviation of three experiments. B) Western blot to detect CV-N. Lane 1: crude leaf extract from CV-N transformed plant; lanes 2 and 3: hydroponic culture media harvested on days 14 and 24, respectively.