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Expression of the Viral Antigen VP60 in Transgenic Potatoes and Its Effect on the Nutritional Composition of Tubers

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ABSTRACT

Recombinant plant-derived pharmaceuticals have been investigated for the last two decades and some products will soon be brought to market. Since veterinary pharmaceuticals seem to be the front-runners of plant-derived vaccines, we selected one model subunit vaccine, the structural capsid protein VP60 against rabbit haemorrhagic disease, and analyzed the expression of three different sequences representing the VP60 open reading frame in potato plants. The generation of antigenic VP60 molecules in the leaf and tuber tissue of potato was tremendously enhanced by replacing virus-derived sequences with plant-optimized codons. In order to identify potentially undesirable alterations in the composition of these genetically modified food components, we studied their nutrient composition and nutritional value in comparison to two parental conventional breeding varieties (Albatros and Desiree). The largest differences in nutrient composition were found between the two conventional breeds and between conventional Desiree and its near-isogenic genetically modified potato plant, indicating that genetic modification as well as conventional breeding can influence nutrient composition. Nevertheless, most parameters of nutritional value seemed to be more affected by conventional breeding than by genetic modification.

Keywords: *Recombinant Plant-Derived Vaccines, VP60, RHDV, Animal Nutrition, Nutritional Assessment*

1. Introduction

In recent years, predominantly transgenic plants of the so-called first generation, with input traits like insect resistance or herbicide tolerance, were generated, cultivated and analyzed [1]. Soon, plants of the second and third generation, with output traits like specific components of technical interest [2,3], modified nutritional values [4,5], or plant-based pharmaceuticals [6] are supposed to enter the market. Remarkably, after 20 years of research, recombinant pharmaceutical plants or plant cells are still not on the market [7]. This might either be due to the inadequate expression level or uptake of the pharmaceuticals, as well as to the high production costs, which are mainly caused by the application process and the extensive risk assessment. Nevertheless, some plant-derived recombinant pharmaceuticals established for the

treatment of human diseases and veterinary vaccines are now approaching commercialization [8].

Although research worldwide mainly focuses on human therapeutics [9], important improvements have been achieved in the sector of animal health products [10,11]. Since the application of transgenic plant products to humans is still under great discussion, we can speculate that veterinary pharmaceuticals, and especially vaccines, will be the front runners in the process of commercialisation. Therefore, we decided to concentrate on plant-derived animal immunogens. In a model study we generated antigen-producing potato tubers expressing the major capsid protein VP60 of the Rabbit haemorrhagic disease virus (RHDV) as an interesting veterinary vaccine candidate. VP60 was shown to induce a strong immune response and to protect rabbits against rabbit haemorrhagic disease (RHD) [12]. RHD, an acute and highly conta-

gious disease in wild and domestic rabbits, was first reported by Liu *et al.* 1984 in the People's Republic of China, after which it spread throughout other parts of Asia, Europe, Africa and the Central and North American continent [13,14]. The characteristic pathological signs of RHD are liver damage and haemorrhages in the lung, trachea, liver, spleen, cardiac muscle, and occasionally in the kidneys [15]. Because of the lack of a cell culture system to propagate RHD virus (RHDV) *in vitro*, so far, all commercially available vaccines against RHDV are based on liver homogenates from RHDV-infected rabbits.

In recent years, VP60 has been successfully expressed in several heterologous systems such as *Escherichia coli* [16], *Saccharomyces cerevisiae* [17], baculovirus [18-20], poxvirus [21-23], and plants [24-26]. The oral applications of baculovirus-expressed VP60 [27] and potato-produced VP60 [28] in rabbits gave positive results, a promising indication for the replacement of traditional RHD vaccine production in the near future.

However, such genetically modified (GM) plants or organisms have given rise to an increased concern about the safety of these products in the food chain [29]. "Unintended effects" were proposed to result from the genetic modification process. Although all results available to date reveal no significant differences in the safety and nutritional value of feedstuffs containing material derived from the so-called first generation of genetically modified plants [30], allergenic effects have been reported for soy beans expressing brazil-nut 2S albumin [31] and for peas expressing α-amylase inhibitor-1 from the common bean [32]. Different nutritional composition has also been observed in GM plants as compared to near-isogenic parental control plants (Momma *et al.*, 1999: increased protein content in GM rice; Hashimoto *et al.*, 1999: augmented glycoalkaloid content in GM potatoes).

In order to ensure that no unintended changes in nutrient composition or nutritional value occurred in our GM potato, we compared it to its near-isogenic conventional potato counterpart and carried out a feeding experiment with rats. Taking into consideration that not only the transgene itself but also other factors like potato variety, location of transgene integration and somaclonal variation can affect nutrient composition and nutritional value and can have physiological impacts, two breeds of conventional potatoes plus three transgenic potato plants were tested.

2. Materials and Methods

2.1. DNA Constructs and Construction of Plant Expression Vectors

The coding sequence of *vp60* was designed according to the codon usage of *Nicotiana tabacum* and based on the VP60 sequence of the RHDV isolate "Eisenhüttenstadt" (accession number Y15440), 2/18 (R590 2/18). The motif GCTTCCTCC was inserted immediately after the VP60 start codon and the motif TCTGAAAAGGAT-GAACTT immediately in front of the stop codon. The synthesis was carried out using synthetic nucleotides in the LCR (ligase chain reaction). The natural and synthetic VP60 coding regions (**Figure 1**) were fused to the CaMV 35S promoter and 35S-terminator and integrated into the binary plasmid pGK (Keil, unpublished), leading to pGK-*vp60*-2/18 and pGK-*vp60*synSEKDEL. pGK, pGK-*vp60*-2/18 and pGK-*vp60*synSEKDEL were introduced into the *Agrobacterium tumefaciens* strain LBA4404.

Moreover, the synthetic *vp60* sequence was fused to a synthetic sequence of the non-toxic subunit B of cholera toxin and integrated between the regulatory regions of GBSS promoter and t-nos terminator in the binary plasmid pPGB-1S [33] to obtain tuber-specific expression. The plasmids pPGB-1S and pP-*ctbvp60*synSEKDEL were introduced into the *Agrobacterium tumefaciens* strain LBA4404.

2.2. Bacterial Strains and Plant Transformation

Agrobacterium tumefaciens strain LBA4404, containing the binary plasmids pGK, pGK-*vp60*-2/18, pGK-*vp60*synSEKDEL, pPGB-1S or pP-*ctBvp60*synSEKDEL were propagated under selective pressure and used to infect potato plants using a transformation and regeneration protocol according to [34] with slight modifications. Potato leaves were wounded with razor blades and co-cultivated for three days with Agrobacteria. To induce callus material explants were cultivated for 10 days on MS medium supplemented with 5 mg L^{-1} α-naphthaleneacetic (NAA), 0.1 mg L^{-1} 6-benzylaminopurine (BAP), 100 mg L^{-1} kanamycin and 250 mg L^{-1} cefotaxime. Explants were transferred to MS medium supplemented with 2 mg L^{-1} zeatinribose, 0.02 mg L^{-1} α-naphthaleneacetic (NAA), 0.002 gibberellic acid, (GA$_3$), 100 mg L^{-1} kanamycin and 250 mg L^{-1} cefotaxime. The explants were transferred to fresh medium every two weeks. Shoots were transferred to hormone-free MS medium containing 100 mg L^{-1} kanamycin and 250 mg L^{-1} cefotaxime for root induction. pGK and pGK*vp60*SEKDEL were introduced into the cultivars Desiree and Albatros, while pPGB-1S and pP-*ctbvp 60*SEKDEL were introduced into Albatros potatoes.

2.3. Detection of vp60, ctb and npt II Genes in Transformed Plants

Genomic DNA was isolated from young leaves of transformed potato plants using Qiagen DNeasy Plant Mini Kit, according to the manufacturer's instructions (Qiagen, Hilden, Germany). The presence of the genes was deter-

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bases          1            10
vp60-2/18      ATG
vp60syn SEKDEL ATG GCT TCC TCC
aa-sequence    M   A   S   S

bases              10      19      28      37
vp60-2/18      GAG GGC AAA GCC CGC ACA GCG CCG CAA GCA GCA
vp60syn SEKDEL GAA GGA AAG GCT AGA ACT GCT CCA CAA GCT GGA GCT
aa-sequence    E   G   K   A   R   T   A   P   Q   A   G   A

bases          40      49      58      67      76
vp60-2/18      GCA GGC ACT GCC ACC ACA GCA TCA GTC CCT GGA ACC ACA
vp60syn SEKDEL GCT GGA ACT GCT ACT ACT GCT TCT GTT CCA GGA ACT ACT
aa-sequence    A   G   T   A   T   T   A   S   V   P   G   T   T

bases          79      88      97      106     115
vp60-2/18      ACC GAT GGC ATG GAT CCT GGC GTT GTG GCC ACT ACC AGC
vp60syn SEKDEL ACT GAT GGA ATG GAC CCA GGA GTT GTT GCT ACT ACT TCT
aa-sequence    T   D   G   M   D   P   G   V   V   A   T   T   S

bases          118     127     136     145     154
vp60-2/18      GTG GTC ACT GCA GAG AAC TCA TCC GCA TCG ATT GCA ACG
vp60syn SEKDEL GTT GTT ACT GCT GAA AAT TCT TCT GCT TCT ATT GCT ACT
aa-sequence    V   V   T   A   E   N   S   S   A   S   I   A   T

bases          157     166     175     184     193
vp60-2/18      GCA GGG ATT GGC GGA CCA CCC CAA CAG GTG GAC CAA CAA
vp60syn SEKDEL GCT GGA ATT GGA GGA CCA CCA CAA CAA GTT GAT CAA CAA
aa-sequence    A   G   I   G   G   P   P   Q   Q   V   D   Q   Q

bases          196     205     214     223     232
vp60-2/18      GAG ACA TGG AGA ACG AAC TTT TAT TAT AAT GAT GTT TTC
vp60syn SEKDEL GAA ACT TGG AGA ACT AAT TTT TAT TAT AAT GAT GTT TTT
aa-sequence    E   T   W   R   T   N   F   Y   Y   N   D   V   F

bases          235     244     253     262     271
vp60-2/18      ACT TGG TCA GTC GCG GAT GCC CCT GGC AGC ATA CTT TAC
vp60syn SEKDEL ACT TGG TCT GTT GCT GAT GCT CCA GGA TCT ATT CTT TAT
aa-sequence    T   W   S   V   A   D   A   P   G   S   I   L   Y

bases          274     283     292     301     310
vp60-2/18      ACC GTT CAA CAT TCT CCA CAG AAC AAC CCA TTC ACA GCC
vp60syn SEKDEL ACT GTT CAA CAT TCT CCA CAA AAT AAT CCA TTC ACT GCT
aa-sequence    T   V   Q   H   S   P   Q   N   N   P   F   T   A

bases          313     322     331     340     349
vp60-2/18      GTG CTG AGC CAG ATG TAT GCT GGC TGG GCT GGT GGC ATG
vp60syn SEKDEL GTT CTT TCT CAA ATG TAT GCT GGA TGG GCT GGA GGA ATG
aa-sequence    V   L   S   Q   M   Y   A   G   W   A   G   G   M

bases          352     361     370     379     388
vp60-2/18      CAG TTT CGC TTC ATA GTT GCC GGA TCG GGT GTG TTT GGT
vp60syn SEKDEL CAA TTC AGA TTC ATT GTT GCT GGA TCT GGA GTT TTT GGA
aa-sequence    Q   F   R   F   I   V   A   G   S   G   V   F   G

bases          391     400     409     418     427
vp60-2/18      GGG CGG TTG GTT GCG GCC GTG ATA CCA CCG GGC ATC GAG
vp60syn SEKDEL GGA AGA CTT GTT GCT GCT GTT ATT CCA CCA GGA ATT GAA
aa-sequence    G   R   L   V   A   A   V   I   P   P   G   I   E

bases          430     439     448     457     466
vp60-2/18      ATT GGA CCA GGG CTG GAG GTC AGG CAA TTC CCC CAT GTT
vp60syn SEKDEL ATT GGA CCA GGA CTT GAA GTT AGG CAA TTC CCA CAT GTT
aa-sequence    I   G   P   G   L   E   V   R   Q   F   P   H   V

bases          469     478     487     496     505
vp60-2/18      GTC ATC GAC GCT CGT TCA CTT GAA CCT GTT ACC ATC ACC
vp60syn SEKDEL GTT ATT GAT GCT AGG TCT CTT GAA CCA GTT ACT ATT ACT
aa-sequence    V   I   D   A   R   S   L   E   P   V   T   I   T

bases          508     517     526     535     544
vp60-2/18      ATG CCA GAC TTG CGT CCC AAC ATG TAC CAT CCA ACT GGT
vp60syn SEKDEL ATG CCA GAC CTT AGA CCA AAT ATG TAT CAT CCA ACT GGA
aa-sequence    M   P   D   L   R   P   N   M   Y   H   P   T   G

bases          547     556     565     574     573
vp60-2/18      GAC CCT GGC CTT GTT CCC AAC CTA GTC CTT AGT GTT TAT
vp60syn SEKDEL GAT CCA GGA CTT GTT CCA ACT CTT GTT CTT TCT GTT TAT
aa-sequence    D   P   G   L   V   P   T   L   V   L   S   V   Y

bases          586     595     604     603     622
vp60-2/18      AAC AAC CTC ATC AAC CGC TTT GGT GGG TCC ACC AGC GCA
vp60syn SEKDEL AAT AAT CTT ATT AAT CCA TTT GGA GGA TCT ACT TCT GCT
aa-sequence    N   N   L   I   N   P   F   G   G   S   T   S   A

bases          625     634     643     652     661
vp60-2/18      ATC CAG GTG ACA GTG GAA ACA AGG CCA AGT GAA GAT TTT
vp60syn SEKDEL ATT CAA GTT ACT GTT GAA ACT AGA CCA TCT GAA GAT TTT
aa-sequence    I   Q   V   T   V   E   T   R   P   S   E   D   F

bases          664     673     682     691     700
vp60-2/18      GAG TTC GTG ATG ATT CGA ACC CCC TCC AGC AAG ACT GTT
vp60syn SEKDEL GAA TTT GTT ATG ATT AGA ACT CCA TCT TCT AAG ACT GTT
aa-sequence    E   F   V   M   I   R   T   P   S   S   K   T   V

bases          703     712     721     730     739
vp60-2/18      GAC TCA ATT TAC CCT GCA GGC CTC CTC ACG ACC CCA GTC
vp60syn SEKDEL GAT TCT ATC TAT CCA GCT GGA CTT CTT ACT ACT CCA GTT
aa-sequence    D   S   I   Y   P   A   G   L   L   T   T   P   V

bases          742     751     760     769     778
vp60-2/18      CTC ACT GGG GTT GGC AAT GAC AAC AGG TGG AAT GGC CAA
vp60syn SEKDEL CTT ACT GGA GTT GGA AAT GAT AAT AGA TGG AAT GGA CAA
aa-sequence    L   T   G   V   G   N   D   N   R   W   N   G   Q

bases          781     790     799     808     817
vp60-2/18      ATA GTG GGA CTC CAA CCA GTA CCT GGA GGG TTC TCT ACG
vp60syn SEKDEL ATT GTT GGA CTT CAA CCA GTT CCA GGA GGA TTT TCT ACT
aa-sequence    I   V   G   L   Q   P   V   P   G   G   F   S   T

bases          820     829     838     847     856
vp60-2/18      TGC AAC AGG CAT TGG AAC TTG AAT GGC AGC ACA TAT GGC
vp60syn SEKDEL TGT AAT AGA CAT TGG AAC CTT AAT GGA TCT ACT TAT GGA
aa-sequence    C   N   R   H   W   N   L   N   G   S   T   Y   G

bases          859     868     877     886     895
vp60-2/18      TGG TCA AGC CCC CGG TTT GCC GAC ATT GAC CAT CGA AGA
vp60syn SEKDEL TGG TCT TCT CCA CGT TTT GCT GAT ATC GAT CAT AGA AGA
aa-sequence    W   S   S   P   R   F   A   D   I   D   H   R   R


bases          898     907     916     925     934
vp60-2/18      GGC AGT GCA AGT TAC CCT GGG AGC AAC GCA ACC AAC GTG
vp60syn SEKDEL GGA TCT GCT TCT TAT CCA GGA TCT AAT GCT ACT AAT GTT
aa-sequence    G   S   A   S   Y   P   G   S   N   A   T   N   V

bases          937     946     955     964     973
vp60-2/18      CTT CAG TTT TGG TAT GCC AAT GCT GGA TCT GCA ATC GAC
vp60syn SEKDEL CTT CAA TTT TGG TAT GCT AAT GCT GGA TCT GCT ATT GAT
aa-sequence    L   Q   F   W   Y   A   N   A   G   S   A   I   D

bases          976     985     994     1003    1012
vp60-2/18      AAT CCC ATC TCC CAG GTT GCA CCA GAC GGC TTT CCT GAT
vp60syn SEKDEL AAT CCA ATT TCT CAA GTT GCT CCA GAT GGA TTT CCA GAT
aa-sequence    N   P   I   S   Q   V   A   P   D   G   F   P   D

bases          1015    1024    1033    1042    1051
vp60-2/18      ATG TCG TTC GTG CCC TTT AAT GGC CCT GGC ATT CCA GCC
vp60syn SEKDEL ATG TCT TTT GTT CCA TTC AAT GGA CCA GGT ATT CCA GCT
aa-sequence    M   S   F   V   P   F   N   G   P   G   I   P   A

bases          1054    1063    1072    1081    1090
vp60-2/18      GCG GGG TGG GTC GTC GGA TTT GGT GGA GCT ATT TGG AAC AGT AAC
vp60syn SEKDEL GCT GGA TGG GTT GGA TTT GGA GCT ATT TGG AAC TCT AAT
aa-sequence    A   G   W   V   G   F   G   A   I   W   N   S   N

bases          1093    1102    1111    1120    1129
vp60-2/18      AGC GGT GCC CCC AAC GTT ACG ACT GTG CAG GCT TAT GAG
vp60syn SEKDEL TCT GGA GCT CCA AAT GTT ACT ACT GTT CAA GCT TAT GAA
aa-sequence    S   G   A   P   N   V   T   T   V   Q   A   Y   E

bases          1132    1141    1150    1159    1168
vp60-2/18      TTA GGT TTT GCC ACT GGG GCA CCA GGC AAC CTC CAG CCC
vp60syn SEKDEL CTT GGA TTT GCT ACT GGA GCT CCA GGA AAT CTT CAA CCA
aa-sequence    L   G   F   A   T   G   A   P   G   N   L   Q   P

bases          1171    1180    1189    1198    1207
vp60-2/18      ACC ACC AAC ACT CCA GGT TCA CAG ACT GTC GCC AAG TCC
vp60syn SEKDEL ACT AAT AAT ACT CCA GGA TCT CAA ACT GTT GCT AAG TCT
aa-sequence    T   T   N   T   P   G   S   Q   T   V   A   K   S

bases          1210    1219    1228    1237    1246
vp60-2/18      ATA TAT GCC GTG GTA ACT GGC ACA GCC CAA AAC CCC GCC
vp60syn SEKDEL ATC TAT GCT GTT GTT ACT GGA ACT GCT CAA AAT CCA GCT
aa-sequence    I   Y   A   V   V   T   G   T   A   Q   N   P   A

bases          1249    1258    1267    1276    1285
vp60-2/18      GGA TTG TTT GTG ATG GCC TCG GGT GTT ATC TCC ACC CCA
vp60syn SEKDEL GGA CTT TTT GTT ATG GCT TCT GGT GTT ATT TCT ACT CCA
aa-sequence    G   L   F   V   M   A   S   G   V   I   S   T   P

bases          1288    1297    1306    1315    1324
vp60-2/18      AGT GCC AAC GCC ATC ACA TAT ACG CCC CAA CCA GAC AGA
vp60syn SEKDEL TCT GCT AAT GCT ATT ACT TAT ACT CCA CAA CCA GAT AGA
aa-sequence    S   A   N   A   I   T   Y   T   P   Q   P   D   R

bases          1327    1336    1345    1354    1363
vp60-2/18      ATT GTA ACC ACA CCC GGC ACT CCT GCC GCC GCA CCT GTG
A1 (vp60syn)   ATT GCT ACT ACT CCA GGA ACT CCA GCT GCA GCA CCA GTT
aa-sequence    I   V   T   T   P   G   T   P   A   A   A   P   V

bases          1366    1375    1384    1393    1402
vp60-2/18      GGT AAG AAC AAC ACA CCC ATC ATG TTC GCG TCT GTC GTC AGG
vp60syn SEKDEL GGA AAG AAT ACT CCA ATT ATG TTT GCT TCT GTT GTT AGA
aa-sequence    G   K   N   T   P   I   M   F   A   S   V   V   R

bases          1405    1414    1423    1432    1441
vp60-2/18      CGC ACC GGT GAC GTC AAC GCC ACA GCT GGG TCA GCT AAC
vp60syn SEKDEL AGA ACT GGA GAT GTT AAC GCT ACT GCT ACT GGA TCT GCT AAT
aa-sequence    R   T   G   D   V   N   A   T   A   G   S   A   N

bases          1444    1453    1462    1471    1480
vp60-2/18      GGG ACC CAG TAC GGC ACA GGC TCT CAA CCA CTG CCA GTA
vp60syn SEKDEL GGA ACT CAA TAT GGA ACT GGA TCT CAA CCA CTT CCA GTT
aa-sequence    G   T   Q   Y   G   T   G   S   Q   P   L   P   V

bases          1483    1492    1501    1510    1519
vp60-2/18      ACA ATT GGA CTT TCG CTC AAC AAC TAC TCG TCA GCA CTT
vp60syn SEKDEL ACT ATT GGA CTT TCT CTT AAT AAT TAT TCT TCT GCT CTT
aa-sequence    T   I   G   L   S   L   N   N   Y   S   S   A   L

bases          1522    1531    1540    1549    1558
vp60-2/18      ATG CCC GGA CAG TTT TTC GTT TGG CAG TTA ACC TTT GCA
vp60syn SEKDEL ATG CCA GGA CAA TTT TTC GTT TGG CAA CTT ACT TTT GCT
aa-sequence    M   P   G   Q   F   F   V   W   Q   L   T   F   A

bases          1561    1570    1579    1588    1597
vp60-2/18      TCT GGT TTC ATG GAG ATT GGT TTA AGT GTG GAC GGG TAT
vp60syn SEKDEL TCT GGA TTC ATG GAA ATT GGA CTT TCT GTT GAT GGA TAT
aa-sequence    S   G   F   M   E   I   G   L   S   V   D   G   Y

bases          1600    1609    1618    1627    1636
vp60-2/18      TTT TAT GCT GGA ACC GGA GCC TCA ACC ACA CTC ATT GAC
vp60syn SEKDEL TTT TAT GCT GGA ACT GGA GCT TCT ACC ACA CTT ATT GAC
aa-sequence    F   Y   A   G   T   G   A   S   T   T   L   I   D

bases          1639    1648    1657    1666    1675
vp60-2/18      TTG ACT GAA CTC ATT GAC GTA CGC CCT GTG GGA CCC AGG
vp60syn SEKDEL CTT ACT GAA CTT ATT GAT GTT AGA CCA GTT GGA CCA AGA
aa-sequence    L   T   E   L   I   D   V   R   P   V   G   P   R

bases          1678    1687    1696    1705    1714
vp60-2/18      CCA TCC AAG AGC ACA CTC GTG TTC AAC CTG GGG GGC ACA
vp60syn SEKDEL CCA TCT AAG TCT ACT CTT GTT TTC AAT CTT GGA GGA ACT
aa-sequence    P   S   K   S   T   L   V   F   N   L   G   G   T

bases          1717    1726    1735
vp60-2/18      GCC AAT GGC TTT TCT TAT GTC TGA
vp60syn SEKDEL GCT AAT GGA TTT TCA TAT GTT TCT GAA AAG GAT GAA CTT TAA
aa-sequence    A   N   G   F   S   Y   V   S   E   K   D   E   L
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Figure 1. Sequence of the natural VP60 coding region (virus isolate R590-2/18) and the derived synthetic sequence (tobacco codon preference) with the corresponding amino acid sequence.

mined by PCR analysis using primers VP60-2/18-fw and VP60-rv, VP60syn-fw and VP60syn-rv, 2npt-fw and 2npt-rv, ctBfw_l and ctBrv_l (**Table 1**). Cycling conditions were as follows: 95˚C for 5 min, followed by 37 cycles of 95˚C for 1 min, 54˚C for 1 min and 72˚C for 1 min, and a final elongation at 72˚C for 10 min. The PCR products were analyzed on a 2% agarose gel.

2.4. Analysis of RNA from Transformed Tomato Plants

Total RNA from leaves and tubers of transformed potato plants was isolated according to the method of Logemann *et al*. (1987). 1 µg of total RNA was used for DNase treatment, followed by a reverse transcription assay using the Qiagen Omniscript RT kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). PCR was subsequently used, as described above, to identify the mRNA of the vp60, ctb and npt II genes. The RT-PCR products were analyzed on a 2% agarose gel.

2.5. RHDV Enzyme-Linked-Immunosorbent Assay (ELISA) of Plant Material

Plant material was ground in liquid nitrogen and the resulting powder was extracted three times with phosphate-buffered saline PBS (pH 7.2) by mixing and subsequent centrifugation (5 min, 16,000 g). The total soluble protein content of each extract was determined by a Bradford assay [35].

ELISA was performed as previously described [36]. Briefly, plates were coated with RHDV antiserum raised in guinea pigs. After the plant extract was added, a mix of three murine monoclonal antibodies against VP60 was used to detect recombinant VP60. Visualisation was performed using peroxidase-labelled anti-mouse IgG (Sigma) and o-phenylenediamine (Sigma) as the substrate. Between the incubation steps the plates were washed three times with PBST [PBS, 0.05 % Tween20 (v/v)]. Finally, the plate was measured at 492 nm using an ELISA reader (Anthos Lucy2). To calculate the relative amount of VP60 in the plant material the following equation was used, Index = $(OD_{sample} - OD_{neg}) / (OD_{pos} - OD_{neg}) \cdot 100$

Table 1. Oligonucleotides used for detection of *vp60*, *ctb* and *npt*II sequences.

Primer	Sequence
VP60-2/18-fw	5'-AAGCAGGAGCAGCAGGCACT-3'
VP60-rv	5'-TGTCACCTGGATTGCGCTGG-3'
VP60syn-fw	5'-AGGCTAGAACTGCTCCACAA-3'
VP60syn-rv	5'-CCATTAGCAGTTCCTCCAAG-3'
ctBfw_l	5'-TTCTTATACTGAATCTCTTGCTGGA-3'
ctBrv_l	5'-CGGATCCAAGCTTTTAAGAAA-3'
2npt-fw	5'-TCCGGCCGCTTGGGTGGAGAG-3'
2npt-rv	5'-CTGGCGCGAGCCCCTGATGCT-3'

ng VP60 produced by a Baculovirus system served as standard.

2.6. CTB ELISA of Plant Material

Isolation and determination of total soluble protein occurred as described above. Detection and quantification of CTB protein in crude leaf extracts, as well as in potato tuber material was done by coating plates with rabbit antiserum against CTB (Natutec) diluted 1:2,000 in carbonate buffer (50 mM N_2CO_3, pH 9), incubation for 2 h and blocking with 1% BSA solution for 1 h. Plant samples were incubated in duplicated samples for two hours on coated plates, followed by incubation with goat antiserum against CTB diluted 1:5000 in PBS. After incubation with the anti-goat IgG-horseradish peroxidase conjugate (Dianova) diluted 1:5000 in PBS, visualisation was performed with a peroxidase labelled anti-rabbit IgG (Sigma) o-phenylenediamine as a substrate (Sigma). All incubation steps were done at 37˚C and the intervening washing steps were repeated three times with PBST. Finally, the plate was measured at 405 nm using an ELISA reader (Anthos Lucy2). All plant samples were analysed three times, and an analysis of variance was carried out using the statistical analysis program Excel (Microsoft®).

2.7. NPTII ELISA of Plant Material

Isolation and determination of total soluble protein occurred as described above. Detection and quantification of NPTII protein in crude leaf extracts, as well as potato tuber material was performed by means of NPTII ELISA (Biofords) [37].

2.8. Preparation and Analysis of Transgenic Dry Tuber Material

Tubers of the three transgenic potato plants, Desiree/ GKvp60$_{SEKDEL}$ #6, Albatros/pPGB-1S #210 and Albatros/pP*ctbvp60*$_{synSEKDEL}$ #202 and the corresponding isogenic plants, Desiree and Albatros, were harvested and the raw material was lyophilised to concentrate the protein. The concentrations of VP60 and CTB were estimated using quantitative VP60- and CTB-ELISA. Additionally the content of neomycin-phosphotransferase II was determined.

2.9. Animals, Diets and Experimental Design

The feeding study was carried out with 40 young male Wistar rats (Charles River Laboratories, Germany), each weighing approximately 75g at the beginning of the experiment. Animals were kept under standardized humidity (60 ± 5%) and temperature (21.5 ± 1˚C) with a 12 hr light: 12 hr dark regime in metabolism cages for 14 days. The animals were divided into five groups and were all

fed a half-synthetic basic diet supplemented with casein (3% supplementation of methionine) as the sole protein source. Additionally, the animals received five different potatoes as fodder additives (D-niv, A-niv, A-NPTII, A-CTP: VP60, D-VP60, see **Table 2**; for detailed explanation see **Figure 2**), one potato construct per group. Potatoes were given in amounts so that 15% of dietary protein (based on N) was supplied by the investigated potato plant.

All diets were iso-energetic and iso-nitrogenous, containing 150 mg nitrogen per 10 g dry matter. Animals were fed for 14 days. Feed amount for each animal was adjusted according to the body weight (10 g dry matter per 100g body weight) and was fed once a day. Free access to food and water was allowed. Body weight (BW) was recorded on days 1, 8 and 14 of the feeding experiment, before feeding. From day 8 to day 14 the daily food intake was recorded, and the urine and feces were collected daily. HCl was added to urine and feces samples for storage at 4°C until analyses were done. At the end of the experiment the animals were sacrificed under inhalant isofluran (IsoFlo®) anesthesia 12 hours after the last meal.

2.10. Nutritional Value of Potato Tubers

Amounts of principal nutrient components (dry matter (DM), crude ash (cA), crude fibre (cF), ether extract (EE), and nitrogen free extract (NfE)) in the five strains of potato plants were determined using the Weender standard procedure [38]. Crude protein (cP) was calculated from N-content analyzed in the elementary analyzer LECO CNS 2000 applying the Dumas combustion technique [39,40].

2.11. Nitrogen Balance

N-content of all fecal samples as well as of pure potato lyophilisates (feed and uneaten feed) was determined as described above and was used for the evaluation of N balance. N-balance was further used for to calculate total tract apparent crude protein (TTACPD) and amino acids digestibility (TTACAAD), net protein utilization (NPU) and the protein efficiency ratio (PER) [41]. Endogenous and metabolic nitrogen were calculated using regression formulas based on rats' BW and DM intake, which were obtained in previous nitrogen-free experiments on rats that had been studied at the Research Institute for the Biology of Farm Animals [41].

2.12. Amino Acid Composition

The amount of total amino acids was determined in lyophilised potatoes and in feces by the Biochrom 20 Plus analyzer (Biochrom Ltd, Cambridge, UK) using automated ion exchange chromatography according to [42]

and [43].

2.13. Statistics

Statistical analysis of parameter differences between animal groups and potato plants was done by means of one-factor ANOVA, followed by the HSD-Tukey Test (SAS®, 2002). A p-value ≤ 0.05 was considered significant.

3. Results

3.1. Enhancement of VP60 Content in Transgenic Potato Plants

The expression of genes coding for pharmaceuticals is often too low to obtain sufficient amounts of protein in the plant cell, hence the content of transgene-encoded proteins should be increased in the plant cell. The amount of these proteins depends on the promoter used, the sequence of the coding region influencing RNA stability and translation, and the localisation of the protein in the plant cell that influences protein stability. One possibility for enhancing protein expression is the adoption of transgenes to the plants codon usage (e.g., Peng *et al*. 2006). Therefore we designed a coding region adapted to *Nicotiana tabacum* codon usage and compared its expression to the wild type gene (**Figure 1**). For a promoter, we chose the strong and constitutively expressed CaMV35S promoter which still is one of the strongest in potato leaves and quite strong in tubers, explaining its frequent application in potato (e.g., [44,45]. Additionally a GCTTCCTCC motif [46] was inserted immediately after the start codon ATG to stabilize the *vp60* mRNA. To reduce protein degradation the sequence TCTGAAAAGGATGAACTT coding for the retention signal SEKDEL of the endoplasmatic reticulum [47] was linked 3' of the coding region. Three populations of transgenic potato plants expressing a) neomycin-phosph -otransferase II (NPTII), b) neomycin-phos- photransferase II and VP60 (natural sequence background) as well as c) neomycin-phosphotransferase II and VP60$_{SEKDEL}$ (synthetic sequence background) were generated via *Agrobacterium*-mediated transformation. The T-DNAs used for transforming plants are illustrated in **Figure 2**.

The cultivar Desiree was used to introduce pGK-*vp60*-2/18. Eight independent potato clones were established for further investigation. The cultivars Desiree and Albatros were used to introduce pGK and pGK*vp60*$_{synSEKDEL}$. Out of the many shoots regenerated on selective medium, 11 independent transgenic GK*vp60*$_{synSEKDEL}$ potato plants were established (seven for the variety Desiree and four for Albatros). The presence of the two different *vp60* coding regions was confirmed by PCR amplification of the genomic DNA (data not shown). Transcription was confirmed using RT-PCR. Transgenic plants constitu-

tively expressing the neomycin-phosphotransferase II (GK) were used together with non-transgenic plants (near isogenic variety, niv) as controls for mRNA analysis to exclude false positive signals (**Figure 3**). In order to obtain VP60-producing potato material transgenic potato clones were grown in the greenhouse for tuberization. Afterwards, the tubers and leaves were analysed for the presence of VP60 using the VP60-ELISA. As shown in **Figure 4**, four transgenic Desiree/GK-$vp60_{SEKDEL}$ and four Albatros/GK-$vp60_{SEKDEL}$ plants showed high amounts of VP60 in leaves as well as in tuber material. Plants with the natural $vp60$-2/18 coding sequence produced detectable VP60 (**Figure 4**). Compared to the viral sequence, the synthetic sequence resulted in a tremendous increase in VP60 production.

3.2. Enhancement of the Antigenic Potential of Potatoes Producing VP60

The biological activity of the antigen VP60 should be enhanced by the fusion of the $vp60$ sequence to a synthetic *ctB* sequence (**Figure 2**). This effect has already been demonstrated for several different antigens [48-50]. Nevertheless, in some cases this fusion led to reduced expression levels or even to the failure of protein fusion complex production in plants [51,52]. Since the weight of CTB::VP60 is approximately 355 kDa we worried about reduced levels of the fusion protein CTB::VP60 compared to VP60. In order to adjust the expression of the fusion protein to levels obtained for VP60 alone, the coding region was driven by the tuber specific GBSS promoter [53]. According to Visser *et al*. (1991), the GBSS promoter leads to higher ß-glucorionidase activity in transgenic potato tubers compared to CaMV35S-*gus* fusions.

After *Agrobacterium*-mediated Albatros transformation, 17 independent transgenic Albatros/P*ctbvp60*$_{synSEKDEL}$ potato plants were obtained, and the presence of $vp60$ was confirmed by PCR amplification of the genomic DNA (data not shown). Again, total RNA from non-transgenic plants (niv), *npt*II plants (pPGBS121) and seven transgenic plants expressing tuber-specific *ctbvp60*$_{synSEKDEL}$ were assayed for the presence of the $vp60$-specific transcript using RT-PCR (data not shown). In VP60-ELISA tests, no transgenic Albatros/P-*ctbvp60*$_{synSEKDEL}$ plants demonstrated detectable VP60 in leaf and tuber material. Hence, as assumed, the fusion of VP60 with CTB might have led to the drastic reduction of the amount of transgene-encoded protein in the cell, or the GBSS promoter used to enhance the expression was unable to drive VP60 expression. In order to verify the promoter effect, we fused GBSS with the VP60 coding region alone. No VP60 could be detected in the transgenic plants (data not shown).

3.3. Selecting Transgenic Potato Plants Producing VP60 for Feeding Experiments

The plant Desiree/GK$vp60_{SEKDEL}$ #6 (D-VP60) showed one of the highest VP60-Indexes (**Figure 4**). Around 4000 plants were propagated *in vitro* and grown in the greenhouse for tuberization. In addition, the Albatros/P*ctbvp60*$_{synSEKDEL}$ #202 (A-CTB::VP60) and Albatros/PGB-1S #210 (A-NPTII) were selected for feeding experiments. Even if no detectable amounts of VP60 could be demonstrated, mRNA specific to the *ctb* and $vp60_{synSEKDEL}$ coding regions could be detected in tuber material of Albatros/P*ctbvp60*$_{synSEKDEL}$ #202 (**Figure 5**). These plants were used for tuber production, with 700 plants per event. Harvested potato tubers were milled and then lyophilised. ELISA analyses of the powdered potato tuber material were used to verify VP60, CTB and NPTII content in the produced powdered potato material. Consequently, three transgenic plants containing different inserts (**Table 2**) and the corresponding near-isogenic variety, Desiree (D-niv) and Albatros (A-niv) were used for feeding experiments.

3.4. Nutrient Composition and Nutritional Value of Transgenic and Conventionally Bred Potatoes

Contents of the main nutrients of the two conventionally bred plants (A-niv, D-niv), their VP60/NPTII-expressing transgenic counterparts (A-CTB::VP60 and D-VP60) and a NPTII transgenic plant (A-NPTII) were comparatively analysed. In addition, the nutritional value of these potatoes was studied in a feeding experiment with rats. Based on a comparison of the conventional Albatros potato with all other varieties, the putative effects of conventional breeding including somatic cloning (A-niv *vs.* D-niv), the insertion and expression of the foreign *vp60* gene (A-niv *vs.* A-CTB::VP60 and D-niv vs. D-VP60) and the insertion of the *npt*II gene (A-niv *vs.* A-NPTII) were estimated. The differences between the both conventional varieties Desiree and Albatros were set to 100%. Differences between all other plants are given in % of the difference between both conventional varieties Desiree and Albatros. Thus, relative differences of less than 100% (columns 2-4 in **Tables 3** and **4**) indicate that the differences between the transgenic plant and its corresponding conventionally bred variety were lower than the differences between the two varieties of conventionally bred plants.

3.5. Nutrient Composition of Different Potato Varieties

In **Table 3** differences in the main nutrient components

Table 2. Protein content of VP60, CTB and NPTII in freeze-dried potato tuber material, prepared for feeding experiments.

Plant	Abbr.	VP60 µg/mg TSP	CTB µg/mg TSP	NPTII µg/mg TSP
Desiree, near isogenic variety	D-niv	0	0	0
Albatros, near isogenic variety	A-niv	0	0	0
Desiree/GK$vp60_{synSEKDEL}$ #6	D-VP60	0.21 ± 0.140	0	5.96 ± 0.273
Albatros/PGB1S #210	A-NPTII	0	0	0.053 ± 0.0004
Albatros/P$ctbvp60_{synSEKDEL}$ #202	A-CTB::VP60	0.00042 ± 0.000230	non detectable	0.026± 0.0050

Table 3. Effects of conventional breeding and genetic modification on nutrient composition in different potato lines and varieties.

Parameter	Means of conventional breeding variety		D-niv - A-niv	Comparison of lines (absolute differences)		
	A-niv	D-niv		A-NPTII - A-niv	A-CTB::VP60 - A-niv	D-VP60 - D-niv
Dry matter (DM) [%]	93.02	95.36	2.34 (2.52%)	0.11 (0.12%)	0.18 (0.19%)	0.82 (0.86%)
Crude ash [% DM]	3.74	4.20	0.46 (12.30%)	–0.01 (–0.27%)	–0.17 (–4.55%)	0.66 (15.71%)
Crude protein [% DM]	9.62	9.15	–0.47 (–4.89%)	–0.68 (–7.07%)	–0.02 (–0.21%)	6.23 (68.09%)
Crude fiber [% DM]	2.23	2.98	0.75 (33.63%)	0.27 (12.11%)	0.03 (1.35%)	6.22 (208.72%)
Ether soluble extract [% DM]	0.28	0.41	0.13 (46.43%)	–0.02 (–7.14%)	–0.02 (–7.14%)	–0.05 (–12.20%)
Nitrogen free extract [% DM]	85.63	83.15	–2.48 (–2.9%)	–0.30 (–0.35%)	0.70 (0.82%)	–13.50 (–16.24%)
Total amino acids [g/16 g N]	84.57	70.33	–14.24 (–16.84%)	–7.26 (–8.85%)	–8.38 (–9.91%)	1.73 (2.46%)

Table 4. Effects of conventional breeding and genetic modification in different potato lines and varieties on weight gain and parameters of nutritional value determined in rats.

Parameter	Absolute means of conventional breeding varieties		Comparison of lines (absolute differences)			
	A-niv	D-niv	A-nvi - D-niv	A-niv - A-NPTII	A-niv - A-CTB::VP60	D-niv - D-VP60
weight gain [g]	13.66 ± 4.28	16.44 ± 2.12	2.78 (20.33%)	0.39 (2.82%)	–0.58 (–4.22%)	0.47 (2.83%)
N-balance [mg/d]	77.32 ± 6.11	94.21 ± 6.22	16.89* (21.85%)	10.00 (12.93%)	–4.90 (–6.34%)	13.85* (14.70%)
total tract true crude protein digestibility [%]	73.93 ± 3.80	79.33 ± 1.50	5.40* (7.31%)	1.59 (2.15%)	–2.12 (–2.87%)	5.82* (7.34%)
net protein utilization [%]	69.82 ± 2.84	76.59 ± 2.72	6.77* (9.69%)	2.10 (3.01%)	–1.77 (–2.53%)	6.14* (8.02%)
protein efficiency ratio [%]	1.73 ± 0.54	2.01 ± 0.26	0.28 (16.05%)	–0.03 (–1.89%)	–0.07 (–4.08%)	0.02 (1.06%)
Digestibility ratio of total amino acids [g/16 gN]	74.36 ± 2.97	77.40 ± 2.47	3.04* (4.09%)	–0.40 (–0.54%)	–4.58* (–6.16%)	4.98* (6.43%)

*Absolute differences between potato varieties are significant with $p \leq 0.05$

between the compared potato varieties are shown. Considering both the number of traits exhibiting differences and the extent of differences, the strongest effects were observed between A-niv and D-niv as well as between D-niv and D-VP60. Absolute contents of crude fibre, ether extract, crude ash and total amino acids differed among both conventionally bred potatoes (A-niv and D-niv) between 3 to 46% (ether soluble extract). In relation to these results, the comparison of the A-niv potatoes with both transgenic potatoes (A-CTB::VP60 and A-NPTII) revealed fewer differences (*i.e.* <100%) in nearly all of the traits of nutrient composition (see **Table 3**, columns 2 and 3). This result indicates that in the Al-

batros potatoes investigated, neither the integration of foreign DNA nor the expression of *ctbvp60*synSEKDEL and *npt*II caused changes in nutrient composition that were any greater than those observed between conventional breeding and somatic cloning. Only the difference in crude protein content between the NPTII-expressing Albatros and the conventional Albatros plant material was higher than that between Desiree and Albatros. In contrast, genetic modification of Desiree potatoes caused distinctive changes in nutrient composition. Differences in crude ash (143.5%), crude protein (–1325.5%), crude fibre (829%) and nitrogen free extract content (544.4%) considerably exceeded the differences (100%) between
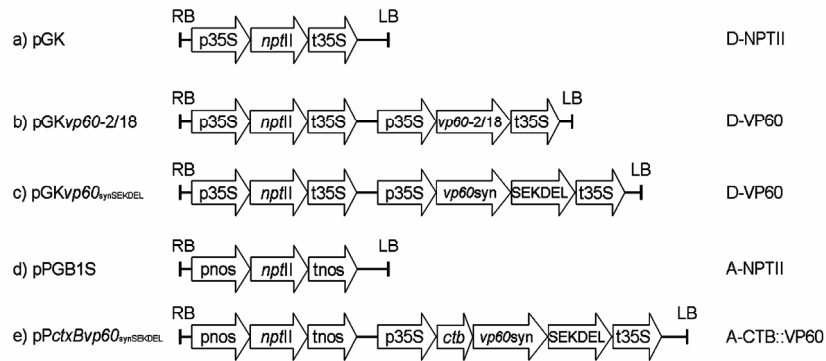
Figure 2. Schematic diagram of the constructs used for potato transformation to generate VP60-expressing plants. The constructs contain two different *vp60* genes, one based on the natural sequence of the specific RHDV isolate "Eisenhüttenstadt" (*accession number* Y15440), 2/18 (R590 2/18) and the second a synthetic vp60-sequence with tobacco codon usage as well as a synthetic *ctb* sequence. The coding regions are under the control of the CaMV 35S promoter (p35S) and terminator (t35S). The marker gene neomycine phosphotransferase regulated by p35S and t35S is integrated in all constructs (b-e) and was used as an internal control (a).
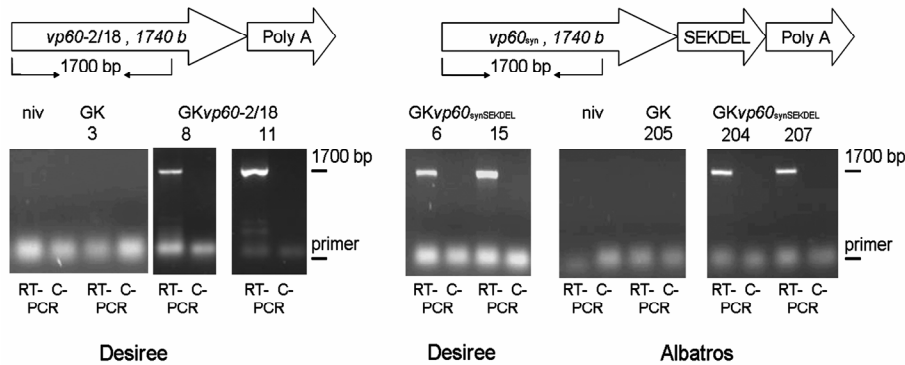
Figure 3. Detection of transgene-specific transcripts by RT-PCR. RT-PCR was carried out using 1 µg of total RNA after DNase treatment, by using the Qiagen Omniscript RT kit. Oligonucleotides VP60$_{syn}$fw and VP60$_{syn}$rv (Table 1) were used for amplification of *vp60*-specific cDNA.
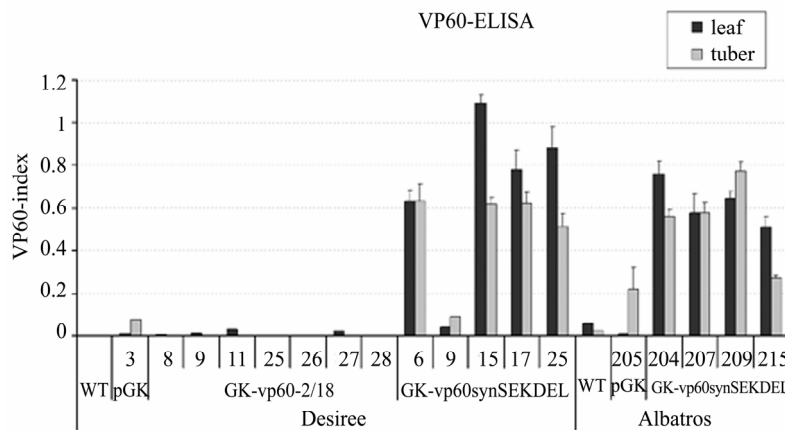
Figure 4. Detection of VP60 in transgenic potato plants Desiree and Albatros carrying the native and the synthetic *vp*60 coding region. The Index was generated by using the optical density at 405 nm of purified CTB, PBS, non-transformed plant material (near isogenic variety, niv) and plant material of the transgenic plants (OD $_{transgenic\ line}$ - OD $_{wild\ type}$) / (OD $_{purified\ CTB}$ - OD $_{PBS}$).
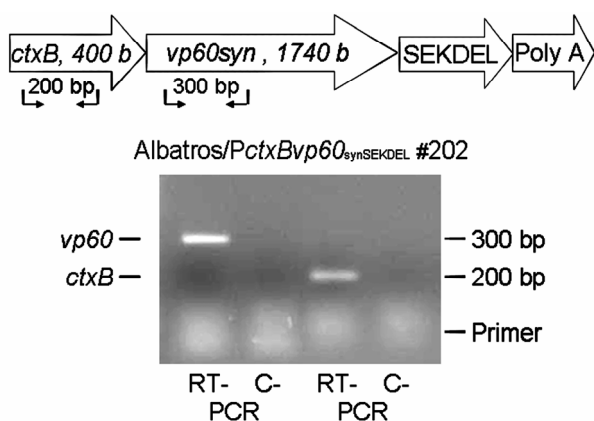
Figure 5. Detection of transgene-specific transcripts by RT-PCR. RT-PCR was carried out using 1 µg of total RNA after DNase treatment, by using the Qiagen Omniscript RT kit. Oligonucleotides VP60$_{syn}$fw, VP60$_{syn}$rv, ctb-fwl and ctb-rvl (Table 1) were used for the amplification of *vp60*- and *ctb*-specific cDNA.

the two conventional varieties (see **Table 3**, column 4). In particular, crude protein content seemed to be strongly affected.

3.6. Nutritional Value of Different Transgenic Potatoes

As expected from the different nutritional composition, both parental potato varieties Desiree and Albatros as well as VP60-expressing Desiree tubers exhibited significantly different nutritional values ($p < 0.05$) as revealed in a model feeding experiment with rats. When comparing D-niv with A-niv potatoes ingested by rats, a significantly lower nitrogen balance, total tract true crude protein digestibility, net protein utilization and digestibility ratio of total amino acids were observed in A-niv potatoes (see **Table 4**, column 1). Nevertheless, the weight gain of rats that ingested both potatoes did not differ significantly.

In comparison with the A-niv potato, the transgenic potato which expressed the VP60-antigen (A-CTB::VP60) and the NPTII-expressing transgenic potato (A-NPTII) did not exhibit significant differences in almost all parameters of nutritional value when fed to rats. Only the digestibility ratio of total amino acids of A-CTB::VP60 potatoes was significantly reduced (A-niv: 74.4 g/16 g N; A-CTB::VP60: 69.9 g/16 g N). Correspondingly, the extent of differences between the A-niv and the corresponding transgenic potatoes was smaller (<100%) in relation to the variance between both niv-potatoes (see **Table 4**, columns 2 and 3). Similar results were observed for Desiree potatoes. Changes in most nutritional parameters between conventional Desiree and transgenic Desiree potatoes were smaller or equal in relation to the differences between the two parental potatoes (see **Table 4**, column 4), although compositional analysis had revealed distinctive variation.

4. Discussion

Codon adaptation of viral-derived *vp60* open reading frame was essential to detect structural capsid protein VP60 in leaves and tubers of transgenic potato plants. In contrast to previously reported data [24,25], expression of virus-derived sequences in the potato variety Desiree was close to detection limit of VP60-ELISA and not sufficient for further analysis of nutritional parameters. Castanon and coworkers demonstrated a maximal expression of 0.3% TSP in leaves of transgenic Desiree plants by using a *vp60* open reading frame from the Spanish isolate AST/89 [16] under the control of the 35S promoter [24]. This is higher then the detected level in the powdered tuber material (**Table 2**). The published Spanish isolate AST/89 [16] and RHDV-isolate "Eisen-hüttenstadt" used in our work differ only in around 5% of the *vp60* open reading frame. Moreover, the nos-terminator was used [24] instead of the 35S terminator applied in our expression analysis. Apparently, these small differences cause this tremendous difference in the expression of VP60 in potato. However, by using the synthetic *vp60* sequence with codon adaptation to tobacco and the regulatory motif described by Sawant and coworkers [54], we demonstrated a tremendous enhancement of antigenic VP60 molecules in the leaves of transgenic Desiree plants. We assume this higher amount of accumulated viral proteins is due to the better availability of necessary t-RNAs [55] which compensate for the plant's uncommonly high frequency of specific amino acids like valine, alanine, glycine and proline in the amino acid chain of VP60.

In contrast to codon adaptation, the selected tuber-specific GBSS promoter did not increase the expression of VP60 in potato tubers, a result which also differs from that reported for the reporter protein GUS by others [56;57]. We assume that low expression can be caused by the negative effect of CTB::VP60 during first plant regeneration steps. This idea was confirmed by very low regeneration rates—16%, 38% and 48%—obtained in three independent transformations [58]. In concordance, nearly the same reduction in regeneration rates—20%, 43% and 60%—was demonstrated for the expression of VP60 under the control of the GBSS promoter [58]. Strong induction of transgenes harbouring the GBSS promoter by sucrose during first stages of plant regeneration seems to have a higher negative influence compared with constitutive induction by 35S promoter. On the

other hand, different sequences in both untranslated regions (5' and 3' UTR) of the gene, due to cloning procedure, can cause high variation in expression. As shown in many studies, changes in these regulatory regions can dramatically affect the expression of coding regions [59;60]. The genetic fusion of the *ctb* and *vp60* open reading frames under the control of the GBSS promoter did not lead to an accumulation of immunogenic CTB::VP60 molecules in potato plants. However, in tobacco and pea plants, expressed CTB::VP60 could demonstrate a tremendous enhancement of immunogenicity as compared to VP60 [61]. Because of the fact that only a few nanograms of VP60 in the complex of the fusion antigen CTB::VP60 are able to induce immunogenicity in rabbits, we integrated this construct into Albatros, expressed under the GBSS control in compositional analysis and feeding trials.

The objective of compositional analysis and the feeding experiments with rats was to investigate the effect of the integration of different transgenes, *npt*II, *npt*II + *vp60* and *npt*II + *ctbvp60*$_{synSEKDEL}$, on the nutrient composition of the potato tuber, on its nutritional value and on weight gain in rats. Taking into account that conventional breeding can change the nutritional value distinctively and is never trusted to be safe, we related all our data to the measured difference between two conventional potatoes varieties (Albatros and Desiree). These changes were set as 100%.

Both conventional varieties (A-niv and D-niv) varied in nutrient composition (DM, cA, cP, cF, EE, NfE, total amino acids) and in nutritional value (N-balance, TTtPD, NPU, PER, digestibility of amino acids), indicating that normal breeding procedures modify nutrient composition and nutritional value of the potato varieties studied in this experiment. Moreover the weight gain of rats was slightly affected. These effects are probably due to intentional breeding for certain traits but can also be caused unintentionally by somaclonal variation if *in vitro* plant regeneration is used [62]. Analogous results were observed when comparing the nutrient composition and nutritional value of transgenic Albatros potatoes expressing either the *npt*II gene (A-NPTII) or the *ctb-vp60*$_{synSEKDEL}$ + *npt*II genes with its near-isogenic parental wildtype potato (A-niv), but these differences were less distinct. Distinctive changes were found when comparing D-niv with its VP60-expressing counterpart (D-VP60). It was especially notable that crude protein content was considerably elevated in the transgenic tubers. Nevertheless, the nutritional parameters were hardly affected by these strong differences. They were within the variance range of conventional breeding effects (comparison of A-niv vs. D-niv) and in most parameters these effects seemed to be even stronger than

those resulting from genetic modification. The reason for these observed occurrences are difficult to identify. It is known that DNA integration is random and gene disruption, sequence changes or the production of new proteins can occur as a consequence of the recombination event [63]. Whether the mentioned results are due to such interactions or due to somaclonal variation—a reprogramming during plant regeneration [64]—cannot be judged by this study. Nevertheless, it becomes clear that any process involved in genetic modification may possibly lead to compositional and nutritional changes. However, it can be also concluded that conventional breeding methods can do so as well. In our opinion, the reported differences in nutrient composition and nutritional value of the genetically modified potatoes are not drastic in reference to Albatros potatoes. Many differences did not even exceed the variation caused by normal conventional breeding methods. These results are in agreement with findings of Catchpole *et al*. [65], who observed that metabolic changes caused through conventional breeding were at least of a comparable magnitude to those that resulted as an unintended effect of genetic engineering techniques. Genetic modification of Desiree potatoes led to stronger differences in nutrient composition. Nevertheless, parameters of nutritional value were hardly affected and the weight gain of rats that ingested the potatoes was comparable to differences between the conventional varieties. Taking into account that these potatoes are supposed to be applied as food additives and not as main nutrient source, we conclude that the observed changes in the investigated potatoes do not point out to any harmful effect on the animals in short time application. The results of [66] (2007), who demonstrated that blood and serum parameters of rats that ingested the same transgenic potatoes did not show significant differences compared to rats fed the corresponding conventional potato varieties, support our conclusion. Regardless, it becomes evident that changes in nutrient composition and nutritional value may occur and that studies investigating these side effects are essential when introducing genetically modified plants or even new varieties into the food chain.

5. Acknowledgements

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Abbreviations:

ELISA—enzyme-linked immunosorbent assay; TSP—total soluble protein; niv—near-isogenic variety; A-niv—Albatros near-isogenic variety; D-niv—Desiree near-isogenic variety; DM—dry matter; cA—crude ash; cP—crude protein; cF—crude fibre; EE—ether extract; NfE—nitrogen-free extract; TTtPD—total tract crude protein; NPU—net protein utilization; PER—protein efficiency ratio; vs—versus.