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Structure of Two *Solanum bulbocastanum* Polyubiquitin Genes and Expression of Their Promoters in Transgenic Potatoes

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Abstract Two polyubiquitin genes, *bul409* and *bul427*, were isolated from a *Solanum bulbocastanum* BAC library. The *bul409* and *bul427* genes encode hexameric and heptameric polyproteins, respectively. *bul427* exhibits a number of features suggestive of a pseudogene: (1) The last ubiquitin monomer of *bul427* is interrupted by a frame shift mutation. (2) The coding sequence is flanked 3′ by mitochondrial and chloroplast sequences and 5′ by a protein kinase pseudogene. However, characterization of cDNAs amplified using *bul427*-based primers demonstrated that *bul427* is transcriptionally active. Chimeric transgenes encoding β-glucuronidase (GUS) translationally fused to the first ubiquitin-coding units of *bul409, a truncated version 409s,* and *bul427* were constructed and introduced into potato. In transgenic potato lines both *S. bulbocastanum* promoters were weakly transcribed in tubers and efficiently transcribed in leaves. In leaves, *bul409s* was wound-induced, while *bul427* was not. In tubers both promoters were wound-induced. In unwounded leaves and tubers, the steady state mRNA levels from both *bul* promoters were lower than the steady state mRNA levels from the Cauliflower Mosaic Virus *35S* promoter. However, in the leaves and tubers of many of the transgenic lines the GUS activity was significantly higher in the *bul* lines compared to the *35S* lines. The apparent inconsistency of higher enzymatic activity correlated with lower steady state levels of mRNA demonstrates the enhanced protein expression observed with ubiquitin fusion proteins.

Resumen Dos genes de poliubiquitina, *bul409* y *bul427*, fueron aislados de una librería BAC de *Solanum bulbocastanum*. Los genes *bul409* y *bul427* codifican poliproteínas hexámera y heptámera, respectivamente. El *bul427* exhibe varios rasgos que sugieren un seudo gen: (1) El último monómero de *bul427* es interrumpido por una mutación con desplazamiento de la pauta de lectura. (2) La secuencia codificante es flanqueada en 3′ por secuencias mitocondriales y de cloroplastos y en 5′ por un seudogen de proteína quinasa. Sin embargo, la caracterización de cDNAs amplificadas usando iniciadores basados en *bul427* demostró que *bul427* es transcripcionalmente activo. Se construyeron e introdujeron en papa transgenes quiméricos que codifican β-glucuronidasa (GUS) translacionalmente fusionados a las primeras unidades que codifican ubiquitina de *bul409*, una versión truncada de *409s* y *bul427*. En las líneas de papa transgénica de *S. bulbocastanum*, los promotores fueron débilmente transcritos en los tubérculos y eficientemente transcritos en hojas. En hojas el *bul409s* fue inducido por heridas, mientras que *bul427* no fue. En tubérculos, ambos promotores fueron inducidos por heridas. En hojas y tubérculos sin herir, los niveles mRNA en estado de equilibrio para ambos promotores *bul* fueron más bajos que los niveles de mRNA en estado de equilibrio del promotor 35S del virus del Mosaico de la Coliflor. Sin embargo, en hojas y tubérculos de muchas líneas transgénicas, la actividad de GUS fue significativamente más alta en las líneas *bul* en comparación con las 35S. La aparente inconsistencia de una actividad enzimática mayor,

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correlacionada con bajos niveles de mRNA en estado de equilibrio demuestra el incremento de la expresión de proteína observada con proteínas fusión ubiquitina.

Keywords β-glucuronidase · Intron · Leaf · Tuber · Wound-induced

Abbreviations

GUS *E. coli* β-glucuronidase
BAC bacterial artificial chromosome
UTR untranslated region

Introduction

Ubiquitin is a small (76 amino acids), highly conserved protein that serves as recognition signal for protein degradation via the 26S proteasome pathway (Smalle and Vierstra 2004). The ubiquitin gene family contains two types of structures, polyubiquitin and ubiquitin extension protein genes. Polyubiquitin genes contain several tandem repeats of the ubiquitin-coding unit while the extension protein genes contain a single ubiquitin-coding unit fused in frame to the coding region for ribosomal proteins (Ozkaynak et al. 1987). Both types of genes are transcribed as gene fusions and translated as polyproteins that undergo rapid enzymatic hydrolysis to give free ubiquitin or ubiquitin and ribosomal proteins. To date, active polyubiquitin genes isolated from eukaryotes contain conserved architectural features. While these genes contain an intron between the 5′ untranslated region and the ATG start codon of the first ubiquitin monomer, in no cases are the polyprotein coding sequences interrupted by additional introns.

Promoter elements derived from polyubiquitin genes from a number of plant sources have been characterized. Previously described elements include those from *Arabidopsis* (Burke et al. 1988), maize (Christensen and Quail 1996), potato (Garbarino et al. 1995), tobacco (Plesse et al. 2001), rice (Wang et al. 2000), Gladiolus (Joung and Kamo 2006) and sunflower (Binet et al. 1989; Binet et al. 1991). These control elements have been employed in the expression of a wide variety of transgenes in monocots and dicots (Atkinson et al. 2004; Chen et al. 2004; Kang et al. 2006; Panahi et al. 2004; Ramesh et al. 2004; Rommens et al. 2004; Schultheiss et al. 2005; Tiimonen et al. 2005; Yang et al. 2007).

Of particular utility are transgenes in which coding regions are translationally fused to the first ubiquitin monomer (Garbarino et al. 1995; Hondred et al. 1999; Sivamani and Qu 2006). Not only are these translational fusions highly expressed (Sivamani and Qu 2006), they are efficiently processed by ubiquitin-specific proteases releasing the transgene product. In addition, this type of construct does not interfere with appropriate cellular *protein* targeting (Hondred et al. 1999).

Two new polyubiquitin genes from the wild potato species, *Solanum bulbocastanum,* are reported here. The first, *bul427*, is related to the potato *ubi7* gene (Garbarino et al. 1995). The second, *bul409,* has similarity to a tomato polyubiquitin gene (Rollfinke et al. 1998). Control elements from both genes have potential applications in expressing transgenes in potato.

Materials and Methods

Plant Material

Solanum bulbocastanum and potato cv. Lenape (Akeley et al. 1968) were obtained from the Inter-Regional Potato Introduction Station (NRSP-6), Sturgeon Bay, Wisconsin. Plants were maintained in tissue culture or grown in a glasshouse in Albany, CA.

Isolation and Characterization of Polyubiquitin Genes

A *S. bulbocastanum* Bacterial Artificial Chromosome (BAC) library (Song et al. 2000) was probed with random primed (GE Healthcare) coding sequence from the potato polyubiquitin cDNA *ubi9* (Garbarino et al. 1992). To identify unique polyubiquitin genes, DNA prepared (Qiagen) from positive clones was digested (*Bam*HI, *Hin*DIII and *Eco*R1), separated by agarose gel electrophoresis, blotted to charged nylon membranes and hybridized with the polyubiquitin probe described above. Hybridizing bands were subcloned into the plasmid BluescriptII SK(+) (Stratagene) for further characterization. Subcloned restriction fragments were subjected to DNA sequence analysis.

Pustell matrix analysis (Pustell and Kafatos 1982) was used to compare DNA sequences (MacVector8.0). Matrix alignments presented in the figures were downloaded from MacVector. Similar sequences in the available database were identified using the BLAST Network Service of the National Center for Biotechnology Information (Altschul et al. 1990).

cDNA Isolation

Total RNA was prepared from wounded *S. bulbocastanum* tubers as previously described (Verwoerd et al. 1989). Polyadenylated RNA was then isolated using magnetic beads (Promega Z5300 Polytract Isolation System), subjected to reverse transcriptase PCR (BD Biosciences Marathon cDNA Amplification Kit) for first stand synthesis, and amplification using *bul427*-specific primers (forward ATCCCCAAATCA TCATCC and reverse AAAACAACAGAACATAACACA

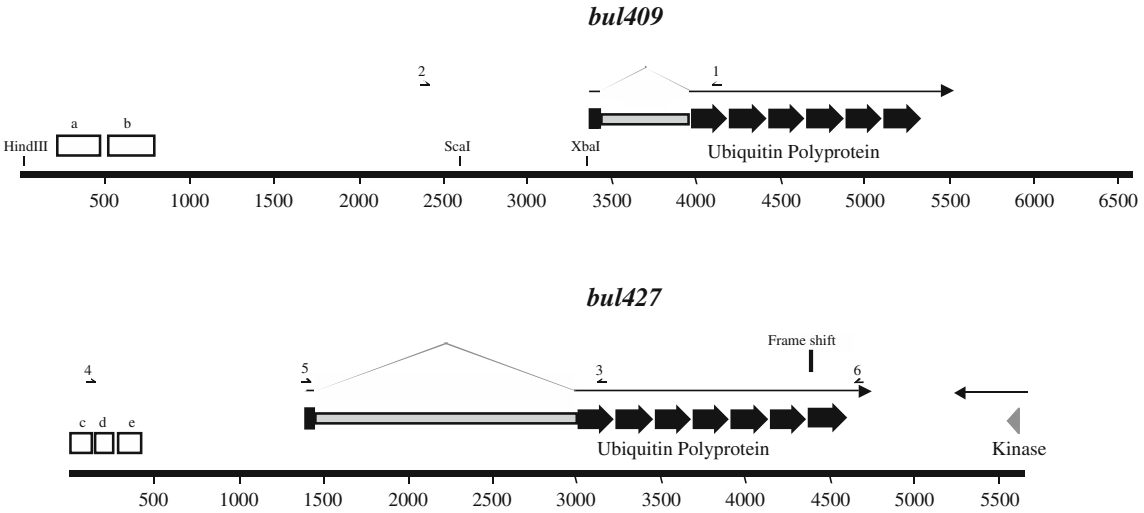

Fig. 1 Structures of *S. bulbocastanum* polyubiquitin genes *bul409* and *bul427*. Sequences encoding ubiquitin monomers (*black*) and S-receptor protein kinase (*gray*) proteins are designated with *large arrows with lines/arrows above* indicating their associated transcripts indicated above. *Shaded boxes* indicate positions of 5′-UTR domains and introns indicated by *hatched boxes*. *Small arrows* indicate the locations of primer pairs for PCR amplification of promoters (1–2, 3–4) or reverse transcriptase PCR amplifications of cDNAs (5–6). Domains *a–e* indicate locations of flanking recombination events described in text: *a* transposon-related sequence; *b* and *c* repeats of unknown origin; *d* chloroplast genome sequences; *e* mitochondrial genome sequences

TTACAAGGTCTCT). The 1.8 kb amplification products *(*Fisher Scientific Taq DNA Polymerase*)* were cloned into the pCR2.1 vector using the TA Cloning Kit (Invitrogen) and subjected to DNA sequence analysis.

Transgene Construction

Three chimeric genes were constructed. The first (*bul409*-GUS) contains the first 4.2 kb of the *bul409* genomic clone

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            D  G  R  T  L  A  D  Y  N  I  Q  K  E  S  T  L  H  L  V  L  R  L  R  G  G  M  Q  I  F  V  K  T    D
bul427      GACGGGAGGACTCTTGCAGACTATAACATCCAGAAGGAGTCAACTCTCCATTTGGTGTTGCGCTTGAGAGGAGGGATGCAGATCTTTGTGAAGACT--GACT 4300
            || |||||||||||||||||||| |||||||||||||||||||||||||||||| |||||||||| |||||||||||||||||||||||||||||||||   |||
LEPUR       GATGGGAGGACTCTTGCAGACTACAACATCCAGAAGGAGTCAACTCTCCATCTGGTGTTGCGTTTGAGAGGAGGGATGCAGATCTTTGTGAAGACTCTTACT 3170
            D  G  R  T  L  A  D  Y  N  I  Q  K  E  S  T  L  H  L  V  L  R  L  R  G  G  M  Q  I  F  V  K  T  L  T

            W  E  D  N  H  I  G  G  G  E  L  *
bul427      GGGAAGACAATCACATTGGAGGTGGAGAGCTCTGATACTATTGACAATGTGAAAGCAAAGATACAGGACAAGGAAGGGATCCCACCAGATCAACAGAGGC 4400
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
LEPUR       GGGAAGACAATCACATTGGAGGTGGAGAGCTCTGATACTATTGACAATGTGAAAGCAAAGATACAGGACAAGGAAGGGATCCCACCAGATCAACAGAGGC 3270
            G  K  T  I  T  L  E  V  E  S  S  D  T  I  D  N  V  K  A  K  I  Q  D  K  E  G  I  P  P  D  Q  Q  R

bul427      TTATCTTTGCTGGTAAGCAGCTTGAGGATGGTCGCACCCTTGCAGACTACAATATCCAGAAAGAGTCTACTCTTCATCTTGTCCTCAGGCTCCGTGGCGG 4500
            | |||||||||||||||||||||||||||||| |||||||||||||||| || |||||||||||||||||||||||||||||||| |||||||||||||||
LEPUR       TCATCTTTGCTGGTAAGCAGCTTGAGGATGGCCGCACCCTTGCAGACTATAACATCCAGAAAGAGTCTACTCTTCATCTTGTCCTGAGGCTCCGTGGCGG 3370
            L  I  F  A  G  K  Q  L  E  D  G  R  T  L  A  D  Y  N  I  Q  K  E  S  T  L  H  L  V  L  R  L  R  G  G

bul427      GGACTTCTGAATGTCCTGTGTGCT-------------------------TTGTTGTTTTATTTCCAGAC-TCAAGTGTTTTTCGTTGTAGTTCTATCTTT 4668
             |||||  |||||||||||||||||                        ||||||||||||||  ||| |  |||||| |||||| |||| ||||||||
LEPUR       TGACTTGTGAATGTCCTGTGTGCTGCTGATTTCATGATGGCTGTGTGCTTTGTTGTTTTTATTTCATGACTTTTAGTGTTCTTCGTTATAGTACTATCTTT 3470
              D  L  *

bul427      CTTTTAAGAGACCTTGTAATGTGTTATGTTCTGTTGTTTTT-GTTGCAACCTAAATAAATAAAGATTAGCCGATAAATGTGTTG---------------C 4747
            |||||||||||||||||||||||||||||||||||||||| | |||||||| |||||||| |||||||||||||||||||               |
LEPUR       CTTTTAAGAGACCTTGTAATGTGTTATGTTCTGTTGTTTTTTGCTGCAACCTTAATAAATAAATATTAGCCGATAAATGTGTTGGTGACTCCTCAGATGC 3570

bul427      ATTGTGAACTTA--ACACACACTCTCACCCTCCCCCCCTTCTCCCCCCCCCCCCCCCCCC 4809
            |||||||  ||||  ||||||  |  ||||||  ||    |   |
LEPUR       ATTGTGAGCTTAGGACACACTCATTCACCCCCCAAACACACGTGTGTTATGGTGGTGGT 3629
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Fig. 2 Alignment of the 3′ end of the *bul427* endogenous gene with the tomato *LEPUR* polyubiquitin gene (GenBank Accession X73156). *Dashes* indicate deletions. Translations of *bul427* and *LEPUR* are indicated. *Underlined sequences* indicate position of the *LEPUR* 3′-UTR

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bul427.4      M  Q  I  F  V  K  T  L  T  G  K  T  I  T  L  E  V  E  S  S  D  T  I  D  N  V  K  A  K  I  Q  D  K
bul427.4     ATGCAAATTTTTGTCAAGACTTTGACTGGGAAGACAATTACATTGGAGGTTGAGAGTTCCGATACCATTGACAACGTCAAAGCAAAGATTCAAGACAAGG 1348
bul427.3     ATGCAAATTTTTGTCAAGACTTTGACTGGGAAGACAATTACATTGGAGGTTGAGAGTTCCGATACCATTGACAACGTCAAAGCAAAGATTCAAGACAAGG 1348
427 gen      ATGCAAATTTTTGTCAAGACTTTGACTGGGAAGACAATTACATTGGAGGTTGAGAGTTCCGATACCATTGACAACGTCAAAGCAAAGATTCAAGACAAGG 4241
427 gen       M  Q  I  F  V  K  T  L  T  G  K  T  I  T  L  E  V  E  S  S  D  T  I  D  N  V  K  A  K  I  Q  D  K

bul427.4      E  G  I  P  P  D  Q  Q  R  L  I  F  A  G  K  Q  L  E  D  G  R  T  L  A  D  Y  N  I  Q  K  E  S  T  L
bul427.4     AGGGTATTCCCCCAGACCAGCAGCGTTTGATATTTGCTGGTAAACAACTTGAGGACGGGAGGACTCTTGCAGACTATAACATCCAGAAGGAGTCAACTCT 1448
bul427.3     AGGGTATTCCCCCAGACCAGCAGCGTTTGATATTTGCTGGTAAACAACTTGAGGACGGGAGGACTCTTGCAGACTATAACATCCAGAAGGAGTCAACTCT 1448
427 gen      AGGGTATTCCCCCAGACCAGCAGCGTTTGATATTTGCTGGTAAACAACTTGAGGACGGGAGGACTCTTGCAGACTATAACATCCAGAAGGAGTCAACTCT 4341
427 gen      E  G  I  P  P  D  Q  Q  R  L  I  F  A  G  K  Q  L  E  D  G  R  T  L  A  D  Y  N  I  Q  K  E  S  T  L

bul427.4       H  L  V  L  R  L  R  G  G  M  Q  I  F  V  K  T  L  T  G  K  T  I  T  L  E  V  E  S  S  D  T  I  D
bul427.4     CCATTTGGTGTTGCGCTTGAGAGGAGGGATGCAGATCTTTGTGAAGACTctGACTGGGAAGACAATCACATTGGAGGTGGAGAGCTCTGATACTATTGAC 1548
bul427.3     CCATTTGGTGTTGCGCTTGAGAGGAGGGATGCAGATCTTTGTGAAGACT--GACTGGGAAGACAATCACATTGGAGGTGGAGAGCTCTGATACTATTGAC 1546
427 gen      CCATTTGGTGTTGCGCTTGAGAGGAGGGATGCAGATCTTTGTGAAGACT--GACTGGGAAGACAATCACATTGGAGGTGGAGAGCTCTGATACTATTGAC 4439
427 gen        H  L  V  L  R  L  R  G  G  M  Q  I  F  V  K  T    D  W  E  D  N  H  I  G  G  G  E  L  *

bul427.4      N  V  K  A  K  I  Q  D  K  E  G  I  P  P  D  Q  Q  R  L  I  F  A  G  K  Q  L  E  D  G  R  T  L  A
bul427.4     AATGTGAAAGCAAAGATACAGGACAAGGAAGGGATCCCACCAGATCAACAGAGGCTTATCTTTGCTGGTAAGCAGCTTGAGGATGGcCGCACCCTTGCAG 1648
bul427.3     AATGTGAAAGCAAAGATACAGGACAAGGAAGGGATCCCACCAGATCAACAGAGGCTTATCTTTGCTGGTAAGCAGCTTGAGGATGGTCGCACCCTTGCAG 1646
427 gen      AATGTGAAAGCAAAGATACAGGACAAGGAAGGGATCCCACCAGATCAACAGAGGCTTATCTTTGCTGGTAAGCAGCTTGAGGATGGTCGCACCCTTGCAG 4539

bul427.4      D  Y  N  I  Q  K  E  S  T  L  H  L  V  L  R  L  R  G  G  D  F  *
bul427.4     ACTACAATATCCAGAAAGAGTCTACTCTTCATCTTGTCCTCAGGCTCCGTGGCGGGGACTTCTGAATGTCCTGTGTGCTTTGTTGTTTTATTTCCAGACT 1748
bul427.3     ACTACAATATCCAGAAAGAGTCTACTCTTCATCTTGTCCTCAGGCTCCGTGGCGGGGACTTCTGAATGTCCTGTGTGCTTTGTTGTTTTATTTCCAGACT 17486
427 gen      ACTACAATATCCAGAAAGAGTCTACTCTTCATCTTGTCCTCAGGCTCCGTGGCGGGGACTTCTGAATGTCCTGTGTGCTTTGTTGTTTTATTTCCAGACT 4639

bul427.4     CAAGTGTTTTTCGTTGTAGTTCTATCTTTCTTTTAAGAGACCTTGTAATGTGTTATGTTCTGTTGTTTT 1817
bul427.3     CAAGTGTTTTTCGTTGTAGTTCTATCTTTCTTTTAAGAGACCTTGTAATGTGTTATGTTCTGTTGTTTT 1815
427 gen      CAAGTGTTTTTCGTTGTAGTTCTATCTTTCTTTTAAGAGACCTTGTAATGTGTTATGTTCTGTTGTTTTTGTTGCAACCTAAATAAATAAAGATTAGCCG 4739
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Fig. 3 Alignment of *bul427* genomic sequence flanking the frame shift mutation with two *S. bulbocastanum* polyubiquitin cDNAs. *Dashes* indicate relative deletions. Translations of the *bul427* genomic DNA and the *427.4* cDNA are indicated. At 4526 on bul427 genomic sequence there is a mismatched base relative to cDNA *427.4* indicated with *lower case letter*. The binding site of the 3′ primer used to amplify the cDNAs is *underlined*

indicated in Fig. 1 including the full-length promoter, intron and first ubiquitin monomer translationally fused to the GUS marker gene. Attempts to generate a full length promoter by PCR failed, potentially due to repeated domains within the intron. This construct was initiated by generating a 1.8 kb PCR product from the *bul409* genomic subclone employing 5′ (CCGGATCCAATATTACTTAAA TATCATGAAATAAACTA) and 3′ (CCGGATCCACCAC CACGTAGACGG) primers as indicated in Fig. 1. Both primers contain 5′ *Bam*HI sites, the location of the *Bam*HI site on the 3′ primer allows construction of GUS translational fusions identical to those employed previously (Garbarino and Belknap 1994; Garbarino et al. 1995). The *bul409*-GUS transgene was constructed by fusing the 3′ end of *bul409* (3.3 kb *Hind*III/*Xba*I, Fig. 1) to the 0.9 kb *Xba*I/*Bam*HI from the above PCR product (containing 5′-UTR, intron, ubiquitin monomer) and the GUS-NOS (*Agrobacterium* nopaline synthase polyadenylation signal) sequence employed previously (Garbarino and Belknap 1994; Garbarino et al. 1995) in BluescriptII SK(+). The resulting transgene with a 3.4 kb promoter, 536 bp intron and ubiquitin monomer fused to GUS-NOS was then mobilized into the binary plant transformation vector pBINPlus/ARS (McCue et al. 2006).

In the second transgene (*bul409s*-GUS, Fig. 4) 2.6 kb at the 5′ end of the *bul409* promoter was deleted by fusing

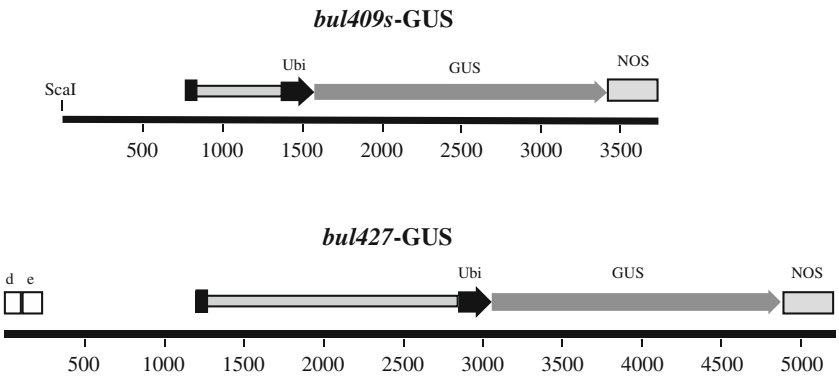

Fig. 4 Structures of promoter-GUS fusion transgenes. Ubiquitin coding sequences, introns, 5′-UTRs and recombination sites are as in Fig. 1. The GUS coding sequence translationally fused to the ubiquitin monomer and the NOS (*Agrobacterium* nopaline synthase) polyadenylation signal are indicated

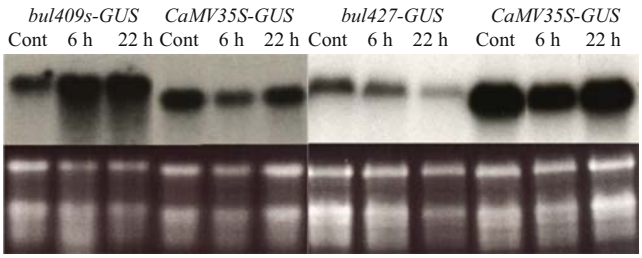

Fig. 5 Wound-regulated GUS mRNA levels in transgenic potato leaves. Total RNA was prepared from leaves, separated by agarose gel electrophoresis and stained with ethidium bromide (*lower panel*). RNA was transferred to a nylon membrane and hybridized to a probe for GUS. Time post injury is indicated and plant lines characterized include *bul409s*-GUS #14 (a high level expressing clone), *bul427*-GUS #1 and *CaMV35S*-GUS #8

unique *Hin*DIII and *Sca*I sites (Fig. 1) in the *bul409*-GUS binary plasmid, resulting in a 771 bp promoter and intron/ubiquitin sequences as in *bul409*-GUS.

The third transgene (*bul427*-GUS) was constructed by first amplifying the a 3 kb promoter fragment from the *bul427* BAC clone using 5′ (GCTTGGTCTTACTTCATCGTC) and 3′ (CCGGATCCTCCACCACGTAGACGAAGG) primers and cloning the product into plasmid pCR2.1. The *bul427* promoter product (1,278 bp promoter, 1555 bp intron and ubiquitin monomer) was used to construct a GUS translational fusion product with NOS terminator as described above (Fig. 4). The transgene was then mobilized into the binary plant transformation vector pBINPlus/ARS.

For relative promoter strength determination, the Cauliflower Mosaic Virus (*CaMV*) *35S*-GUS-*NOS* transgene from pBI121 (Jefferson et al. 1986) was employed.

Transgenes were mobilized into potato cv. Lenape via *Agrobacterium*-mediated transformation (Snyder and Belknap 1993). Potato leaf and tuber tissues were wounded as described (Garbarino and Belknap 1994; Garbarino et al. 1995).

RNA Blots

Total RNA was prepared from control and wounded leaves and tubers as described previously (Garbarino et al. 1995). RNA was fractionated by agarose gel electrophoresis, and transferred to a nylon membrane and hybridized with a random primed (GE Healthcare) double stranded GUS probe.

GUS Assays

Leaf tissues were taken from 15-week-old plants from the greenhouse. Tuber tissue was obtained from freshly harvested mature plants. Individual transgenic lines were sampled in triplicate, and assayed for GUS activity (μkat mg protein^{-1}) as described previously (Garbarino et al. 1995; Jefferson et al. 1986). Standard deviations were determined using Excel (Microsoft) and pairwise multiple comparison of means (Holm–Sidak method) in one-way ANOVA using SigmaStat (Jandel).

Results and Discussion

Structure of *bul409* and *bul427* Endogenous Genes

The coding sequence of a highly expressed potato polyubiquitin cDNA, *ubi9* (Garbarino et al. 1992) was used to probe a BAC library containing genomic *S. bulbocastanum* DNA inserts. Hybridizing BACs were identified and characterized by restriction enzyme analysis. Two BACs displaying distinct ubiquitin-hybridizing restriction band profiles and patterns indicating a single ubiquitin-hybridizing locus were selected for further characterization. Both BACs contained approximately 100 kb inserts (Song et al. 2000) and polyubiquitin-hybridizing restriction fragments from each

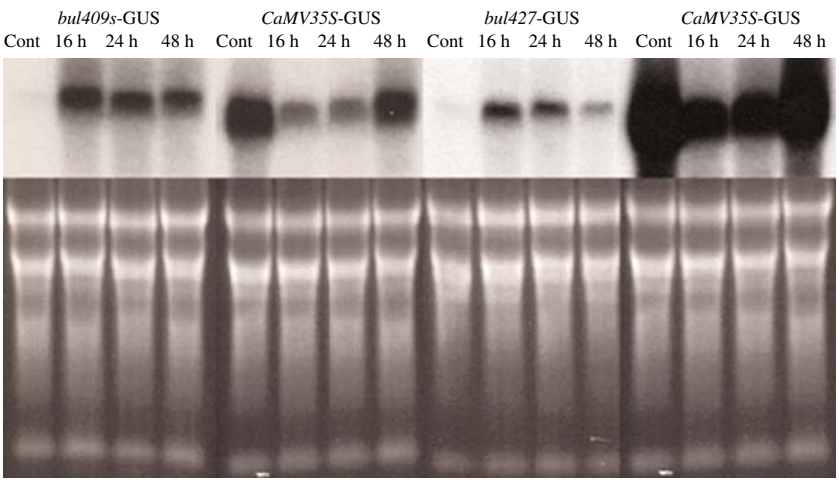

Fig. 6 Wound-regulated GUS mRNA expression in transgenic potato tubers. Total RNA was prepared from tubers, separated by agarose gel electrophoresis and stained with ethidium bromide (*lower panel*). RNA was transferred to a nylon membrane and hybridized to a probe for GUS. Time post injury is indicated and plant lines characterized include *bul409s*-GUS #14 (a high level expressing clone), *bul427*-GUS #1 and *CaMV35S*-GUS #8

were subcloned and subjected to sequence analysis. The structures of the two genes are shown in Fig. 1.

The *bul409* gene appears as a standard hexameric polyubiquitin gene. The 5′-UTR, coding sequence and 3′-UTR of this gene have considerable identity (>95%) to a tomato cDNA (BT012698) and to the potato *ubi7* polyubiquitin cDNA (Garbarino et al. 1992). The *bul409* and *ubi7* promoter regions also share high (~80%) identity (Garbarino et al. 1995). In addition, the *bul409* promoter region shown in Fig. 1 contains two repetitive domains found in *Solanum* BACs. Repeated domain "a" (Fig. 1) represents an apparent terminal inverted repeat flanking transposon found in *S. demissum* accession AC149301 (see related rice transposon; Fujino et al. 2005). The "b" repeated domain in Fig. 1 is of unknown origin and is found in a single *S. demissum* BAC accession (AC149287).

In contrast to *bul409*, the heptaubiquitin *bul427* gene (Fig. 1) has a number of features suggesting it represents a pseudogene. This gene has significant similarity to the tomato heptaubiquitin gene LEPUR locus (accession X73156) (Rollfinke et al. 1998), While the *bul427* promoter domain shares less than 80% identity with the similar *LEPUR* domain, these two genes share 96% identity in the coding sequence. The most striking feature of *bul427* is the presence of a two bp deletion in the seventh ubiquitin monomer, resulting in a frame shift and truncated protein (Fig. 2). Several other features were consistent with *bul427* representing a pseudogene. First, similarity to the *LEPUR* gene ends abruptly following the *LEPUR* polyadenylation site (Fig. 2) at an unusual sequence (the extent of this C-rich domain is unique in the available database) consistent with a rearrangement event. Second, the *bul427* has a cluster of recombination events within 900 bp of the transcription start site (Fig. 1): "c", an unknown element found in a variety of *Solanum* loci (GenBank accessions AC149266, AC149291, and AC144791*)*; domain "d" is 92% identical to sequences 3′ the to chloroplast 16S rRNA (GenBank Accession AM087200); domain "e" has 80% identity to a sequence 5′ to the mitochondrial apocytochrome b gene (GenBank Accession AF095724). Finally, an apparent S-receptor protein kinase pseudogene is located in extremely close proximity (Fig. 1), 600 bp 3′ to the *bul427* 3′ UTR (89% identity to tomato EST GenBank Accession A1896901).

bul427-related cDNAs

In order to determine if *bul427* is expressed, cDNAs related to the gene were amplified by reverse transcriptase PCR using primers indicated in Fig. 1. Two classes of transcripts were amplified; those with complete heptaubiquitin open reading frames and those with the 2bp deletion. Alignment of the 3′ ends of cDNAs from both classes to the *bul427* genomic sequence is shown in Fig. 3. These data are consistent with expression of both *bul427* and closely related polyubiquitin genes that are not interrupted by a deletion.

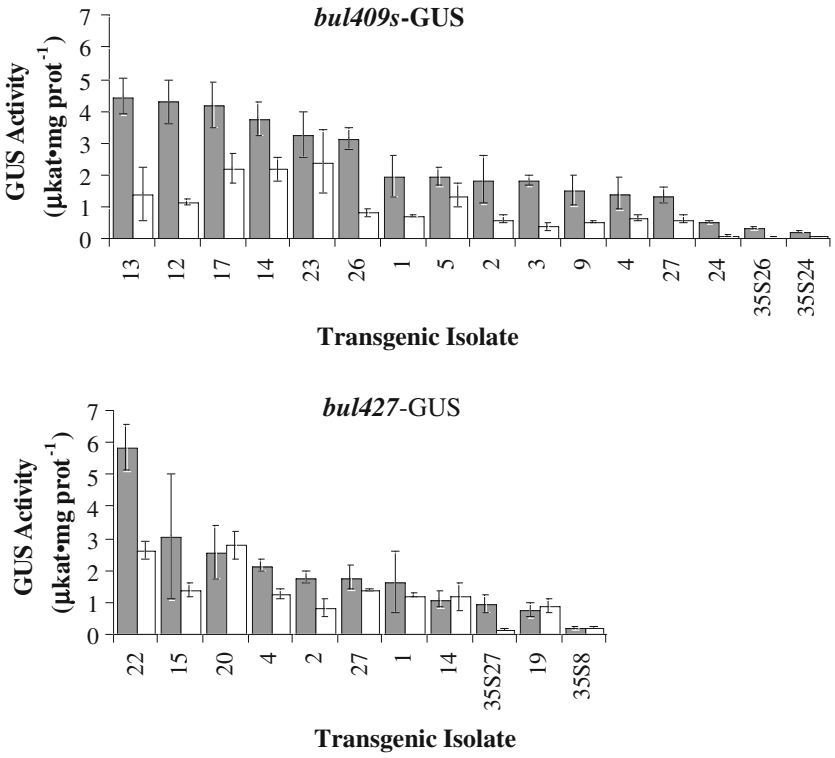

Fig. 7 GUS activity in unwounded leaves (gray) and tubers (white) of individual transgenic potato clones containing *bul409s-* (*top*), *bul427-* (*bottom*), or *CaMV35S*-GUS (*35S#*) transgenes. *Bars* indicate standard deviation

Transgene Expression Profiles

Three chimeric genes were constructed to evaluate the expression profiles of the *bul409* and *bul427* promoters. The transgenes included the introns and the first ubiquitin monomer translationally fused to the marker gene GUS (Jefferson et al. 1987) for optimized expression (Hondred et al. 1999; Sivamani and Qu 2006). The first transgene, *bul409*-GUS, contained the 4.2 kb of the *bul409* genomic clone (Fig. 1) including the full length promoter, intron and first ubiquitin monomer. While this promoter directed efficient wound-induced expression in transgenic leaves and tubers (data not shown), its excessive size limits its applicability. For this reason, a second transgene, *bul409s*-GUS, (Fig. 4) employing 1.6 kb of the *bul409* gene (771 bp promoter, intron and first ubiquitin monomer) was constructed. The third transgene (*bul427*-GUS) was constructed by amplifying a 3 kb promoter/intron/ubiquitin fragment from the *bul427* BAC (Fig. 4).

In unwounded transgenic leaves, the *bul409s* promoter directs expression of GUS mRNA at levels similar to the *CaMV35S* promoter (Kay et al. 1987) (Fig. 5) and expression is increased by wounding. In contrast, levels of expression from the *bul409s* promoter are lower in unwounded tubers and significantly induced by injury (Fig. 6), similar to the *ubi7* profile (Garbarino et al. 1995). The GUS mRNA levels in leaves of plants expressing the *bul427*-GUS transgene were significantly lower than observed with the *CaMV35S* promoter (Fig. 5). While expression of *bul427*-GUS in leaves was not wound-induced (Fig. 5), expression was strongly induced by wounding in tubers (Fig. 6).

GUS Expression

GUS assays were performed on three individual leaves or tubers for a series of transgenic isolates and the data is arranged by descending values for GUS activity in the leaves (Fig. 7). Each set of plant lines were grown, harvested and assayed with two independent *CaMV35S*-GUS lines for comparison (with the exception of *bul409s*-GUS#14 that was included in the *bul427* set). As expected (Hondred et al. 1999; Sivamani and Qu 2006), GUS activity in transgenic plants expressing ubiquitin translational fusions was significantly higher than that of the *CaMV35S* transgenes when compared to the steady state GUS mRNA levels (Figs. 5, 6 and 7). The activity of *bul427*-GUS #1 in leaves was not significantly higher, but resulted from a much lower steady state mRNA level (Fig. 5). In *bul409s*-GUS leaves, the activity of the four highest lines (3, 12, 17 and 14) was significantly greater than the rest ($P \leq 0.001$). The activity of the 4 lowest lines (9, 4, 27 and 24) was not significantly different than both of the *CaMV35S*-GUS lines. In *bul409s*-GUS tubers, the activity of the five highest lines (12, 17, 14, 13 and 5) was significantly different than the *CaMV35S*-GUS lines. In *bul427*-GUS leaves, the activity of only the highest line (22) was significantly different than both *CaMV35S*-GUS lines. In *bul427*-GUS tubers, the activity in all but the two lowest lines (29 and 2) was significantly higher than the *CaMV35S*-GUS lines. In both leaves and tubers, mean levels of GUS activity controlled by both promoters were comparable to, or up to 30-fold greater than observed with the *CaMV35S* promoter.

Conclusions

The *bul427* gene contains structural features common to pseudogenes (Balakirev and Ayala 2003) including a frame shift mutation and numerous potential recombination events proximal to the coding sequence. However, these recombination events have not resulted in the inactivation of gene transcription (Figs. 3 and 7) and the frame shift mutation does not prevent the synthesis of active protein from the mRNA due to the unique duplicate polyprotein nature of polyubiquitin coding sequences. Rather than gene inactivation, the presence of the frame shift mutation in this case suggests a novel mechanism for fine-tuning the levels of accumulated gene product in cells.

The promoter employed in the construction of a transgene largely determines the magnitude and condition/tissue-specificity of its expression. In addition, plant promoter elements capable of directing levels of transgene expression suitable for commercial applications have generally proven difficult to isolate. The promoter elements derived from both the *bul409* and *bul427* polyubiquitin genes described here are capable of directing expression of transgenic protein at levels significantly greater than observed with the *CaMV35S* promoter (Fig. 7). These elements represent useful alternatives to currently employed promoter sequences, with specific utility in construction of transgenic potatoes containing only native Solanaceous DNA (Rommens et al. 2004).

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