

Integration, stability and expression of the *E. coli* phytase transgene in the Cassie line of Yorkshire EnviropigTM

Cecil W. Forsberg · Roy G. Meidinger ·
Mingfu Liu · Michael Cottrill · Serguei Golovan ·
John P. Phillips

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Abstract The genomic structure and generational stability of the transgene carried by the Cassie (CA) line of the transgenic EnviropigTM, a prospective food animal, are reported here. This transgene is composed of the *Escherichia coli* phytase coding sequence regulated by the mouse parotid secretory protein promoter to direct secretion of phytase in the saliva. In the CA line the transgene integrated in chromosome 4 is present as a concatemer of three copies, two in a head to tail orientation and the third in a reverse orientation 3' to the other copies with a 6 kbp deletion in the 5' promoter

region. The overall size of the integrated transgene complex is 46 kbp. During integration a 66 kbp segment of the chromosome was deleted, but a BLAST search of the segment from a GenBank clone did not reveal any essential genes. The transgene integration site was stable through 9 generations analyzed. Phytase activity in the saliva was similar among 11 day old hemizygous boars and gilts and remained relatively constant through nine generations of hemizygous pigs. However, as the pigs grew there generally was a gradual decrease in activity that stabilized when pigs reached the finisher phase of growth (4–6 months old). Homozygous pigs exhibited 1.5 fold higher phytase activity ($P < 0.0001$) than that of hemizygous littermates. Moreover, no differential salivary phytase activity was seen in hemizygotes arising from CA-Yorkshire and CA-Duroc breed outcrosses, suggesting that expression of the transgene is unaffected by genetic background. This data demonstrates that an exogenous phytase gene can be stably transmitted and expressed in the salivary glands of a domestic food animal.

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C. W. Forsberg (✉) · R. G. Meidinger ·
M. Cottrill · J. P. Phillips
Department of Molecular and Cellular Biology,
University of Guelph, Guelph, ON N1G 2W1, Canada
e-mail: cforsber@uoguelph.ca

Present Address:
M. Liu
LA Biomedical Research Institute, Harbor-UCLA
Medical Center, 1224 W Carson St, Torrance, CA 90745,
USA

Present Address:
S. Golovan
Department of Animal and Food Science,
University of Delaware, 531 South College Ave.,
046 Townsend Hall, Newark, DE 19716-2150, USA

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Introduction

Transgenic technology has been envisioned as one route to addressing issues in animal agriculture including problems surrounding the use of animal

manures as fertilizer. The high phosphorus content of hog manure can become a serious global environmental pollutant in areas of intense hog production (Carpenter 2008; Gerber et al. 2010; Key et al. 2011). Phosphorus enriched manure arises because the indigestible plant phytate that accounts for 59–79 % of the total phosphorus present in cereal grains and plant protein supplements becomes enriched in manure due to digestion and absorption of other nutrients (Viveros et al. 2000). Inclusion of phytase supplements in the diet has been the conventional approach to enable digestion of phytate with release of inorganic phosphorus that is readily absorbed and metabolized by the pig. To eliminate the need for continuous addition of phytase to diets, transgenic pigs with the novel capability to secrete phytase in the saliva were developed by Golovan et al. (2001b). Using pronuclear microinjection of a transgene consisting of the *Mus musculus* parotid secretory protein promoter linked to the *Escherichia coli appA* phytase gene (Lama2/APPA), they generated 33 different lines of transgenic pigs (Golovan et al. 2001b). Fourteen lines of transgenic piglets 7–11 days old produced between 5 to 6,000 U of phytase per millilitre in the saliva. All lines producing phytase were crossed with conventional Yorkshire pigs to obtain the second and subsequent generations. The WA line had the highest salivary phytase and it had a true phosphorus digestibility of 99 % (Golovan et al. 2001b). Two other tested lines, the JA and GO lines also showed high plant phosphorus digestibility. To resolve which line should be studied in detail to meet regulatory requirements for commercial production, preliminary studies were conducted on all of the transgenic lines exhibiting high salivary phytase activity. Here we report the selection of the line of transgenic phytase pigs for detailed characterization and describe the site of integration of the transgene, stability of the transgene and salivary phytase production over many generations, and in different genetic backgrounds.

Materials and methods

Transgenic pig breeding and out crossing

Transgenic pigs producing phytase in the saliva were created by pronuclear microinjection using the Lama2/APPA transgene composed of the promoter

from the mouse parotid secretory protein gene fused to the *E. coli* phytase gene (Golovan et al. 2001a; Golovan et al. 2001b). New generations of the Cassie (CA) line of pigs were maintained in the hemizygous state for the phytase transgene insertion site by outbreeding hemizygous CA gilts or sows with commercial semen from high performance Yorkshire or Duroc boars. Pigs homozygous for the transgene insertion site were produced by crossing male and female CA pigs hemizygous for the phytase insertion site. The experimental protocols were in accordance with the “Canadian Council for Animal Care (CCAC) guidelines on: the care and use of farm animals in research, teaching and testing” (2009) and “CCAC guidelines on: transgenic animals (1997) available at http://www.ccac.ca/en/_standards. The confinement measures used were described in the Canada Gazette February 20, 2010 (<http://www.gazette.gc.ca/rp-pr/p1/2010/2010-02-20/html/notice-avis-eng.html>).

The transgenic pigs were fed diets corresponding to those for conventional pigs except no supplemental phosphorus was added (ONLINE RESOURCE 1). Water was available ad libitum.

Enzyme assays and analytical procedures

Saliva was collected by placing a cotton swab into the buccal cavity and allowing the saliva to soak into the swab. Swabs were then placed into microcentrifuge tubes and centrifuged for 5 min at 15,000×*g* and the resulting liquid used for assays. Phytase was assayed as described by Golovan et al. (2000), using sodium phytate as the substrate. One unit of phytase activity was defined as the amount of enzyme liberating one μmol of phosphate per min. SDS-PAGE was performed according to standard methods using a 10 % (w/v) resolving gel. Protein concentrations in saliva were determined using the BCA method described by Smith et al. (1985) with fraction V bovine serum albumin (Sigma) as the standard. Monoclonal antibodies were produced using the purified *E. coli* AppA phytase as the antigen following the procedure described by Lam and Mutharia (1994).

PCR screening and Southern blot

Genomic DNA was extracted from blood as described by Higuchi (1989). Homozygous, hemizygous, and non-

Table 1 PCR primers designed for the amplification of different segments of the clone H11A9F8

Primer	Sequence	Location ^a	Junction	Restriction endonuclease
mr-1	5'-CAGCCAGGTCCACCCTATACGCACT-3'	21,047		
mr-2	5'-ACGCACTCATCTGTATCTCTCCTTTGGA-3'	21,029		
mr-3	5'-CTGAAAGATTTGCGGGTGAGGGATT-3'	20,969		
Up1	5'-GTGCTGCTCCAAGTTTGGTG-3'	32,982, 50,711, 62,054		
Down4	5'-CACGCACACCATGACGACTGACAATCACC-3'	33,650, 51,379, 61,886		
L1A	5'-CCGCTCACAATCCACACAA-3'			<i>DdeI</i>
R1B	5'-CTTCCCTCCTTTCTCGCCA-3'			
R2A	5'-CGGTACCCAATTCGCCCTAT-3'			<i>PvuI</i>
Amp1	5'-GGCACCTATCTCAGCGATCT-3'			
Amp2	5'-CTGGATCTCAACAGCGTAAG-3'			
H11-1F	5'-ACAACITTTTAAACATATTCTGAGC-3'	100,107		<i>SstI</i>
H11-1R	5'-GGACAATAGGGATAAATGTAATAAA-3'	99,974		
H11-2F	5'-TGTCCAGCAGAATGCAATTG-3'	345		<i>NcoI</i>
H11-2R	5'-GGAGTCATCTTTGAACTGGG – 3'	463		
6F	5'-CAGTTTGAAGAAAGCAGGAGTCCC-3'	67,331		<i>EcoRI</i>
6R	5'-CTGATGTGGATCAATGGGATTGGT-3'	67,876		
mr-10	5'-CCTCCACTGGGGGCTCTGTGTTTTA-3'	38,417, 56,146, 56,619		
who3	5'-TGCTGAAAGGACCCTGATATAGCTGGC-3'	38,531, 56,260, 56,505		
cas-f3 (F)	5'-GGTTTTCAACCAAAGCTAGCCCTGTAAGGT-3'	20,442	1	<i>BglIII</i>
cas-r7	5'-GGT GTC AGG GTG ATT TTT GTC AGA TCA-3'	(wild type seq.)		
J2 F	5'-ATACCCTCCCACCTCCCTGC-3'	38,171	2	<i>SstI</i>
J2 R	5'-GGTGTCTCCTCAGCAGCCAG-3'	38,770	2	
J3 F	5'-CCTACACACCCACTCCCACTTT-3'	55,923	3	<i>SstI</i>
J3 R	5'-AAGCCTCTTCAGCACCATGTATG-3'	56,398	3	
J4 F	5'-ACATGGTGCTGAAGAGGCTTAGAG-3'	56,401	4	<i>SstI</i>
J4 R	5'-CTATACCCTCCCACCTCCCTG-3'	56,847	4	
J5 F	5'-TGACCTTGGCCTGGAAGATG-3'	66,916	5	<i>XbaI</i>
J5 R	5'-CGTGGAACATTTGGCAGTGT-3'	67365	5	

^a The number is the base on the clone template at which new strand synthesis is initiated

transgenic pigs were differentiated by PCR using three primers cas-f3 (base pair 20,442), mr-1 (base pair 21,047) and cas-r7 (wild type sequence) in the reaction mix and DNA template isolated from a blood sample (Table 1). The presence of a single 602 bp band from primers cas-f3/mr-1, visualized by ethidium bromide staining, indicated a homozygote while the presence of both 602 and 816 bp bands indicated a hemizygote. The presence of an 816 bp band from primers cas-f3/cas-r7 and none from other primers indicated an individual negative for the transgene. Screening for vector sequences by PCR used Platinum Taq (Invitrogen) and

β -lactamase primers Amp1 and Amp2, pUC ori primers L1A and R1B and fl(–) region primers R2A and R1B (Table 1). Each reaction contained 2 μ L of DNA, 1.5 mM MgCl₂, 0.2 mM dNTP, 40 pmol of each primer and 5 U Taq in a final volume of 40 μ L. After the initial denaturation at 95 °C for 3 min, a cycle consisted of denaturation for 30 s at 95 °C, annealing at 58 °C for 30 s and extension for 1 min at 72 °C. The procedure continued for 40 cycles with the final extension continuing for 5 min. Amplification products were visualized by electrophoresis on a 1.5 % agarose gel containing ethidium bromide.

The Southern blot was performed using spleen cell genomic DNA from the CA line and from control Yorkshire pigs along with Hind III λ markers and XhoI linearized pLama 2/APPA. Samples were prepared using the CHEF Genomic DNA Plug Kit (Bio-Rad). The genomic DNA samples were cut with SmaI. Pulse field gel electrophoresis (PFGE) was performed on a CHEF-II (Bio-Rad) with running conditions of 6 V/cm, initial time = 0.2 s, final time 35 s, 14 °C for 18 h. The DNA in the gel was depurinated with 0.25 M HCl and denatured with 0.5 M NaOH in 1.5 M NaCl and then transferred to a positively charged Nylon membrane (Roche cat# 1-209-299). The membrane was stained using DIG labeled phage λ DNA and XhoI linearized pLama2/APPA with the DIG Luminescent Detection kit (Roche Applied Science).

Sequencing of the transgene insert

The site of insertion of transgene copies in the chromosome was identified by asymmetric PCR amplification using the APATM reverse primer set from Bio S&T Inc. (Montreal, Canada; <http://www.biost.com/en/APAwalk.aspx>) with three nested transgene primers mr-1, mr-2 and mr-3 for the 5' terminus of the phytase transgene (Table 1). Primer mr-1 was used for the first round of PCR with CA DNA as template, mr-2 and mr-3 were used for the 2nd and 3rd rounds of PCR each using the product from the previous round PCR as template. The major bands from the 3rd PCR were sequenced, and that enabled the production of a primer cas-f3 to the pig sequence 5' to the transgene insertion site.

Liver tissue was collected from a fifth generation (G₅) female pig hemizygous for the transgene and sent to BioS&T (Montreal, Quebec) for extraction of genomic DNA and production of a BAC library. The genomic DNA was partially digested with HindIII and ligated into a similarly digested pIndigoBAC-HindIII vector (Epicentre Technologies; www.epicentre.com). These clones were propagated by transformation into *E. coli* DH10B (*A(mrr-hsd RMS-mcrBC)mcrA recA 1*). Identification of the BAC clones was based on the synthesis of PCR products using Up1/Down4 primers and cas-f3/mr-1 primers (Table 1). The former primer pair produces a product that spans the transgene promoter—phytase coding region junction. The latter primer pair produces a product that spans the

previously identified pig—5' transgene insert junction. One clone, H11A9F8, was positive for both PCR reactions.

Sequencing of the BAC clone H11A9F8 was also done by Bio S&T Inc (Montreal, Quebec). BAC DNA was purified from the clone using a Qiagen Large Construct kit (Qiagen Inc, Mississauga, ON). The clone was verified by BAC end sequencing using the BigDye Terminator v3.1 on ABI Prism 3730XL DNA Analyser (Applied Biosystems, Foster City, CA) and by pulse field gel electrophoresis.

Shotgun libraries were produced by ultrasonication of the BAC DNA, filling in the DNA fragments with a combination of T4 DNA polymerase and Klenow DNA polymerase, selection of 1.5–3 kb fragments from an agarose gel and ligation into vector pUC18 treated with SmaI/shrimp alkaline phosphatase for blunt end ligation. Plasmid DNA was isolated by a modified version of common alkaline lysis using Multiscreen HTS plates (Millipore, Billerica, MA).

The subclones were sequenced from the M13 forward direction by the dideoxy chain termination method using BigDye Terminator Cycle sequencing v3.1 Ready Reaction (ABI) or DYEnamic ET Dye Terminator kit (MegaBACE; GE Healthcare, Baie d'Urfe, Quebec) with the analyses done on ABI 3730XL or MegaBACE 1000 capillary sequencing instruments.

A Phred/Phrap/Consed software package (Ewing and Green 1998; Ewing et al. 1998) was used for quality assessment and sequence assembly of the 5,027 sequences generated. Gaps and low quality regions were assessed in two ways; (1) re-sequencing low quality reads and (2) primer walking based on initial sequence data. Results were refined by manual verification of any potential misalignment caused by repeats. Transgene regions were assembled with the aid of a physical map. Sequence quality was enhanced by (1) two independent high quality reads as minimal coverage; (2) sequence coverage on both strands and (3) a Phred quality value >Q40 for each given base. Based on Phrap, the error rate is <1:10,000.

Statistical analysis

Statistical analysis was done using the GLM procedure of SAS (Version 9.1; SAS Institute, Cary, North Carolina, USA). Least square means for each treatment were separated using the pdiff option of SAS. Significant differences were accepted when $P < 0.05$.

Results

Selection of founder line for experimental characterization

The 33 founder lines of transgenic phytase pigs reported by Golovan et al. (2001b) were produced using an *XhoI/NotI* fragment of the phytase transgene pLama2/APPA. These restriction enzymes excised the pBluescript plasmid from the promoter/phytase transgene in the construct. The vector sequence was separated from the transgene insert by electrophoresis. However, as discovered later, some pLama2/APPA plasmid was cleaved at only one of the *XhoI* and *NotI* restriction sites. Because the linearized plasmid was not adequately separated from the transgene by electrophoresis it was present in the final transgene preparation used for pronuclear microinjection of pigs. The WA line of pigs described in detail by Golovan et al. (2001b) contained the β -lactamase gene and the linearized plasmid as documented by PCR detection of both *XhoI* and *NotI* restriction sites from the pLama2/APPA plasmid in WA DNA. Because organisms containing antibiotic resistance factors are not acceptable for regulatory approval by government agencies all lines of transgenic pigs exhibiting salivary phytase activity were screened for both the presence of the β -lactamase gene and the phytase protein. As shown in Fig. 1a transgenic pig lines #4 CA, #7 PA, #9 ST and #12 TE lacked the β -lactamase gene, although PCR efficiency was not determined. Of these lines CA, PA and TE showed the presence of phytase by western blotting using antibodies specific for the phytase (Fig. 1b). The CA line was chosen for further characterization because it exhibited the most desirable combination of salivary phytase activity and performance. This line was also shown by PCR analysis to lack the f1(–) region and the pUC ori regions of the vector pBluescript KS, in addition to lacking the *XhoI* and *NotI* restriction sites used to excise the transgene from the original plasmid.

Characterization of the transgene integration site

Initial sequencing of the transgene insert was done on fragments resulting from Asymmetrical PCR Amplification (APATM kit; Bio S&T Inc, Montreal, Quebec) obtained by ‘walking’ from the 5′ end of the transgene into the upstream flanking pig sequence (Fig. 2).

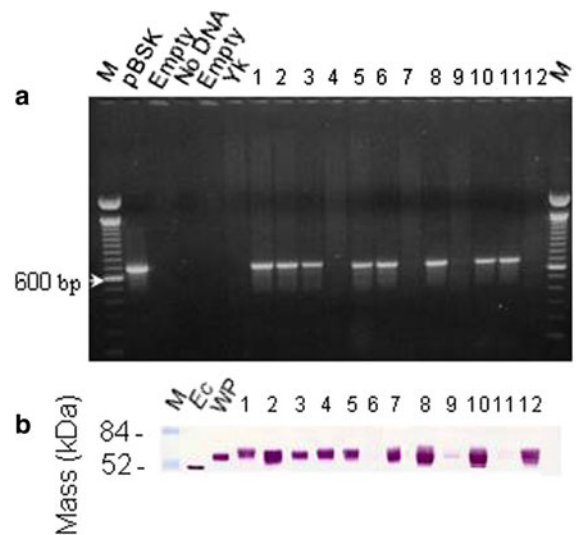
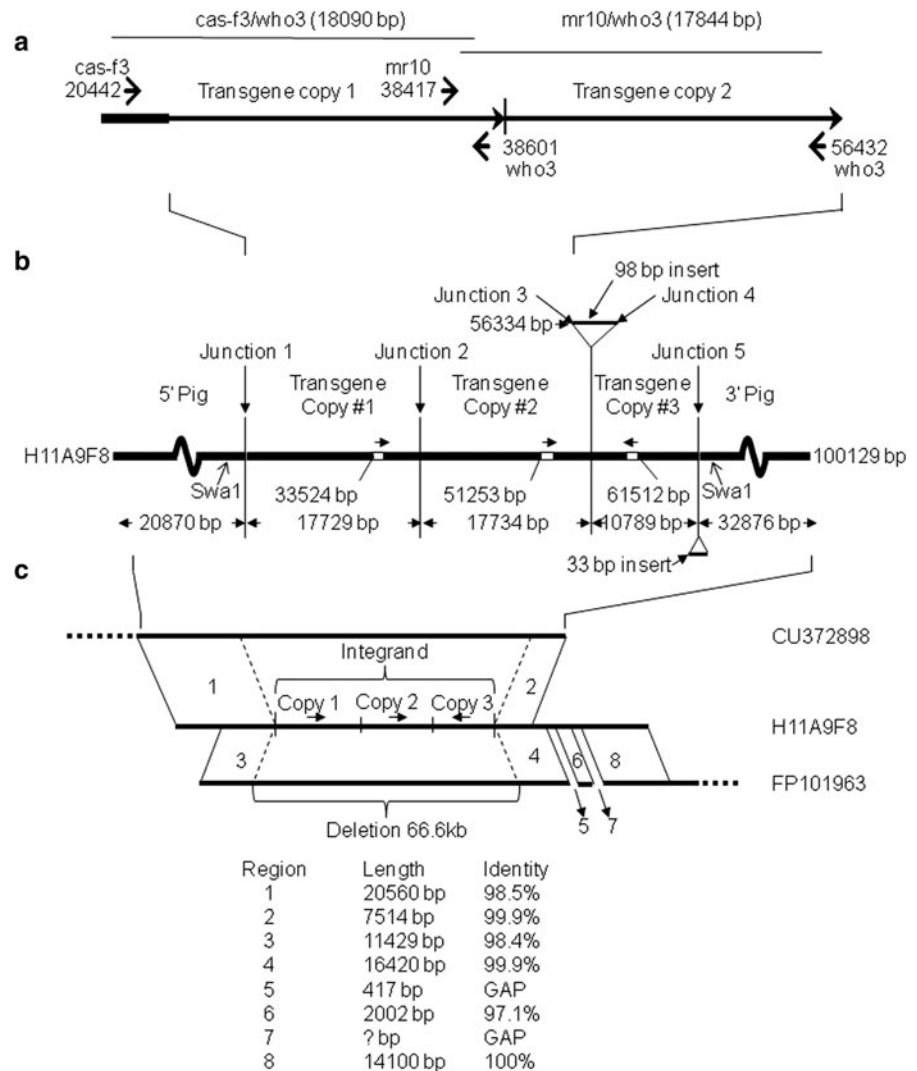


Fig. 1 **a** Agarose gel electrophoresis profile showing the β -lactamase gene product from PCR amplification of genomic DNA from 12 lines of pigs containing the phytase transgene using primers Amp1 and Amp2. A PCR product of 683 bp indicates the presence of the β -lactamase gene. M, 100 bp markers; pBSK, pBluescript; Empty, empty well, Yk, Yorkshire pig DNA. The lane numbers correspond to those in **b**. **b** Immunoblot of saliva from different lines of the first generation transgenic pigs for the presence of the phytase protein using an anti-phytase monoclonal antibody. Lane numbers reference pig identification, μ g protein applied to each well, and phytase specific activity. Lane M, pre-stained molecular mass marker; lane Ec, *E. coli* phytase, 0.5 μ g at 1,200 U mg^{-1} ; lane WP, purified WA phytase, 0.5 μ g at 1,400 U mg^{-1} ; lane 1, WA gilt, 1.6 μ g at 260 U mg^{-1} ; lane 2, JA gilt, 10 μ g at 54 U mg^{-1} ; lane 3, GO boar, 2.5 μ g at 216 U mg^{-1} ; lane 4, CA gilt, 10 μ g at 72 U mg^{-1} ; lane 5, BO boar, 3.3 μ g at 124 U mg^{-1} ; lane 6, MO gilt 15 μ g at 8.7 U mg^{-1} ; lane 7, PA boar 10 μ g at 124 U mg^{-1} ; lane 8, JE boar, 10 μ g at 84 U mg^{-1} ; lane 9, ST boar, 15 μ g at 2 U mg^{-1} ; lane 10 LE boar, 5 μ g at 111 U mg^{-1} ; lane 11, BI gilt, 15 μ g at 1.7 U mg^{-1} ; lane 12, TE boar, 15 μ g at 54 U mg^{-1}

Using this technique with transgene specific primers mr-1, mr-2 and mr-3 that began at base pairs 21,047, 21,029, and 20,969, respectively, a 736 bp segment of sequence 5′ of the pig–transgene junction (Junction 1; Fig. 2b) was isolated and subsequently sequenced. In addition, a second PCR product that began at base pair 38,417 was isolated, cloned and sequenced. This 498 bp segment contained a junction between two head to tail copies of the transgene (Junction 2), 431 bp being from the 3′ end of copy 1 and 67 bp of the 5′ end of copy 2.

To link the first and second copies of the transgene at the integration site two primers were designed for

Fig. 2 Structure of the phytase transgene integration site in chromosome 4. **a** Illustration of the strategy by which the location of transgene copy 2 was identified. **b** Diagram illustrating the organization of the clone H11A9F8 containing the phytase transgene concatemer inserted in the pig chromosome. The vertical bars illustrate the junctions between the porcine chromosome and transgene copies, and among the three transgene copies. The arrows illustrate the locations of the phytase gene and orientation in each transgene insert with the nucleotide number of the start site shown immediately below the transgene. **c** Alignment of *Sus scrofa* GenBank clones CU372898 and FP101963 with H11A9F8 showing the identity with various segments of clone H11A9F8



the 3' end of transgene Copy 1, mr-10 extending 3' from bp 38,417, which would also start at base pairs 56,146 and 56,619 of Copy 2 and Copy 3 (reverse orientation), respectively (Fig. 2a). The second primer to extend 5', who-3 starting at base pairs 38,531, 56,260 and 56,505, respectively of copies 1, 2 and 3 (forward orientation). PCR amplification using the cas-f3/who-3 and mr-10/who-3 primer pairs using Expand™ Long Template PCR Kit (www.roche-applied-science.com) each gave products of approximately 18 kb, which represented copy 1 and copy 2 of the transgene in a head to tail linkage. *HindIII/EcoRI* digestion of these products gave fragments of 2.16 and 1.92 kb, respectively which contained the junction 2 between copies 1 and 2.

To identify the clone in the CA DNA BAC library containing the transgene insertion site the library was screened by PCR using primers Up1/Down4 primers and cas-f3/mr-1 that span the transgene promoter—phytase coding region junction and the pig-5' insertion junction, respectively, Clone H11A9F8 gave rise to PCR products for both primer sets. To determine whether the clone contained the complete insertion site it was checked for pig sequence at both the 5' and 3' by producing primer pairs H11-1F and 1R, and H11-2F and 2R for the 5' and 3' termini of the clone, respectively. These primer pairs were then tested for amplification using CA DNA and conventional Yorkshire DNA as the templates. The primers gave products of 200 and 295 bp, respectively, which showed that

both termini of the clone were of porcine origin and therefore the complete transgene insertion site was cloned. Pulse field gel electrophoresis of *NotI* restriction fragments of the clone H11A9F8 gave a size of approximately 97 kb (ONLINE RESOURCE 2).

The assembled sequence of clone H11A9F8 was 100,129 bp (GenBank Accession number JX481346). A graphic representation of the sequence is shown in Fig. 2b. The complete sequence assembly was verified by digestion of H11A9F8 with *SmaI* followed by agarose gel electrophoresis. Fragments resolved by electrophoresis were unambiguously identified. The sequence consisted of 20,870 bp of pig sequence followed by three transgene integrands. The first and second copies were in ‘forward’ orientation and complete with the exception of a small number of base pairs deleted at the junctions of pig and copy 1 and the junction of copies 1 and 2 (ONLINE RESOURCE 3). The third insert was in the ‘reverse’ orientation to the first two and was truncated with a loss of 6,950 base pairs of the distal promoter region. In addition, a 98 bp insert was found between transgene copies 2 and 3 and a 33 bp insert between the third copy of the truncated transgene and 32,876 bp of downstream porcine sequence. The 98 bp insert did not show similarity to other sequences using it as a query for searches of the (i) phytase transgene, (ii) deleted sequence nucleotide database in GenBank (see later), and (iii) *Sus scrofa* databases (a) Other, (b) EST and (c) WGS. The 33 bp insert exhibited as much as 75 % similarity to numerous sequences in the same databases used for the screening the 98 bp insert. Because it was much smaller than the 98 bp sequence we did not describe it as a junction. The size of the transgene integration site was limited to three copies as documented by the size of the insert shown by pulse field gel electrophoresis and by agarose gel electrophoresis of *SmaI* fragments. Further confirmation of the insert size and its location at one site was provided by a Southern blot of CA DNA digested with *SwaI* followed by pulse field gel electrophoresis and identification using a Lama 2/AppA probe that gave a single band of 66 kb (Fig. 3).

Scanning of H11A9F8 for open reading frames (ORFs) greater than 100 bp revealed a new ORF of 282 bp in a reverse orientation between bp 56,722 to 56,441 that spans the junction between copies 2 and 3. None of the common regulatory elements (Goldberg Hogness, CCAAT box or polyA signal) were found. A TBASTX search of GenBank with it as the query

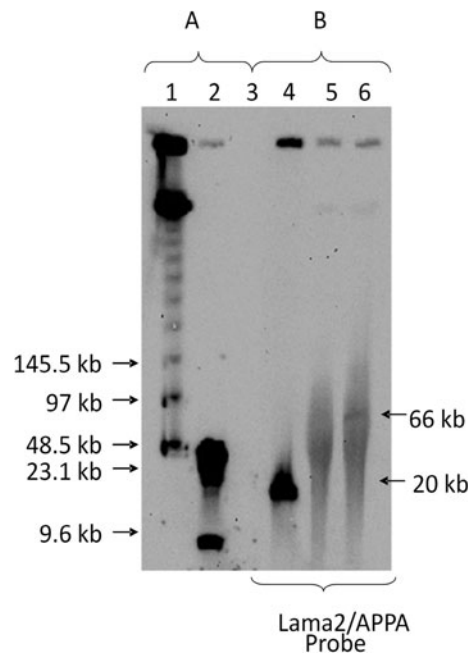


Fig. 3 Southern transfer autoradiogram of *SwaI* digested CA and Yorkshire genomic DNA after pulse field gel electrophoresis using a 17.7 kb *NotI/XhoI* pLama 2/APPA fragments as the probe. Lane 1: λ DNA concatemer markers; Lane 2: *HindIII* digested λ DNA markers; Lane 3: Blank; Lane 4: *XhoI* digested pLama 2/APPA; Lane 5: *SwaI* digested Yorkshire genomic DNA; Lane 6: *SwaI* digested CA genomic DNA

protein did not reveal any similarity above background. Using H11A9F8 as the query in a GenBank BLASTX analysis demonstrated the presence of the three copies of the transgene and a sequence similar to a partial reverse transcriptase in the 3' pig sequence. The partial reverse transcriptase sequence in the pig segment of the clone exhibited 66 % identity with a 275 amino acid segment of the 1,272 amino acid *Bos taurus* reverse transcriptase (AAY53484.1). Using the *Bos taurus* reverse transcriptase as the query in a BLASTX search of the H11A9F8 sequence, no identity to other amino acid sequences of the reverse transcriptase was detected indicating that no intact reverse transcriptase exons was present in the clone H11A9F8. Furthermore, the pig query sequence did not contain the YXDD motif that is characteristic of the active site of reverse transcriptases (Sharma et al. 2005).

Location of the transgene integrand on chromosome 4

The pig sequence portions of H11A9F8 matched to a high degree two overlapping pig chromosome 4 high

throughput genomic clones (CU372898 and FP101963) in GenBank (Fig. 2c). The porcine sequences of H11A9F8 aligned with CU372898 and FP101963 and showed similarities from a high of 99.9 % to a low of 97.4 %. The sequence FP101963 consists of three fragments with two gaps in the sequence with high similarity to H11A9F8, however, these gaps were outside of the transgene integration site. The alignment also revealed that ~ 66.6 kb of pig sequence has been lost during the transgene integration. Using the UCSC Blat program (<http://genome.ucsc.edu/cgi-bin/hgBlat?command=start>, accessed 15th May 2012) the deleted sequence was found to extend from bp 17,740,837 to bp 17,807,829 of chromosome 4.

To determine whether there were essential genes in the deleted 66.6 kb sequence the non-redundant nucleotide database file nt.**.Tar.gz (20-March-2012) was downloaded from NCBI and a BLAST search conducted using the program ncbi-blast-2.2.26 + -ia32-win32.tar.gar on a standalone computer with the 66.6 kb deleted sequence as the query with all options at their default values. A total of 501 similarity matches to GenBank sequences were identified and of these only 8 were *Sus scrofa* sequencing clones. These included AB457789.1 (TRDV gene segments), AB513624 (Immunoglobulin heavy chain), AB221038.1 (cd1b, cd1e, or 10t1, or 10j1, or 10k1, and more), AY208121 (myostatin gene), AB182371.1 (TCR-alpha/delta chain genes), AB280947.1 (GPC6 genes for Dopachrome tautomer), AB291812.1 (TLR3 gene for Toll-like receptor 3 and AB182372.1 (TCR-alpha/delta chain genes). Within these sequencing clones 95 regions of similarity either flanked a gene, or were in introns, and therefore would not be expected to code for functional genes.

Transgene stability

To determine whether there were modifications to the transgene structure during the 9 generations of the CA line, the junctions between the pig chromosome and transgene inserts and among the transgene inserts, the junctions were amplified by PCR using primer pairs cas-f3/mr-1, J2 F/R, J3 F/R, J4 F/R and J5 F/R (Table 1). The data illustrated in Fig. 4 shows the identical sizes of PCR products for junction one for each of the first 6 generations. Identical results were obtained for generations 7, 8 and 9 (Not shown). The

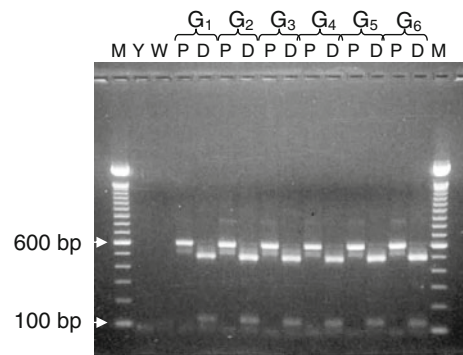


Fig. 4 PCR amplification of junction 1 in CA DNA samples from pigs representing generations G_1 to G_6 using primers cas-f3 and mr-1. A 10 μ L aliquot of each PCR reaction was then digested with *Bgl*III restriction endonuclease and upon completion, a 10 μ L aliquot of each PCR reaction and the digested PCR product were subjected to electrophoresis in a 2 % (w/v) agarose gel and the products visualized using ethidium bromide. The expected PCR product of 606 bp and the *Bgl*III 485 and 121 bp fragments were observed on the electrophorogram. Lane labels: M: 100 bp markers; Y: Yorkshire DNA as template; W: water used instead of DNA; P: PCR product; D: PCR product digested with *Bgl*III

fact that the product from each of the PCR reactions was predictably cleaved by *Bgl*III confirmed the identity of each of the PCR products and documented the absence of base substitutions around junction 1. PCR analysis of junctions 2 through 5 gave identical results for each junction through each of the 9 generations tested. Identical results were also obtained for the transgene integration site of pigs homozygous for the phytase transgene.

Phytase production during growth and through nine generations

Phytase activities in saliva were determined for all 11 day old piglets from transgenic crosses. The data in Fig. 5a illustrates the salivary phytase of 11 day old hemizygous pigs for each of 9 generations and for homozygous pigs resulting from crosses of generation 4, 6 and 9 hemizygous pigs. Statistical analysis showed no differences between boars and gilts of the hemizygous pigs ($P \geq 0.05$) within each generation, except in generation 5 ($P = 0.019$) where male salivary phytase was greater and in generation 8 ($P = 0.016$) where female saliva had greater phytase activity. There were numerous significant differences in the hemizygous pig phytase activities among

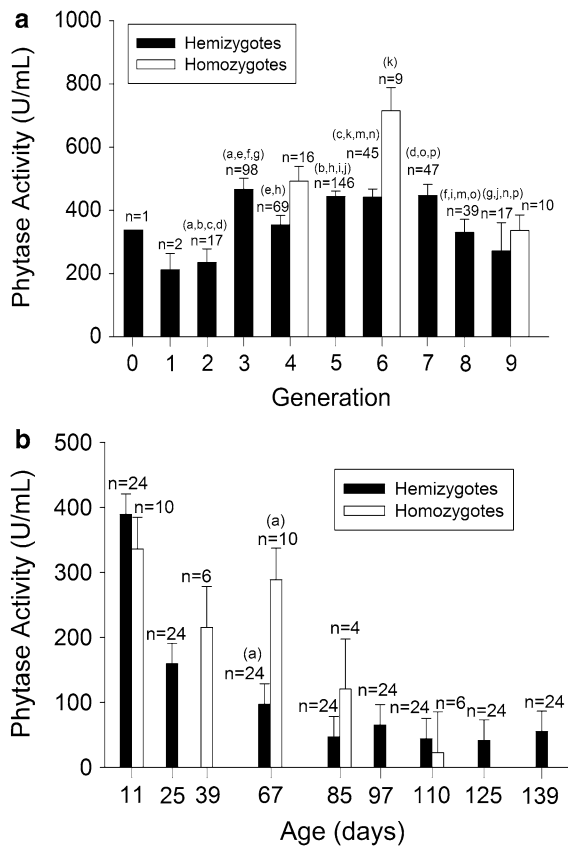


Fig. 5 **a** Phytase activity in saliva from 11 day old piglets from each of the 9 generations. The top bars represent the standard error and the same letters in the parentheses above different bars indicates that the phytase activities are significantly different ($P < 0.05$). Number (n) above the bar indicates number of animals tested. **b** Salivary phytase activities of CA pigs during the weanling, growing and finishing phases of growth. The vertical bars represent the standard error of the mean

generations as illustrated in Fig. 5a. The salivary phytase activities of the pigs homozygous for the phytase gene were generally greater than those of hemizygous pigs of the same generation, but were significantly different in the 6th generation only ($P < 0.05$; data not shown). When hemizygous and homozygous littermates were compared the mean hemizygous value was 330 ± 17 U/mL ($n = 51$) of saliva while that for homozygous pigs was 505 ± 30 U/mL ($n = 35$; $P < 0.0001$). The phytase activities are an approximation of overall phytase activity for 11 day old pigs because of the variables that could affect each reading including whether the pigs have suckled the sow, drank water, or consumed dry feed immediately prior to analysis, therefore the preference

is to take a broader view, which basically indicates that there was no major change in phytase production over the nine generations.

The phytase activity in the saliva was highest in 11 day old pigs and decreased with increasing age to the finisher phase of growth for age (4–6 months old) and weight matched hemizygous pigs of the same generation (Fig. 5b). There was no difference in salivary phytase activity between hemizygous boars and gilts assayed on the same day at all stages of growth and the same was true for homozygous boars and gilts ($P \geq 0.05$). However, the phytase activity of hemizygous pigs at day 11 was significantly higher than that measured at all later days ($P < 0.05$). Similarly, the phytase activity of hemizygous pigs at 25 days was significantly higher than that measured on all subsequently days ($P < 0.05$) for hemizygous pigs. The salivary phytase activities of pigs homozygous for the phytase transgene was significantly greater from that of hemizygous pigs at day 67 ($P = 0.001$) only, otherwise there was no difference. Phytase activity reached a minimum at 111–125 days of age. Phytase activities of mature hemizygous boars and sows (>6 months) were 82.2 ± 33.4 ($n = 4$) and 75.1 ± 16.5 ($n = 11$), respectively.

Two litters of pigs from 7th generation hemizygous CA sows bred with Duroc semen were tested for salivary phytase activities. Ten transgenic pigs were assayed for salivary phytase at 11 days of age, 3 at 58 days 11 at 87 days and 13 at 123 days. No difference was observed in the salivary phytase activities of the CA/Duroc cross pigs from that of 7th generation hemizygous CA pigs of a similar age.

Discussion

We have documented that the transgenic CA line of Yorkshire pigs contains a single site of integration of the phytase transgene on Chromosome 4. There are two copies of the transgene in a head to tail arrangement and a third copy in a reverse orientation that is missing approximately 6 kb of downstream PSP sequence. There were modifications at each junction with a 98 bp insertion presumably of pig sequence between copies 2 and 3 and a 33 bp insertion between copy 3 and porcine sequence and nibbling of nucleotides at junctions. Transgene copies often have host sequences between copies (Covarrubias et al. 1987;

Brinster et al. 1985) and nibbling of a few nucleotides at transgene junctions is commonly observed (Rohan et al. 1990; Hamada et al. 1993). It has been noted that integration of a transgene usually occurs at a single site forming a concatemer in a head to tail orientation (Bishop 1996), consistent with our finding. The copy number of an integrand is highly variable with up to 100 copies of the transgene being inserted as a concatemer (Hammer et al. 1985; Wagner et al. 1981) and copy number may not be related to expression levels (Garrick et al. 1998). Therefore the integration site of the transgenes fits with the general observations made by others.

The transgene integration site in chromosome 4 was stably maintained over 9 generations. This exceeds the number of generations suggested by the Food and Drug Administration (<http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM052463.pdf>; accessed 08 May 2012) to document stability of a transgene, and showed similar stability to that of the Pro347L37 rhodopsin transgene in pigs reported by Sommer et al. (2011). Phytase activity in the saliva of 11 day old transgenic pigs was maintained during 9 generations although it was age dependent, decreasing with the age of the pig approaching a basal level during the finisher phase of growth. Despite the decreased phytase activity in the saliva with age, the volume of saliva reportedly is increased with the quantity of dry feed consumed to as much as 0.5 L of saliva per kg of dry feed consumed (Coring 1980). Because of the larger volume of saliva reportedly produced by older and larger transgenic pigs, they produce sufficient phytase to hydrolyze phytate to satisfy their phosphorus requirement for growth (Unpublished data).

During integration of the phytase transgene into porcine chromosome 4, a 66.6 kb sequence was deleted. This often occurs with non-homologous insertion of foreign DNA into a genome (Wurtele et al. 2003). The extent and nature of the deletion became apparent when the sequence of clone H11A9F8 containing the integrated transgenes was aligned with chromosome 4 clones CU372898 and FP101963 (Fig. 2). No intact genes were detected in the deleted sequence. Furthermore, the deletion did not affect reproduction of the CA pigs documented by similar litter sizes, and growth of pigs either hemizygous or homozygous for the transgene as compared to that of conventional Yorkshire pigs (Unpublished data).

This research has documented the stability of the phytase transgene and its expression over many generations and in different genetic backgrounds. Globally, pigs are an important food animal and will continue to be for the foreseeable future. Because phytic acid in cereal grains is not digestible in standard-breed pigs without supplementation of commercial phytase, the production of transgenic pigs expressing salivary phytase stands as a proven and inexpensive alternative for addressing the environmental challenges posed by high-phosphorus manure in commercial pig production.

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