Production of human vitronectin in *Nicotiana benthamiana* using the INPACT hyperexpression platform

Benjamin Dugdale1,*; Maiko Kato1; Pradeep Deo1; Manuel Plan1,**; Mark Harrison1; Robyn Lloyd1; Terry Walsh1; Robert Harding1 and James Dale1

1Centre for Tropical Crops and Biocommodities, Queensland University of Technology (QUT), Brisbane, QLD, Australia.

**Correspondence** (Tel +61 7 3138 1661; fax + 61 7 3138 4132; email b.dugdale@qut.edu.au)

*Present address: Metabolomics Australia (UQ Node), Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, St Lucia, QLD, 4072, Australia.

Keywords: biopharming, INPACT, transgene expression, *Nicotiana benthamiana*, vitronectin, inducible.

**Summary**
Human vitronectin (hVN) is a glycoprotein that functions as a cell adhesion molecule and a regulator of coagulation in blood plasma and the extracellular matrix. *In vitro*, hVN is added to serum-free media in order to promote the adhesion of animal cells to tissue culture surfaces and the proliferation of undifferentiated stem cells. Here, we report the production of hVN in *Nicotiana benthamiana* using the inducible In Plant ACTivation (INPACT) hyperexpression platform. *N. benthamiana* plants were transformed with an INPACT expression cassette encoding hVN, and both the Tobacco yellow dwarf virus RepA activator and Tomato bushy stunt virus p19 gene under the transcriptional control of the ethanol-inducible ALCaA gene switch. hVN expression was maximal 4–5 days postactivation of the INPACT platform with a dilute ethanol solution, and crude yields of the recombinant protein reached a maximum of 643 ± 78 mg/kg fresh weight. A three-stage purification protocol was developed using heparin and polyhistidine tag affinity binding and size exclusion filtration, resulting in a plant-made hVN product of >90% purity. Storage conditions for plant-made hVN were identified that maximized the capacity of the recombinant protein to promote cell adhesion. Critically, plant-made hVN was shown to be functionally equivalent to commercial, plasma-derived hVN in promoting one-half maximal attachment of murine fibroblast cells (BALB-C/3T3) in serum-free medium at <0.1 μg/cm² to tissue culture plasticware. The INPACT platform represents an attractive means of producing large quantities of functional, animal-free hVN for *in vitro* applications.

**Introduction**
Human vitronectin (hVN), formerly known as serum spreading factor, is one of the major cell adhesion proteins found in the blood and the extracellular matrix (Conlan et al., 1988; Shaffer et al., 1984). Nascent hVN is converted to its mature form via cleavage of a 19-amino acid secretion signal peptide at the N-terminus. Circulating hVN occurs predominantly as a monomeric polypeptide (75 kDa) or as two polypeptides (65 and 10 kDa) linked by a disulphide bond (Tollefsen et al., 1990) and provides a regulatory link between a wide range of biological activities, including tissue repair, angiogenesis, haemostasis and metastasis (Schwartz et al., 1999). hVN interacts with diverse proteins via conserved regions located throughout the polypeptide chain (Jin and Varner, 2004; Preissner, 1991; Zhou et al., 2003) and the carboxyl-terminal region contains a cryptic, arginine-rich domain that binds heparin, an anticoagulant. This domain is only exposed after hVN binding to the thrombin–antithrombin III complex *in vivo* or denaturation with urea, heat, or acid *in vitro*, and it is this conformational change that activates self-association of hVN into multimers with significantly increased affinity for heparin compared to the monomeric form (Preissner, 1991).

Vitronectin (VN) has been isolated from a range of mammalian sources and utilized for both research and clinical applications because of its adhesive properties. VN is most commonly used in animal cell culture to coat tissue culture surfaces in order to promote cell adhesion and induce cell spreading (Underwood and Bennett, 1989). Recombinant VN and its variants have also been used in combination with defined culture media to maintain pluripotency and growth of pluripotent stem cells (Li et al., 2010). Fusion of hVN to insulin-like growth factor (IGF), IGF-binding proteins and epidermal growth factor significantly enhances proliferation and migration of primary skin keratinocytes and the re-epithelialization of wounds (Hollier et al., 2005; Upton et al., 2008; Xie et al., 2010). As a result, clinical hVN-based treatments for improved wound management and healing have been developed, particularly where cell proliferation is required and/or wound repair has been delayed, such as in burns or ulcers (Upton et al., 2011). Further, hVN and its derivatives have been used to promote osseointegration of implantable devices by improving surface–cell interactions and increasing implant connectivity with surrounding bone (Caccioni et al., 2009). hVN in its native form is traditionally purified from human blood plasma. As such, the protein must undergo strict regulatory testing for such use and is prohibitive for applications in translational animal research.

Plant-based protein production is a convenient means of manufacturing therapeutics and nontherapeutics normally isolated from animal sources, including blood plasma or tissues. Production in plants minimizes the risk of contamination with animal pathogens and obviates the need for expensive serological screening and more extensive purification. Such quality control...
processes are typically required for animal-derived protein products destined for use in animal or human therapeutics. However, the economic feasibility of plant-based protein production relative to conventional bacteria, yeast or insect cell bioreactor systems is fundamentally dependent upon recombinant protein yield. The use of plant viral vectors to amplify transgene copy number and virus-derived gene products to suppress post-transcriptional gene silencing (PTGS), an innate plant defence pathway that can specifically target transgene mRNA for degradation, has improved recombinant protein yields such that plants are becoming a competitive platform for the production of biologically equivalent proteins. Recent ‘deconstruction’ of the genomes of both DNA and RNA plant viruses has allowed the development of plant viral transgene expression vectors adapted for either short-term, transient or long-term, stable protein production in plants (reviewed in Lico et al., 2008 and Mortimer et al., 2015).

We recently described the In Plant ACTivation (INPACT) platform, an inducible, high-level expression system for transgenic plants based upon the disaggregated DNA genome of a geminivirus, Tobacco yellow dwarf virus (TYDV) (Dugdale et al., 2013, 2014). The INPACT platform is unique in that the gene of interest is split and arranged such that its expression only occurs from extrachromosomal episomes that are released from the host chromosome in the presence of the TYDV-encoded replication-associated proteins, Rep and RepA. Temporal control of Rep/RepA expression is achieved using the ethanol-responsive alc gene switch. Essentially, the INPACT platform provides the benefits of transient transgene expression in a stably transformed plant, thereby disconnecting plant growth from recombinant protein production. *Nicotiana benthamiana* transformed with an INPACT platform encoding hVN and activated with a dilute ethanol solution reached maximum crude yields of ~100 mg hVN/kg fresh weight (FW) (Dugdale et al., 2013). In the present study, we have integrated the gene encoding Tomato bushy stunt virus (TBSV) p19, a suppressor of PTGS, into the INPACT platform and describe its effects on recombinant hVN accumulation in *N. benthamiana*. The inclusion of TBSV p19 into the INPACT expression platform significantly increased maximum production of recombinant hVN by ~sixfold. A simple, three-stage hVN purification process was developed, and the ability of plant-made hVN to promote the attachment of murine fibroblast cells to tissue culture surfaces was compared to commercial hVN isolated from human plasma. The results demonstrate the potential of the INPACT expression platform for biopharming animal proteins in planta.

**Results**

**Production of transgenic *N. benthamiana* plants containing an enhanced INPACT platform encoding hVN**

Synchronized activation of transgene amplification and expression from the INPACT platform is strongly dependent on the regulated expression of Rep/RepA activator genes. As such, the identification of elite *N. benthamiana* parent lines transformed with the ethanol-inducible TYDV Rep/RepA cassette (pAlc-Rep/RepA; Figure 1) was critical. Elite lines must satisfy two major criteria: (i) minimal Rep/RepA expression in the absence of the ethanol inducer molecule but rapid activation postethanol application, and (ii) minimal negative physiological impact of Rep/RepA accumulation on the plant, as overexpression of these gene products can be phytotoxic and cause rapid yellowing and necrosis (Dugdale et al., 2013). Transgenic *N. benthamiana* plants (NbAlc-1, -2, -3, -4 and -5) containing the ethanol-inducible TYDV Rep/RepA cassette were generated using *Agrobacterium tumefaciens*-mediated leaf disc transformation, acclimatized in soil and activated with a 5% (v/v) ethanol foliar spray. Three days postethanol application, RNA was extracted from leaves and used as the template in a reverse transcription-polymerase chain reaction (RT-PCR) with primers specific for Rep/RepA gene sequences. Rep/RepA transcripts, indicated by an ~750-bp RT-PCR product, were detected in four of the five plants (NbAlc-1, -2, -4 and -5) following ethanol application (Appendix S1). No PCR product was observed in RT-PCRs without reverse transcriptase, indicating the absence of contaminating gDNA. RNA extracted from *N. tabacum* line NtSRN-2 (a tobacco line containing the same pAlc-Rep/RepA cassette) provided the positive control for the RT-PCR. This tobacco line has been previously shown to express Rep/RepA by quantitative real-time PCR (qRT-PCR) following ethanol induction (Dugdale et al., 2013). Based upon the abundance of Rep/RepA transcripts in the RT-PCR and the absence of an abnormal phenotype associated with Rep/RepA accumulation, NbAlc-1 was selected as the elite line for supertransformation with the modified INPACT platform encoding hVN.

*Agrobacterium*-mediated transformation was used to supertransform leaf discs from NbAlc-1 with the modified INPACT platform encoding hVN and containing the TBSV p19 gene under the transcriptional control of the alcA promoter (pINPACT-hVN2, Figure 1). To facilitate accumulation of hVN, the native N-terminal secretion signal was preserved and an ER

| pINPACT-hVN2 | TYDV LIR | 6XHIS-KDEL | Native secretion signal | TYDV LIR |
|--------------|----------|------------|-----------------------|----------|
| alcA→TBSV p19 | nosT→HVN exon | 2→HVN exon | Syntron 3’ half | TYDV LIR |
| alcA→6XHIS-KDEL | nosT→TYDV SIR | Syntron 5’ half |

**Figure 1** Schematic representation of the ethanol-inducible Rep/RepA activator cassette (pAlc-Rep/RepA) and the modified INPACT cassette encoding hVN and p19 (pINPACT-hVN2). 35SP = CaMV 35S promoter, nosT = nopaline synthase gene terminator, alcA→alcA: minimal CaMV 35S promoter fusion, alcR = gene encoding the alcohol receptor transcription factor, TYDV Rep/RepA = gene encoding the Tobacco yellow dwarf virus Rep/RepA activator proteins, TBSV p19 = gene encoding the Tomato bushy stunt virus 19 K protein, TYDV LIR = Tobacco yellow dwarf virus large intergenic region, syntron = synthetic intron, hVN exon = part of gene encoding the human vitronectin protein, TYDV SIR = Tobacco yellow dwarf virus small intergenic region, 6XHIS = polyhistidine affinity tag, KDEL = ER retention signal.

© 2017 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd., 16, 394–403
retention signal (KDEL) added to the C-terminus. For purification purposes, a C-terminal polyhistidine affinity tag (6XHIS) was also included. Following cleavage of the 19-amino acid secretion signal, plant-made hVN has an approximate size of 469 amino acids and an estimated glycans-free molecular weight of 53.63 kDa. Eleven independent *N. benthamiana* lines were regenerated on media containing both kanamycin and hygromycin.

**Identification of elite INPACT supertransformed lines**

Detached leaves from the eleven transgenic *N. benthamiana* lines were excised and tested for ethanol-induced accumulation of recombinant hVN. Leaves were incubated on MS0 solid media with small wells containing 5% (v/v) ethanol. Total soluble protein (TSP) was extracted 5 days postactivation and recombinant hVN levels determined by immunoblotting with an hVN-specific monoclonal antibody. One line, T0-2, was identified as a high hVN-expressing INPACT plant (results not shown) and grown to maturity. Southern hybridization analysis using probes specific for either the nptII selection gene (within the pAlc-Rep/RepA T-DNA) or hVN gene (within the pINPACT-hVN2 T-DNA) showed this elite line contained a single integrated copy of both the pAlc-Rep/RepA and pINPACT-hVN2 cassettes (Appendix S2). Line T0-2 was selfed, and the resulting 16 T1 generation events were screened for hVN accumulation using the same method as was used to analyse the T0 events. Three events expressing the highest levels of hVN (T1-8, T1-13 and T1-15) were selected by immunoblotting (Appendix S3). These lines were selfed and three T2 generation events (T2-1, T2-2 and T2-3) identified as high-expressing hVN plants. All generations of transgenic plants developed normally and appeared phenotypically similar to wild-type *N. benthamiana* plants in tissue culture and soil. Normal growth and development of transgenic plants was also observed in subsequent T3 generation plants grown in soil (Appendix S4).

**Ethanol-activated expression of p19 and Rep/RepA in elite INPACT plants**

Detached leaves from T2 progeny plants, designated T2-1, T2-2 and T2-3, were activated *in vitro* by incubation in liquid MS0 media containing 0.5% (v/v) ethanol for 5 days. RNA was extracted from leaves pre- (Day 0) and postethanol (Day 5) activation and analysed by RT-PCR (Figure 2). *p19* transcripts (as indicated by a ~550-bp RT-PCR product) were detected in all transgenic progeny at Day 0, suggesting ‘leaky’ expression in the absence of the ethanol inducer molecule (Figure 2a). The relative abundance of these RT-PCR products, however, increased by Day 5 suggesting the addition of ethanol does increase *alcA*-directed *p19* expression. In contrast, no *Rep/RepA* expression was observed prior to ethanol activation, and *Rep/RepA* transcripts were relatively abundant after activation (as indicated by a ~450-bp RT-PCR product; Figure 2b). The sizes of the RT-PCR products for both *p19* and *Rep/RepA* were smaller than the PCR products amplified using plasmid DNA as templates, indicating correct processing of both the synthetic and TYDV Rep introns, respectively. No RT-PCR products were observed in reactions using wild-type *N. benthamiana* RNA as the template. RT-PCR products (~400 bp) were obtained from all plant RNAs using primers designed to amplify the actin housekeeping gene (Figure 2c). No RT-PCR products were observed in the absence of the reverse transcriptase enzyme.

**Kinetics of recombinant hVN accumulation and ethanol dose–response**

The kinetics of recombinant hVN accumulation were assessed by incubating transgenic leaf material from elite T2 generation line T2-2 in liquid MS0 solution containing 0.5% (v/v) ethanol for 3, 4, 5, 6 and 7 days. hVN accumulation in leaf total soluble protein (TSP) extracts was measured by immunoblotting (Figure 3a). Maximum accumulation of the 75-kDa form of recombinant hVN was observed 4–5 days after INPACT activation based on immunoblot signal intensities. Ethanol dose–response was assessed by incubating leaf material from the same plant in liquid MS0 solution containing increasing concentrations of ethanol (0.1%, 0.25%, 0.5%, 1.0% and 2.0% (v/v)). Five days after activation, hVN accumulation was measured in leaf TSP extracts by immunoblotting (Figure 3b). Maximum accumulation of the 75-kDa form of recombinant hVN was observed using 0.5% (v/v) ethanol based on immunoblot signal intensity. Interestingly, high molecular weight hVN forms and an ~60 kDa hVN degradation product were also observed after immunoblotting. These extraneous hVN forms are likely multimers of hVN formed by self-association under non-denaturing extraction conditions and a proteolytic cleavage product as a result of the freeze-and-thaw process prior to SDS-PAGE and immunoblotting, respectively.
Estimation of hVN crude yield

Yield estimates of plant-made hVN were obtained using T₃ generation plantlets. Seed from three independent T₂ generation plants (T₂₁, T₂₂ and T₂₃) were sown onto MS0 media containing both kanamycin and hygromycin antibiotics and 100% of the seed germinated within 1 week. This suggested all T₃ generations plants contained both gene cassettes. Eight seedlings representing each T₂ parent plant were randomly selected. Detached leaves from in vitro plants were activated for 5 days in liquid MS0 media containing 0.5% (v/v) ethanol. Leaf material from the eight seedlings was pooled (designated Batches T₃₁, T₃₂ and T₃₃) and hVN levels in the TSP compared to known quantities of commercial, pure hVN (Promega) by SDS-PAGE and immunoblotting (Figure 3c). The use of denaturing buffer to extract plant-made hVN and the immediate analysis of these extracts by SDS-PAGE resulted in a single 75-kDa band on the immunoblot. The commercial hVN standard appeared as a doublet because human-derived hVN is clipped into two major products of 75 kDa and 65 kDa. A ChemiDoc imaging system (Bio-Rad) was used to estimate the amount of hVN in TSP extracts by comparing signal intensity to the hVN standard curve ranging from 50 to 500 ng. This entire process was performed three times on separate occasions. The estimated hVN yields (expressed as mean ± standard error) from Batches T₃₁, T₃₂ and T₃₃ over three independent experiments were 577 ± 162, 709 ± 155, 642 ± 40 mg/kg (FW) of leaf, respectively (Table 1). Statistical analysis indicated there was no significant difference in yields between batches of T₃ generation seedlings (P > 0.05), suggesting consistent activation and expression in this generation of plants. The overall estimated average hVN yield from T₃ generation plants was 643 ± 78 mg/kg FW of leaf.

Purification of recombinant hVN from N. benthamiana

Human vitronectin was purified from N. benthamiana using a three-stage process based upon both affinity chromatography and size exclusion filtration. Samples from each key step (Figure 4) and all steps (Appendix S5) of the purification process were separated by SDS-PAGE and visualized using Coomassie Blue dye. N. benthamiana leaf proteins were solubilized in 9 M urea to induce the conformational change in hVN that increases heparin affinity (Figure 4, lane 1). Denatured hVN was resolved from the majority of contaminating N. benthamiana leaf proteins by heparin affinity chromatography (Figure 4, lane 2). The purity of hVN after elution from the heparin affinity matrix was ~70%. hVN was further purified using metal affinity chromatography (Figure 4, lane 3). The purity of hVN after elution from the metal affinity matrix with 150 mM imidazole was ~80%–90%. hVN was separated from low molecular weight (<30 kDa) contaminants and concentrated to ~0.5 mg/mL using centrifugal ultrafiltration.
The purity of concentrated hVN was estimated to be >90% at a final yield of between 30 and 128 mg/kg FW of transgenic N. benthamiana leaf.

The identity of the purified protein from transgenic N. benthamiana leaf was confirmed by N-terminal amino acid sequencing (Figure 5). No amino acid was detected on the fifth of seven cycles of Edman degradation. However, the presence of Cys-S-β-propionamidine (Cys-S-Pam), the product of cysteine alkylation by acrylamide under alkaline conditions, after the fifth Edman degradation cycle, suggested the presence of a cysteine residue. The resulting amino terminal sequence is identical to that predicted following cleavage of the native 19-amino acid hVN secretion signal (Figure 5).

Adhesive properties of plant-made hVN

The capacity of plant-made hVN to promote cell adhesion in tissue culture was assessed using murine fibroblast cells (BALB-C/3T3) (Figure 6a). Commercial, plasma-derived hVN (Promega) was used as a control. Fibroblast binding increased with increasing concentrations of both plant-made hVN and commercial hVN. Maximum fibroblast adhesion (4 × 10^6 cells/well) was observed at 0.4 μg hVN/cm², and half-maximum fibroblast adhesion occurred at ~0.05 μg hVN/cm². The capacity of plant-made hVN to promote fibroblast adhesion was statistically equivalent to that of the commercial product over all concentrations tested (P > 0.05).

Plant-made, purified hVN was stored for 7 weeks as a liquid at 4 °C, a frozen liquid at ~80 °C, and freeze dried powder at ~80 °C to determine the effects of long-term storage on protein activity. The capacity of plant-made hVN to promote cell adhesion in tissue culture after storage was compared with commercial, plasma-derived hVN (Promega) (Figure 6b). Maximum fibroblast adhesion for all hVN forms was observed at ~1.0 μg hVN/cm². Half-maximum fibroblast adhesion for stored plant-derived hVN was ~0.2 μg hVN/cm², compared to ~0.1 μg hVN/cm² for the commercial control. The concentrations of both commercial and plant-made hVN that promoted maximum and half-maximum cell adhesion were significantly higher than was observed in Figure 6a, most likely because of variations in tetrazolium salt uptake by cells using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] versus MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]. Of the three conditions tested, storage of plant-made hVN as a frozen liquid at ~80 °C was the most effective at retaining adhesion promoting activity. Activity of hVN stored in this manner was statistically equivalent to the commercial product in the range of 0.2–1.6 μg/cm².

Discussion

Vitronecin is primarily used to promote cell attachment and proliferation in vitro but also has other therapeutic and nontherapeutic applications. These applications, however, are limited by the high cost of the native protein due to the extensive purification and serological testing required to ensure the absence of bloodborne pathogens. As such, in planta production of recombinant hVN that is functionally equivalent and free from animal pathogens by virtue of its source offers a cost-effective alternative. We have previously demonstrated inducible production of recombinant hVN in transgenic N. benthamiana plants using the In Plant ACTivation (INPACT) platform (Dugdale et al., 2013). Soil-acclimatized plants or detached leaves were activated with ethanol, and maximal hVN expression was detected by immunoblotting 4–5 days postapplication. Recombinant hVN was partially purified from leaves using heparin affinity chromatography, and crude yields were estimated at ~100 mg hVN/kg FW of leaf. In the present study, we have dramatically improved hVN yield by modification of the INPACT platform, developed an efficient recombinant hVN purification protocol, and demonstrated that recombinant hVN produced in transgenic N. benthamiana is functionally equivalent to commercial, human plasma-derived VN.

The INPACT platform provides the benefits of high-level, transient transgene expression in a stably transformed plant, whereby extrachromosomal copies of the INPACT transgene expression cassette are released, amplified, and transcribed only in the presence of ethanol and the Rep/RepA activator proteins. However, transient overexpression of heterologous genes can trigger PTGS, an innate plant pathogen defence mechanism that can significantly reduce steady state levels of recombinant protein accumulation (Voinnet, 2001; Voinnet et al., 1998; Waterhouse...
Figure 6 Adhesive properties of plant-made hVN and effects of long-term storage. Panel (a): The ability of plant-made hVN (open circles) to promote the attachment of BALB-C3T3 cells in serum-free medium to tissue culture plasticware was compared to commercial, plasma-derived hVN (closed circles). MTS absorbance readings at 490 nm are directly proportional to the number of viable cells bound to the plasticware surface. Samples were analysed in triplicate, and error bars indicate mean ± standard deviation.

(b) Plant-made hVN was stored long term for 7 weeks as (i) a liquid at 4°C (open triangles), (ii) frozen liquid at −80°C (open circles) or (iii) lyophilized at −80°C (closed triangles) and its ability to promote the attachment of BALB-C3T3 cells to tissue culture plasticware compared to commercial, plasma-derived hVN (closed circles). MTT absorbance readings at 570 nm are directly proportional to the number of viable cells bound to the plasticware surface. Samples were analysed in triplicate, and error bars indicate mean ± standard deviation.

et al., 1998). In order to overcome this, virus-encoded suppressors of PTGS can be co-expressed with the gene of interest (Voinnet et al., 1999). One such suppressor, p19 from Tomato bushy stunt virus (TBSV), is known to sequester double-stranded, small interfering RNA duplexes with high affinity, and co-expression of this protein has been shown to significantly increase transient heterologous gene expression (Sainsbury and Lomonossoff, 2008; Sainsbury et al., 2009). However, p19 itself is phytotoxic and the generation of stably transformed plants expressing p19 is challenging (Scholthof, 2007). While a recombinant p19 mutant (R43W) that does not induce phytotoxicity has been developed (Saxena et al., 2011), this mutant form confers only a modest (1.7-fold) increase in transgene expression levels. Therefore, in order to fully exploit the benefits of the TBSV p19 protein in transgenic plants, we integrated ethanol-inducible expression of the TBSV p19 gene into the INPACT platform for the production of hVN. We observed low level, ‘leaky’ p19 transcription in the absence of ethanol induction but there was no evidence of a negative impact upon phenotypic parameters such as plant growth or development in the T0 to T3 generation events. Further, we demonstrated that co-expression of p19 and hVN increased recombinant hVN accumulation up to sixfold, relative to the unmodified INPACT platform. This suggests that PTGS is likely a limiting factor for INPACT-based recombinant protein expression, but it can be overcome by inducible expression of a PTGS suppressor gene.

Intracellular targeting of recombinant proteins can significantly influence the steady state levels to which they accumulate in planta (Harrison et al., 2011; Streatfield et al., 2003). We previously tested whether localized accumulation of recombinant hVN in intracellular compartments greatly influenced its yield, by targeting the protein to the cytoplasm, endoplasmic reticulum (ER), apoplast, mitochondria or chloroplast. We found that hVN retention in the ER resulted in maximum accumulation in N. tabacum leaves (results not shown). Native hVN is glycosylated at three sites which accounts for as much as 30% of the mass of the mature protein (Schwartz et al., 1999), and changes in hVN glycosylation alter its ability to form multimers and bind collagen (Sano et al., 2007). However, glycosylation does not appear essential for some of its biological activities as glycan removal can increase collagen binding (Sano et al., 2007) and bacteria-made VN has been used in chemically defined animal cell culture systems (Chen et al., 2011). In the present study, the native hVN amino-terminal secretion signal was preserved so that the recombinant protein would enter the ER and an ER retention signal (KDEL) added to the carboxyl-terminus of hVN to enhance accumulation.

We have developed a small-scale production system using leaves harvested from transgenic plants maintained in vitro and activated with ethanol in solution. Elite T0 generation N. benthamiana INPACT events expressing high levels of recombinant hVN were micro-propagated in tissue culture using a rapid axillary shoot induction method (Deo et al., 2015) that allowed generation of ~200 individual plants. We routinely produced ~150 g of N. benthamiana leaf mass per week using a rotating subculture system and accumulated ~3 kg of leaves in a 5-month period. Detached leaves were activated in tissue culture containing a liquid growth media supplemented with 0.5% (v/v) ethanol and harvested for purification after 5 days when hVN accumulation was at its peak. Activated leaves could be stored at −80°C or used immediately for hVN extraction.

Laboratory-scale purification of recombinant hVN was routinely undertaken using 10–30 g of N. benthamiana leaves, but we have successfully scaled up purification to 100 g quantities of leaves in the present study. Interestingly, higher plants encode
hVN analogues that function in plasma membrane–cell wall adhesion (Zhu et al., 1993), bacterium–plant interaction (Wagner and Matthysse, 1992) and pollen tube extension (Sanders et al., 1991). One such protein, tobacco PVN1 (plant vitronectin-like 1), is predominantly localized to the cell wall and has been shown to bind both glass and heparin (Zhu et al., 1994). N. benthamiana encodes a protein with 97% similarity to tobacco PVN1 (Nakasugi et al., 2013) and contains an RYD motif with functional similarity to the hVN RGD binding domain. Therefore, a second affinity chromatography step (i.e. immobilized metal) was included in our hVN purification process to minimize the likelihood of contamination with N. benthamiana PVN1. We observed that recombinant hVN yield was significantly affected by the age and physiological status of plants in vitro. Leaves obtained from freshly propagated N. benthamiana in vitro plants were generally larger, appeared healthier and yielded the maximum level of >90% pure recombinant hVN (128 mg/kg FW). In contrast, leaves from the third harvest and beyond were generally smaller, more chlorotic and had reduced hVN content (30 mg/kg FW).

Optimization of the molecular features of the INPACT platform resulted in a sixfold increase in recombinant hVN accumulation compared to the unmodified INPACT platform, and the development of an efficient extraction protocol produced a plant-made hVN of >90% estimated purity that was functionally equivalent to the native human protein. While transient agroinfiltration-based expression remains the preferred method for rapid recombinant protein production in plants, there is still an obvious need for extended, large-scale production capacities. The elite transgenic INPACT plants and the seed bank generated in this study represent a permanent genetic resource for the consistent and high-level production of this valuable protein into the future.

Materials and methods

Vector construction

The CaMV 35S promoter (35SP) controlling expression of the hygromycin B phosphotransferase (hph) selection gene in pCAMBIA1300 was replaced with the nopaline synthase gene promoter (nosP). The nosP and hph genes were amplified by PCR and fused using overlapping PCR with the following primer pairs: hph-F (5'-TCTCCGCTCATGATCATGAAAAAGCCTGAATCCCCGAC-3') and hph-R (5'-CTTCGAGCTTCCGATCGACATCCGGATCGCGCATC-3'), and nosP-F (5'-GAATTCCTCTAGACGCTGAGAAGGGCTTTTTCATGATCATGAGCGGAGAATTAAGGGAG-3') and nosP-R (5'-TTCCAAGCTTTTCTGATCATGAGCGGAGAAATGAGGAGGAGGAG-3'). The resulting nosP-hph fusion was ligated into pCAMBIA1300 using XhoI and EcoRI restriction sites. An INPACT expression cassette encoding the GUS reporter gene was excised from pINPACT-GUS (Dugdale et al., 2013) and ligated into the above vector using EcoRI/HindIII restriction sites. The nearly complete INPACT cassette encoding hVN with native secretion signal, KDEL retention signal and polyhistidine affinity tag was then excised from pINPACT-hVN (Dugdale et al., 2013) and ligated into the vector using Swal/PacI restriction sites to create pINPACT-hVN-nos. Wild-type TBSV p19 (GenBank Accession M21958.1) was codon modified to include human and N. tabacum first preferred codons and an 84-bp synthetic intron (syntron; Dugdale et al., 2013) between the AG/GT at nucleotide position 201. The modified p19 gene was chemically synthesized by GeneArt® (Life Technologies, Mount Waverley, VIC, Australia) and ligated upstream of the nopaline synthase gene terminator (nosT) in the plasmid pACN2 using unique PstI restriction sites. The final INPACT hVN expression vector was constructed by three-way ligation of the following fragments: Pml/XbaI digested pINPACT-hVN-nos backbone, Pml/BamHI digested alcA promoter sequence and BamHI/XbaI digested p19-nosT sequence from pACN2. The resulting vector was designated pINPACT-hVN2 (Figure 1).

Construction of the vector pAlc-Rep/RepA, a pBIN-based vector backbone containing (i) the TYDV Rep/RepA activator genes downstream of the alcA promoter, (ii) the alcR transcription factor gene under the transcriptional control of 35SP and (iii) the neomycin phosphotransferase (nptII) resistance gene for kanamycin selection of transformed plant cells, has been previously described (Figure 1; Dugdale et al., 2013).

Stable transformation of N. benthamiana

All vectors for stable transformation were mobilized into Agrobacterium tumefaciens (strain LBA4404) by electroporation. A. tumefaciens-mediated transformation of N. benthamiana leaf discs and their regeneration were as described by Horsch et al. (1985). Transgenic plants containing the pAlc-Rep/RepA cassette were selected and regenerated in media containing kanamycin (200 µg/mL). Ethanol-inducible expression of the Rep/RepA genes in these lines was assessed by RT-PCR. Leaves from event NbAlc-1 were subsequently used for supertransformation with recombinant A. tumefaciens harbouring pINPACT-hVN2. To ensure supertransformed plants contained both pAlc-Rep/RepA and pINPACT-hVN2 expression cassettes and were independent events, plantlets were excised from different leaf pieces and regenerated in media containing both kanamycin (200 µg/mL) and hygromycin (25 µg/mL). In vitro or soil-acclimatized plants were maintained in a controlled environment chamber with a 16-h photoperiod at 25 °C and grown to the 8- to 10-leaf stage prior to harvest and ethanol activation.

Reverse transcription PCR (RT-PCR)

Leaf samples were immediately snap-frozen in liquid nitrogen following harvesting. Tri reagent (Sigma-Aldrich, Castle Hill, NSW, Australia) was used to extract total RNA from tissue according to the manufacturer's instructions and the method of Azevedo et al. (2003). Oligo (dT) 18 primer was used to synthesize first-strand complementary DNA from total RNA using M-MLV Reverse Transcriptase (Promega, Alexandria, NSW, Australia) according to the manufacturer's instructions. Reactions were also prepared without reverse transcriptase to confirm the absence of contaminating gDNA. PCRs were performed using GoTag Green master mix (Promega) and the following cycling conditions: 94 °C for 5 min followed by 29 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and 72 °C for 10 min. Primer sets were as follows: TYDV Rep/RepA-R (5'-TCAGACTTGCAACCTTATT-3') and Rep/RepA-F (5'-GGCAATTATTACTACAC-3'), wild-type TBSV p19-F (5'-CCTGGAAAGGCTTTTTCATGATCATGAGCGGAGAATTAAGGGAG-3') and p19-R (5'-GAGGCTCTCAGTGGATTTCTGATGACATCCGGAC-3') and actin-F (5'-TATCTTTCGCGTTTGGAC-3') and Actin-R (5'-AGGACCTCAAGGACAGCGAAACG-3').

Ethanol activation

The INPACT platform was activated in whole or detached leaves using three different methods. For soil-acclimatized plants, 5% (v/v) ethanol in water was applied as a foliar spray and root drench. For rapid screening of transgenic lines, leaves of in vitro N. benthamiana plants were ethanol activated in sealed Petri dishes. Leaves were placed adaxial side down onto solid MS0 media containing a 5-mm-wide and 5-mm-deep well filled with 3 mL of...
5% (v/v) ethanol. For time-course kinetics, dose–response, yield estimates and laboratory-scale hVN purification, leaves from tissue culture N. benthamiana plants were harvested, placed in a sterile 500-mL tissue culture vessel, and immersed in 150 mL of MS0 media (Murashige and Skoog, 1962) containing 0.5% (v/v) ethanol. The vessel was agitated on a flatbed rotary shaker (60 rpm) for 5 days with a 16-h photoperiod at 25 °C. For time-course kinetics, sampling time was varied (3, 4, 5, 6, and 7 days postactivation), and for dose–response, ethanol concentration was varied (0.1%, 0.25%, 0.5%, 1.0%, and 2.0% (v/v)). Excess liquid was removed prior to extraction or freezing in liquid nitrogen and storage at −80 °C.

**Purification of hVN from N. benthamiana leaves**

Leaves were ground to a powder in liquid nitrogen using a mortar and pestle. For rapid hVN detection, TSP was extracted in five volumes of either 200 mM phosphate buffer (pH 7) or extraction buffer (9 M urea, 50 mM phosphate, 10 mM β-mercaptoethanol, pH 7) and separated by SDS-PAGE for immunoblotting. For laboratory-scale purification, extraction buffer containing EDTA-free Complete Protease Cocktail Inhibitor (Roche, Castle Hill, NSW, Australia) was added to the leaf powder at a ratio of 7.5 mL per gram FW. The concentration of urea was increased from 8 M (that was used to isolate hVN from blood plasma (Yatohgo et al., 1988), to 9 M in order to compensate for high leaf water content. The resulting slurry was agitated at 15 rpm on an orbital wheel shaker for 15–30 min at room temperature. Larger plant debris was removed by filtration through Miracloth (VWR, Murarrie, QLD, Australia) prior to clarification by centrifugation at 20 000 × g for 20 min at 20 °C. A Heparin Sepharose 6 Fast Flow (GE Healthcare,曼斯菲尔德, QLD, Australia) column was prepared with a packed bed volume equivalent to 1 mL/S g starting leaf material and equilibrated with three column volumes of HS buffer (8 M urea, 50 mM phosphate, pH 7). The supernatant was loaded directly onto the equilibrated Heparin Sepharose. The Heparin Sepharose was washed with five column volumes of HS buffer and bound hVN eluted using five column volumes of ELUTION buffer (HS Column buffer supplemented with 500 mM NaCl).

A column of TALON™ Superflow Metal Affinity Resin (Sigma) was prepared with a packed bed volume equivalent to 1 mL/S g leaf material and equilibrated with three column volumes of HS Elution buffer. The eluent from the Heparin Sepharose column was loaded directly onto the equilibrated TALON® column. The column was washed with five column volumes of HS Elution buffer and bound HIS-tagged hVN eluted using five column volumes of TALON Elution buffer (HS Column buffer supplemented with 150 mM imidazole).

An Amicon Ultra Centrifugal Filter unit (Ultracel-10K, Millipore) was equilibrated using 3 mL of HS ELUTION buffer and centrifugation at 5000 g for 5 min at 18 °C. The eluent from the TALON® column was diluted 1:2 with HS ELUTION buffer (to decrease the imidazole concentration to 75 mM) then loaded onto the Ultracel-10K filter and centrifuged at 5000 g for 10 min at 18 °C. The filter was washed five to 6 times with 3–4 mL of HS ELUTION buffer and centrifuged at 5000 g for 10 min at 18 °C until the final retained volume was between 100 and 500 mL.

A Puradisc FP 30 PTFE, 0.2-µm sterile syringe filter (Thermo Fisher, Scoresby, VIC, Australia) was equilibrated with 1 mL of HS ELUTION buffer. hVN retained after size exclusion filtration was sterilized using the equilibrated filter. Purified plant-made hVN was either stored short-term at 4 °C or long term at −80 °C with or without freeze drying.

Samples (5 µL) from each step of the purification protocol were collected and stored on ice prior to PAGE analysis. Purified plant-made hVN was quantified using the Bradford Protein Assay microtitre plate procedure (Bio-Rad, Regents Park, NSW, Australia) according to the manufacturer’s instructions and known amounts of commercial purified hVN (Promega). Absorbance at 595 nm was determined using a Beckman Coulter™ AD200 plate reader, and samples were analysed in triplicate.

**PAGE, immunoblotting, N-terminus sequencing and yield estimation**

PAGE and immunoblotting for detection of hVN was carried out as described by Dugdale et al. (2013). For amino-terminal sequencing, 3 µg of plant-made hVN was subjected to SDS-PAGE, transferred to PVDF membrane and stained with Ponceau dye. The major 75-kDa band was excised from the PVDF membrane, and the first seven amino-terminal residues were sequenced using an Applied Biosystems 494 Precise Protein Sequencing System (Australian Proteome Analysis Facility, Macquarie University, NSW). For yield estimation, Clarity Western ECL substrate (Bio-Rad) was formulated according to the manufacturer and applied as a 1:10 dilution to the membrane. Signal strength was detected using a ChemiDoc imaging system (Bio-Rad) and yield calculated from a hVN standard curve ranging from 50 to 500 ng. Average plant-made hVN yield is presented as mean ± standard error.

**Cell adhesion assay**

Plant-made and plasma-derived hVN (Promega) were diluted in Dulbecco’s PBS (DPBS, Life Technologies) to concentrations ranging between 1.6, 0.800, 0.400, 0.200, 0.100, 0.050, 0.0250, 0.0125 and 0 µg/cm² in 100 µL. Wells of a Nunc MaxiSorp® flat-bottom 96-well plate (Sigma) were coated with 100 µL of each hVN dilution in triplicate. Plates were incubated for 2 h at room temperature then rinsed 3 times with 250 µL of DPBS per well. After rinsing, 200 µL of DPBS blocking solution (DPBS with 2 mg/mL bovine serum albumin (BSA)) was added to each well and the plate was incubated for 1 h at room temperature. The BSA blocking solution was removed prior to adding animal cells. Three-day-old BALB-C/J3T3 cells were harvested by trypsinization and pelleted by centrifugation at 200 g for 5 min at room temperature. Cells were resuspended in 1 mL Dulbecco’s Modified Eagle Medium (DMEM) containing 10% (v/v) foetal bovine serum (Life Technologies). Viable cell counts were estimated using trypan blue exclusion dye (Life Technologies) and cell concentrations adjusted to 4 × 10⁵ cells/mL. An aliquot (100 µL) of cell suspension (4 × 10⁵ cells/mL) was added to each hVN-coated well, and the plate was incubated at 37 °C with 5% (v/v) CO₂ for 1 h. Unattached cells were gently aspirated from the wells using a multichannel pipette and the attached cells gently washed 3 times with 250 µL of serum-free DMEM (Sigma) per well. Cell densities were measured using a Beckman Coulter™ plate reader and data averaged using three replicates. Mean absorbance at 490 nm (compared to absorbance at a reference wavelength of 690 nm) was measured using a Beckman Coulter™ plate reader and data averaged using three replicates. Mean absorbance at 490 nm versus the hVN concentration was plotted in order to determine ED₅₀. For the MTT substrate, 10 µL of MTT (5 mg/mL) in DPBS was added to each well and incubated at 37°C for 4 h. The absorbance at 570 nm was measured using a Beckman Coulter™ plate reader and data averaged using three replicates. Mean absorbance at 570 nm versus the hVN concentration was plotted in order to determine ED₅₀. For the MTX substrate, 10 µL of MTX (5 mg/mL) in DPBS was added to each well and incubated at 37°C for 4 h.
37 °C with 5% (v/v) CO₂ for 3–4 h until a dark precipitate formed. The media were then aspirated and 200 μl of 100% (v/v) DMSO added to each well in order to solubilize the precipitate. Absorbance was measured at 570 nm (compared to an absorbance at a reference wavelength of 630 nm) and plotted against hVN concentration to determine ED₅₀ as above.

Statistical analysis

One-way analysis of variance was performed to compare different batch yields or different cell binding activities (P < 0.05 was considered significant). Data values were expressed as mean ± standard error for yield estimates and mean ± standard deviation for cell attachment assays.

Acknowledgements

The authors would like to thank the Queensland Government, Queensland University of Technology, and Leaf Energy Inc., for their financial support. The authors would also like to thank Dr Gary Shooter (IHBL, QUT) for supplying BALB-C/3T3 cells and technical advice. This work was facilitated by access to the Australian Proteome Analysis Facility supported under the Australian Government’s National Collaborative Research Infrastructure Strategy (NCRIS).

Conflict of Interest

Authors declare no conflict of interest.

References

Azevedo, H, Lino-Neto, T and Tavares, RM (2003) An improved method for high-quality RNA isolation from needles of adult maritime pine trees. Plant Mol. Biol. Rep. 21, 333–338.

Cacchioli, A, Ravanetti, F, Bagno, A, Dettin, M and Gabbi, C (2009) Human vitronectin-derived peptide covalently grafted onto titanium surface improves osteogenic activity: a pilot in vivo study on rabbits. Tissue Eng. Part A 15, 2917–2926.

Chen, G, Gulbranson, DR, Hou, Z, Bolin, JM, Ruotti, V, Probasco, MD, Smuga-Oto, K et al. (2011) Chemically defined conditions for human iPSC derivation and culture. Nat Meth. 8, 424–431.

Conlan, MG, Tonasini, BR, Schultz, RL and Mosher, DF (1988) Plasma vitronectin polymorphism in normal subjects and patients with disseminated intravascular coagulation. Blood, 72, 185–190.

Deo, PC, Dugdale, B, Harding, RM, Kato, M and Dale, JL (2015) In vitro micro propagation of Nicotiana benthamiana via auxillary shoots. S. Pac. J. Nat. Sci. 32, 55–60.

Dugdale, B, Mortimer, CL, Kato, M, James, TA, Harding, RM and Dale, JL (2013) In plant activation: an inducible, hyperepression platform for recombinant protein production in plants. Plant Cell, 25, 2429–2443.

Dugdale, B, Mortimer, CL, Kato, M, James, TA, Harding, RM and Dale, JL (2014) Design and construction of an in-plant activation cassette for transgene expression and recombinant protein production in plants. Nat Protoc. 9, 1010–1027.

Harrison, MD, Geisjes, RJ, Coleman, HD, Shand, K, Kinkema, M, Palupe, A, Hassall, R et al. (2011) Accumulation of recombinant cellobiohydrolase and endoglucanase in the leaves of mature transgenic sugar cane. Plant Biotechnol. J. 9, 884–896.

Hollier, B, Harkin, DG, Leavels, D and Upton, Z (2005) Responses of keratinocytes to substrate-bound vitronectin: growth factor complexes. Exp. Cell Res. 305, 221–232.

Horsch, RB, Fry, JE, Hoffmann, NL, Eichholtz, D, Rogers, SG and Fraley, RT (1985) A simple and general method for transferring genes into plants. Science, 227, 1229–1231.

Jin, H and Varner, J (2004) Integrins: roles in cancer development and as treatment targets. Br. J. Cancer, 90, 561–565.

Li, J, Bardy, J, Yap, LY, Chen, A, Nurcombe, V, Cool, SM, Oh, SK et al. (2010) Impact of vitronectin concentration and surface properties on the stable propagation of human embryonic stem cells. Bioinformatics, 5, 132–142.

Lico, C, Chen, Q and Santi, L (2008) Viral vectors for production of recombinant proteins in plants. J. Cell. Physiol. 216, 366–377.

Mortimer, CL, Dugdale, B and Dale, JL (2015) Updates in inducible transgene expression using viral vectors: from transient to stable expression. Curr. Opin. Biotechn. 32, 85–92.

Murashige, T and Skoog, F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15, 473–497.

Nakagui, K, Crowhurst, RN, Bally, J, Wood, CC, Hellens, RP and Waterhouse, PM (2013) De novo transcriptome sequence assembly and analysis of RNA silencing genes of Nicotiana benthamiana. PLoS ONE, 8, e59534.

Preissner, KT (1991) Structure and biological role of vitronectin. Annu. Rev. Cell Biol. 7, 275–310.

Sainsbury, F and Lomonossoff, GP (2008) Extremely high-level and rapid transient protein production in plants without the use of viral replication. Plant Physiol. 148, 1212–1218.

Sainsbury, F, Thuememann, EC and Lomonossoff, GP (2009) pEAG: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. Plant Biotechnol. J. 7, 682–693.

Sanders, LC, Wang, C-S, Walling, LL and Lord, EM (1991) A homolog of the substrate adhesion molecule vitronectin occurs in four species of flowering plants. Plant Cell, 3, 629–635.

Sano, K, Asanuma-Date, K, Arisaka, F, Hattori, S and Ogawa, H (2007) Changes in glycosylation of vitronectin modulate multimerization and collagen binding during liver regeneration. Glycobiology, 17, 784–789.

Saxena, P, Hsieh, YC, Akarada, VY, Sainsbury, F, Saunders, K, Lomonossoff, GP and Scholthof, HB (2011) Improved foreign gene expression in plants using a virus-encoded suppressor of RNA silencing modified to be developmentally harmless. Plant Biotechnol. J. 9, 703–712.

Scholthof, HB (2007) Heterologous expression of viral RNA interference suppressors: RISC management. Plant Physiol. 145, 1110–1117.

Schwartz, I, Seger, D and Shaltiel, S (1999) Vitronectin. Int. J. Biochem. Cell Biol. 31, 539–544.

Shaffer, MC, Foley, TP and Barnes, DW (1984) Quantitation of spreading factor in human biologic fluids. J. Lab. Clin. Med. 103, 783–791.

Streatfield, SJ, Lane, JR, Brooks, CA, Barker, DK, Poage, ML, Mayor, JM, Lamphier, BJ et al. (2003) Com as a production system for human and animal vaccines. Vaccine, 21, 812–815.

Tollefsen, DM, Weigel, CJ and Kabeer, MH (1990) The presence of methionine or threonine at position 381 in vitronectin is correlated with proteolytic cleavage at arginine 379. J. Biol. Chem. 265, 9778–9781.

Underwood, PA and Bennett, FA (1989) A comparison of the biological activities of the cell-adhesive proteins vitronectin and fibronectin. J. Cell Sci. 93, 641–649.

Upton, Z, Cuttle, L, Noble, A, Kempf, M, Topping, G, Malda, J, Xie, Y et al. (2008) Vitronectin: growth factor complexes hold potential as a wound therapy approach. J. Invest. Dermatol. 128, 1535–1544.

Upton, Z, Wallace, HI, Shooter, GK, van Lonkhuyzen, DR, Yeoh-Ellerton, S, Raymond, EA, Fleming, JM et al. (2011) Human pilot studies reveal the potential of a vitronectin: growth factor complex as a treatment for chronic wounds. Int. Wound J. 8, 522–532.

Vonnet, O (2001) RNA silencing as a plant immune system against viruses. Trends Genet. 17, 449–459.

Vonnet, O, Vain, P, Angell, S and Baulcombe, DC (1998) Systemic spread of sequence-specific transgene RNA degradation is initiated by localised introduction of ectopic promoterless DNA. Cell, 95, 177–187.

Vonnet, O, Pinto, YM and Baulcombe, DC (1999) Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses. Proc. Natl Acad. Sci. USA, 96, 14147–14152.

Wagner, VT and Matthysse, AG (1992) Involvement of a vitronectin-like protein in attachment of Agrobacterium tumefaciens to carrot suspension culture cells. J. Bacteriol. 174, 5999–6003.
Waterhouse, PM, Graham, MW and Wang, M-B (1998) Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proc. Natl. Acad. Sci. USA*, 95, 13959–13964.

Xie, Y, Rizzi, SC, Dawson, R, Lynam, E, Richards, S, Leavesley, DI and Upton, Z (2010) Development of a three-dimensional human skin equivalent wound model for investigating novel wound healing therapies. *Tissue Eng. Part C*, 16, 1111–1123.

Yatohgo, T, Izumi, M, Kashiwagi, H and Hayashi, M (1988) Novel purification of vitronectin from human plasma by heparin affinity chromatography. *Cell Struct. Funct.* 13, 281–292.

Zhou, A, Huntington, JA, Pannu, NS, Carrell, RW and Read, RJ (2003) How vitronectin binds PAI-1 to modulate fibrinolysis and cell migration. *Nat. Struct. Biol.* 10, 541–544.

Zhu, J-K, Shi, J, Singh, U, Wyatt, SE, Bressan, RA, Hasegawa, PM and Carpita, NC (1993) Enrichment of vitronectin- and fibronectin-like proteins in NaCl-adapted plant cells and evidence for their involvement in plasma membrane-cell wall adhesion. *Plant J.* 3, 637–646.

Zhu, J-K, Damisz, B, Kononowicz, AK, Bressan, RA and Hasegawa, PM (1994) A higher plant extracellular vitronectin-like adhesion protein is related to the translational elongation Factor-I α. *Plant Cell*, 6, 393–404.

### Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Appendix S1** Reverse Transcription (RT)-PCR to detect *Rep/RepA* expression in transgenic *N. benthamiana* parent lines following ethanol activation.

**Appendix S2** Southern hybridization to determine copy number.

**Appendix S3** Identification of high hVN-expressing T1 generation INPACT lines.

**Appendix S4** Growth and development of transgenic plants in soil.

**Appendix S5** Purification of recombinant hVN from *N. benthamiana* using a three-stage extraction protocol.