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Michigan State University

Information Supporting a Regulatory Status Review of Potato Event Kal91.3 Genetically Modified to Decrease Levels of Invertase Providing Protection from Cold-induced Sweetening

Michigan State University is submitting this information to support a Regulatory Status Review by the USDA Animal and Plant Health Inspection Service under 7 CFR Part 340.4

Submitted on behalf of:
Potato Breeding and Genetics Program
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United States

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No CBI
Michigan State University does not consider any information contained in this document to be confidential business information or to be a trade secret.
December 6, 2023

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Abbreviations and Definitions:

LB: Left Border
MOA: Mechanism of Action
MSU: Michigan State University
NPTII: Neomycin phosphotransferase II
ONT: Oxford Nanopore Technology
PCR: Polymerase Chain Reaction
RB: Right Border
RSR: Regulatory Status Review
T-DNA: Transfer DNA
VINV or Vinv: potato vacuolar invertase gene

1. Confidential Business Information (CBI) Statement

This RSR request does not contain CBI.

2. Product Description and Rationale

2A. Requester's name and contact information

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2B. Description of plant's genus, species

- Order: Solanales
- Family: Solanaceae
- Genus: Solanum
- Species: *Solanum tuberosum* L.

2C. Product Description and Rationale

Vacuolar Invertase silencing is a genetic modification that successfully silences invertase which prevents cold-induced sweetening in potatoes. This modification was demonstrated by Bhaskar, Pudota B., et al. (2010). MSU obtained the vacuolar invertase silencing T-DNA within the plasmid pInvBP1, described in the Bhaskar manuscript from the University of Wisconsin, Madison. The T-DNA was designed to down-regulate the potato vacuolar invertase gene (VInv) transcript through RNAi.

3. Description of Host Potato Variety

The MSU conventionally bred potato variety, Kalkaska, is high-yielding and has valuable traits including scab resistance and tolerance to blackspot bruises (Douches, D.S. et al. 2009). Kalkaska is a round white chip-processing variety with a medium set of uniform tubers. The tubers have a low level of internal defects. The strength of this variety is its resistance to common scab, combined with high yield potential and chip-processing quality across many environments.

4. Description of Modification

4A. Circular map of plasmid vector

A circular map of the plasmid pINVBP1 is represented in Figure 1.

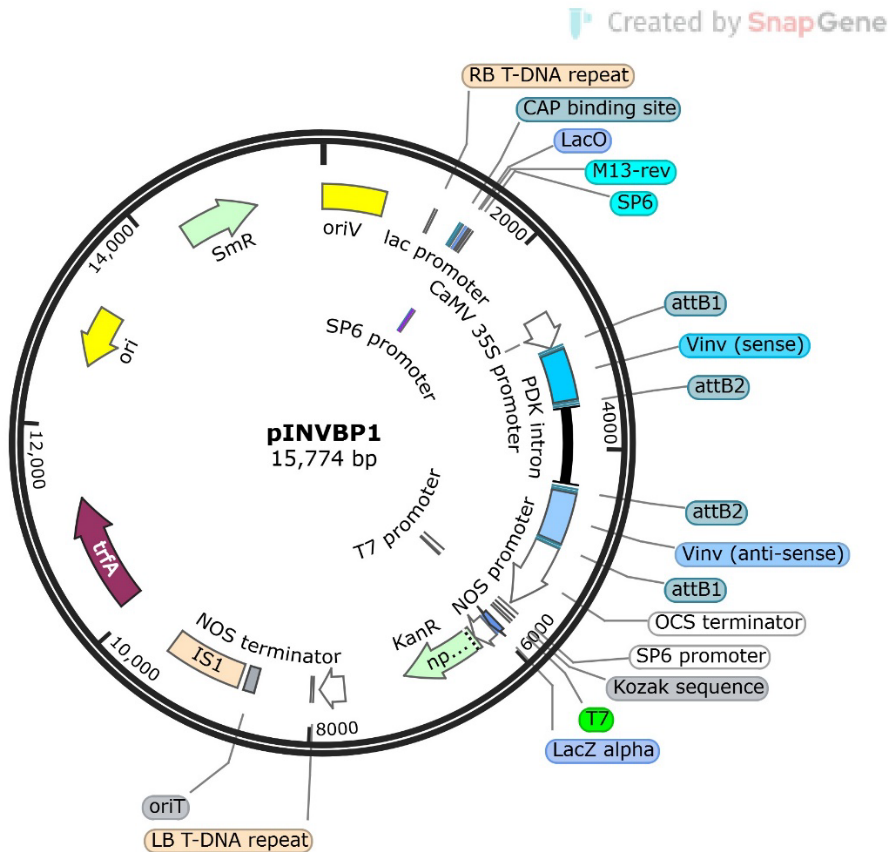


Figure 1. Circular Plasmid Map of pINVBP1.

4B. Description of plasmid vector construction

MSU transformed this Kalkaska variety with a plasmid construct developed at UW-Madison called pInvBP1, which contains 508bp of potato VInv cDNA in the plasmid pHELLSGATE 8 using Agrobacterium-mediated transfer. The 508-bp cDNA fragment (1,310–1,818 bp; construct InvBP1) was amplified using primers 5′CACCGAAAGCTTAAGAGGCGGTGATCC-3′ (forward) and 5′-CTGCTCCATTCACTGCCTTTGTT-3′ (reverse). The amplified PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), gel verified, and cloned into pENTR/D directional TOPO cloning vector (Invitrogen). The VInv cDNA fragments were then transferred into the pHellsGate8 vector using the LR Clonase recombination method (Helliwell et al., 2002). Sequences in the recombinant pHellsGate8-VInv plasmids were confirmed by restriction digestion (*XhaI* and *XbaI*) and sequencing of inserts to ensure that the VInv sequences recombined in sense and antisense orientations. Information on the genetic elements in the plasmid pInvBP1 can be found in Table 1. The Simplot Z6 event, obtained non-regulated status

(Docket No. APHIS–2020–0048), uses a vacuolar invertase silencing cassette containing a tuber-specific promoter called the granule-bound starch synthase (pGbss) gene promoter. Our vacuolar invertase silencing cassette utilizes the commonly used constitutive 35S CaMV promoter.

4C. Sequence of the T-DNA insert

Due to the size of the insert the data has been placed in Section 8. Supplementary Information. Figure 2 on page 13.

4D. Annotation of the T-DNA Inserted Genetic Material

An annotation of the T-DNA of pINVBP1is described in Table 1.

Table 1. Genetic elements of the DNA Insert of pInvBP1. T-DNA Right Border (RB) site to Left Border (LB) highlighted in Blue. pINVBP1 plasmid backbone in grey.

Acronym	Location in pInvBP1	Size (bp)	Direction	Function	Origin
oriV	**1..636**	**636**	==		pHELLSGATE Sequence ID: AJ311874.1
Intervening sequence	**637..1123**	**486**		Used for cloning	pHELLSGATE
RB T-DNA repeat	**1124..1148**	**25**	==	T-DNA right border	*Agrobacterium tumefaciens*
Intervening sequence	**1149...1402**	**254**		Used for cloning	pHELLSGATE
CAP binding site	**1403..1424**	**23**	==	Used for cloning	pHELLSGATE
Intervening sequence	**1425..1438**	**14**		Used for cloning	pHELLSGATE
lac promoter	**1439..1472**	**32**	=>	Used for cloning	pHELLSGATE
Lac operator	**1473..1495**	**24**	==	Used for cloning	pHELLSGATE
Intervening sequence	**1496..1500**	**5**		Used for cloning	pHELLSGATE
M13-rev	**1501..1521**	**21**	=>	Used for cloning	pHELLSGATE
Intervening sequence	**1522..1526**	**5**		Used for cloning	pHELLSGATE
SP6 promoter	**1527..1553**	**27**	=>	Used for cloning	pHELLSGATE

Intervening sequence	**1554..2612**	**1059**			pHELLSGATE
CaMV 35S promoter	**2613..2958**	**347**	=>	35S promoter	Cauliflower mosaic virus
Intervening sequence	**2959..2966**	**7**		Used for cloning	pHELLSGATE
attB1	**2967..2991**	**25**	=>		bacterial attachment site
Intervening sequence	**2992..3007**	**16**		Used for cloning	pHELLSGATE
Vinv (sense)	**3008..3515**	**508**	==	Fragment of the acid invertase (sense orientation)	Generates double-stranded RNA that triggers the degradation of invertase transcripts
Intervening sequence	**3516..3535**	**20**		Used for cloning	pHELLSGATE
attB2	**3536..3560**	**25**	<=		bacterial attachment site
Intervening sequence	**3561..3586**	**26**		Used for cloning	pHELLSGATE
PDK intron	**3587..4355**	**769**	==		Pyruvate orthophosphate dikinase intron from Flaveria trinervia
Intervening sequence	**4356..4397**	**42**		Used for cloning	pHELLSGATE
attB2	**4398..4422**	**25**	=>		bacterial attachment site
Intervening sequence	**4423..4442**	**20**		Used for cloning	pHELLSGATE
Vinv (anti-sense)	**4443..4950**	**508**	==	Fragment of the acid invertase (anti-sense orientation)	Generates double-stranded RNA that triggers the degradation of invertase transcripts
Intervening sequence	**4951..4966**	**16**		Used for cloning	pHELLSGATE
attB1	**4967..4991**	**25**	<=		bacterial attachment site

Intervening sequence	**4992..4999**	**8**		Used for cloning	pHELLSGATE
OCS terminator	**5000..5707**	**708**	=>		pHELLSGATE
Intervening sequence	**5708..5747**	**40**		Used for cloning	pHELLSGATE
SP6 promoter	**5748..5766**	**19**	<=		pHELLSGATE
Intervening sequence	**5767..5794**	**28**		Used for cloning	pHELLSGATE
Kozak sequence	**5795..5804**	**10**	==		pHELLSGATE
Intervening sequence	**5805..5833**	**29**		Used for cloning	pHELLSGATE
T7 promoter	**5834..5860**	**29**	<=		pHELLSGATE
M13-fwd	**5860..5877**	**18**	<=		pHELLSGATE
Intervening sequence	**5878..5947**	**70**		Used for cloning	pHELLSGATE
LacZ alpha	**5948..6016**	**69**	=>		pHELLSGATE
Intervening sequence	**6017..6033**	**17**		Used for cloning	pHELLSGATE
NOS promoter	**6034..6217**	**184**	=>	NOS promoter	*Agrobacterium tumefaciens*
KanR	**6218..7039**	**822**	=>	Kanamycin resistance gene	*Escherichia coli*
Intervening sequence	**7040..7663**	**624**			Cloning vector pHELLSGATE
NOS terminator	**7664..7916**	**253**	=>	NOS terminator	*Agrobacterium tumefaciens*
Intervening sequence	**7917..7978**	**62**		Used for cloning	pHELLSGATE
LB T-DNA repeat	**7979..8003**	**25**	==	T-DNA left border	*Agrobacterium tumefaciens*
Intervening sequence	**8004-8557**	**554**			pHELLSGATE
oriT	**8558..8667**	**110**	==		pHELLSGATE
Intervening sequence	**8668..8726**	**59**			pHELLSGATE
IS1	**8727..9494**	**768**	==		pHELLSGATE
Intervening sequence	**9495..10,118**	**624**			pHELLSGATE
trfA	**10,119..11,267**	**1149**	=>		pHELLSGATE
Intervening sequence	**11,268..12,626**	**1359**			pHELLSGATE
ori	**12,627..13,215**	**589**	<=		pHELLSGATE
Intervening sequence	**13,216..14,311**	**1095**			pHELLSGATE
SmR	**14,312..15,100**	**789**	=>		pHELLSGATE

Intervening sequence	15,101..15,774	673			pHELLSGATE

4E. Description of the Transformation Method

Potato plant events were produced using Agrobacterium transformation as part of the at Michigan State University. The Agrobacterium strain GV3101 carrying pINVBP1, was used to transform potato internode explants following the method described by Douches et al. (1998). Transformed internode explants were regenerated on medium containing 50 mg/l kanamycin to select for lines containing a T-DNA insert.

4F. Molecular Characterization of Kal91.3

T-DNA Insert

A combination of Xdrop™ enrichment technology (Samplix, Denmark) utilizing Nanopore sequencing and followed by Sanger sequencing as well as corroborating studies using droplet digital PCR (ddPCR) showed the presence of two inserts in Kal91.3 following transformation of Kalkaska with pINVBP1Collectively, the data indicated that the two copies of the T-DNA were inserted at separate loci, which were shown to be on chromosome 1 and chromosome 3. One insert has a truncation of 513bp of the left T-DNA region which includes the entire NOS terminator of the NPTII selection marker gene. The second insert also has a truncation of 381bp of the left T-DNA which includes some of the NOS terminator of the *nptII* selection marker gene.

The Kal91.3 T-DNA sequence data was generated by first utilizing Xdrop™ enrichment technology. The enriched DNA was subjected to debranching followed by library generation with the ONT Ligation Sequencing Kit. The libraries were sequenced using GridION (Oxford Nanopore Inc.) sequencing platform to generate long-read sequencing data. Junction-finding scripts using these sequences and the DM potato reference genome (Potato Genome Sequencing Consortium, 2011; Sharma et al., 2013), specifically PGSC *S. tuberosum* group *Phureja* clone DM1-3 pseudomolecules (v4.04), indicated two insertion sites, corroborating T-DNA copy number by ddPCR. Sanger sequencing was performed on PCR products across the junctions including at least 1 kb of flanking DNA near the left border and right border for both of the inserts. These sequences matched the plasmid T-DNA sequence.

Integration Site

There are two T-DNA inserts in Kal91.3. One insert is located in Chromosome 1 at 6820002 and 6820965 with a 963bp chromosomal deletion compared to the PGSC *S. tuberosum* group *Phureja* clone DM1-3 pseudomolecules (v4.04) reference sequence. The insertion does not interrupt or delete any genes. There is a truncation of 513bp of the left T-DNA region which includes the entire NOS terminator of the *nptII* selection marker gene. The second insert is located in chromosome 3 at 55608167 and 55608288 with a 121bp chromosomal deletion. It also does not interrupt or delete any genes when compared with the DM1-3 pseudomolecules (v4.04) reference sequence. There is a truncation of 381bp of the left T-DNA which includes part of the NOS terminator of the NPTII selection marker gene. The NOS terminator defines the end of the

transcription of the *nptII* gene. Since the *nptII* gene is only used in the selection of the event following transformation, its expression does not impact the success of the Kal91.3 event. The NPTII A summary of the Kal91.3 T-DNA inserts is shown in Table 2.

Table 2. A summary of the Kal91.3 T-DNA inserts, T-DNA deletions, location in the T-DNA and impact.

T-DNA Insert in Kal91.3	Deletion of T-DNA	Location in pInvBP1	Function Deleted DNA	Impact of Deleted DNA for Event
T-DNA Insert Chr.1	NOS terminator for the *nptII* gene was deleted	7491-8003 (513bp)	The NOS terminator defines the end of the transcription of the *nptII* gene for Kanamycin resistance.	No impact. NPTII used as a selectable marker during the initial transformation of the event
T-DNA Insert Chr.3	Part of the NOS terminator for the *nptII* gene was deleted	7623-8003 (381bp)	The NOS terminator defines the end of the transcription of the *nptII* gene for Kanamycin resistance.	No impact. NPTII used as a selectable marker during the initial transformation of the event

Absence of Backbone

PCR analysis was used to show the absence of large pINVBP1 plasmid backbone sequences in Kal91.3. This was followed by Southern analysis, using 6 PCR labeled probes that span the entire pINVBP1 backbone region, for the detection of small backbone sequences. The results showed that no backbone from the plasmid was inserted in Kal91.3.

5 Description of New Trait

5A. Intended trait

Invertase silencing:
The potato contains a vacuolar invertase RNAi silencing cassette that successfully down-regulates the invertase which prevents cold-induced sweetening in potatoes.

NPTII:
The potato contains a protein that confers resistance to the antibiotic neomycin and serves as a selectable marker for plant transformation.

5B. Intended phenotype

Invertase silencing:
Potatoes that have successful down-regulation of invertase can be stored in the cold (4ºC) for much longer times than standard 10ºC cold storage durations. Cold-stored potatoes without down-regulation are subject to cold sweetening and French fries and potato chips made from these tubers become brown to black when processed (fry method), taste bitter, and may have elevated levels of acrylamide, a possible carcinogen.

NPTII:
Potatoes are resistant to the antibiotic neomycin and the NPTII protein serves as a selectable marker for plant transformation.

5C. Description of the Mechanism of Action (MOA)

The following describes the mechanism of action of RNAi Invertase silencing.

Down-regulation of invertase slows the conversion of sucrose into fructose and glucose in the vacuole. Baskar et al.(2010) determined that RNAi silencing of the *VInv* gene does not affect potato development. This MOA has been previously reviewed and given non-regulated status (Docket No. APHIS–2020–0048)

The second gene transferred to the potato genome encodes neomycin phosphotransferase II (NPTII). Expression of this protein in plant cells confers resistance to the antibiotics neomycin and kanamycin and serves as a selectable marker for plant transformation. The antibiotics neomycin and kanamycin, bind to the negatively charged backbone of nucleic acids to disrupt protein synthesis and therefore inhibit bacterial cell growth. Neomycin phosphotransferase II catalyzes the addition of phosphate from ATP to the 3'-hydroxyl group of the 4,6-disubstituted aminoglycosides neomycin and kanamycin. The NPTII-mediated phosphorylation of neomycin/kanamycin introduces a phosphate group on the antibiotic that reduces the binding affinity to nucleic acids due to steric hindrances and unfavorable electrostatic interactions and thereby disrupts the mechanism of action of the antibiotic (Wright and Thompson, 1999).

In the absence of neomycin or kanamycin, the NPTII expressed in the potato transformed event is not expected to exhibit any enzymatic activity. Therefore, NPTII is not expected to have any effect on other potato metabolic pathways. Additionally, the food, feed and environmental safety of NPTII has been well established. Neomycin phosphotransferase II has been used as a selectable marker in many different commercial genetically-engineered (GE) crops {e.g. Genuity® DroughtGard™ corn (MON 87460), YieldGard® Rootworm corn (MON 863), Bollgard® cotton (MON 531), Bollgard®II cotton (MON 15985), Roundup Ready® cotton (MON 1445)}, and therefore has a history of safe use in the environment as well as in food and feed uses. Furthermore, the NPTII protein has been fully characterized, and the NPTII protein expressed in GE crops has been shown not to pose any discernable environmental, food, or feed safety concerns (Fuchs et al., 1993a and 1993b; Nap et al., 1992; Flavell et al, 1992).

5D. Metabolism, Physiology, and Development

None of the modifications in Kal91.3 have an impact on the metabolism, physiology, or development of the plant.

6 Proposed plant-trait-MOA language for the website

Plant: *Solanum tuberosum* (potato)
Trait: Invertase gene silencing
Phenotype: Decreased cold-induced sweetening in potatoes.
MOA: Down-regulates the amount of invertase, which slows the conversion of sucrose into fructose and glucose in the vacuole.

7 Literature Cited

Bhaskar, Pudota B., et al. "Suppression of the vacuolar invertase gene prevents cold-induced sweetening in potato." Plant Physiology 154.2 (2010): 939-948. (UW-Madison)

Douches, D.S., Coombs, J., Hammerschmidt, R., Kirk, W.W. and Long, C., 2009. Kalkaska: a round white chip-processing potato variety with common scab resistance. *American Journal of Potato Research*, *86*, pp.347-355.

Douches, D.S., A.L. Westedt, K. A. Zarka, and E.J. Grafius. 1998. Transformation of CryV-Bt transgene combined with natural resistance mechanisms for resistance to tuber moth in potato (*Solanum tuberosum* L.) HortScience 33(6):1053-1056.

Flavell, R.B., Dart, E., Fuchs, R.L. and Fraley, R.T. (1992) Selectable marker genes: Safe for plants? Nature Biotechnology 10: 141-144.

Fuchs, R.L., Ream, J.E., Hammond, B.G., Naylor, M.W., Leimgruber, R.M. and Berberich, S.A., 1993. Safety assessment of the neomycin phosphotransferase II (NPTII) protein. *Bio/technology*, *11*(12), pp.1543-1547.

Nap, J-P., Bijvoet, J. and Stiekema, W.J. (1992) Biosafety of kanamycin-resistant transgenic plants. Transgenic Research 1: 239-249.

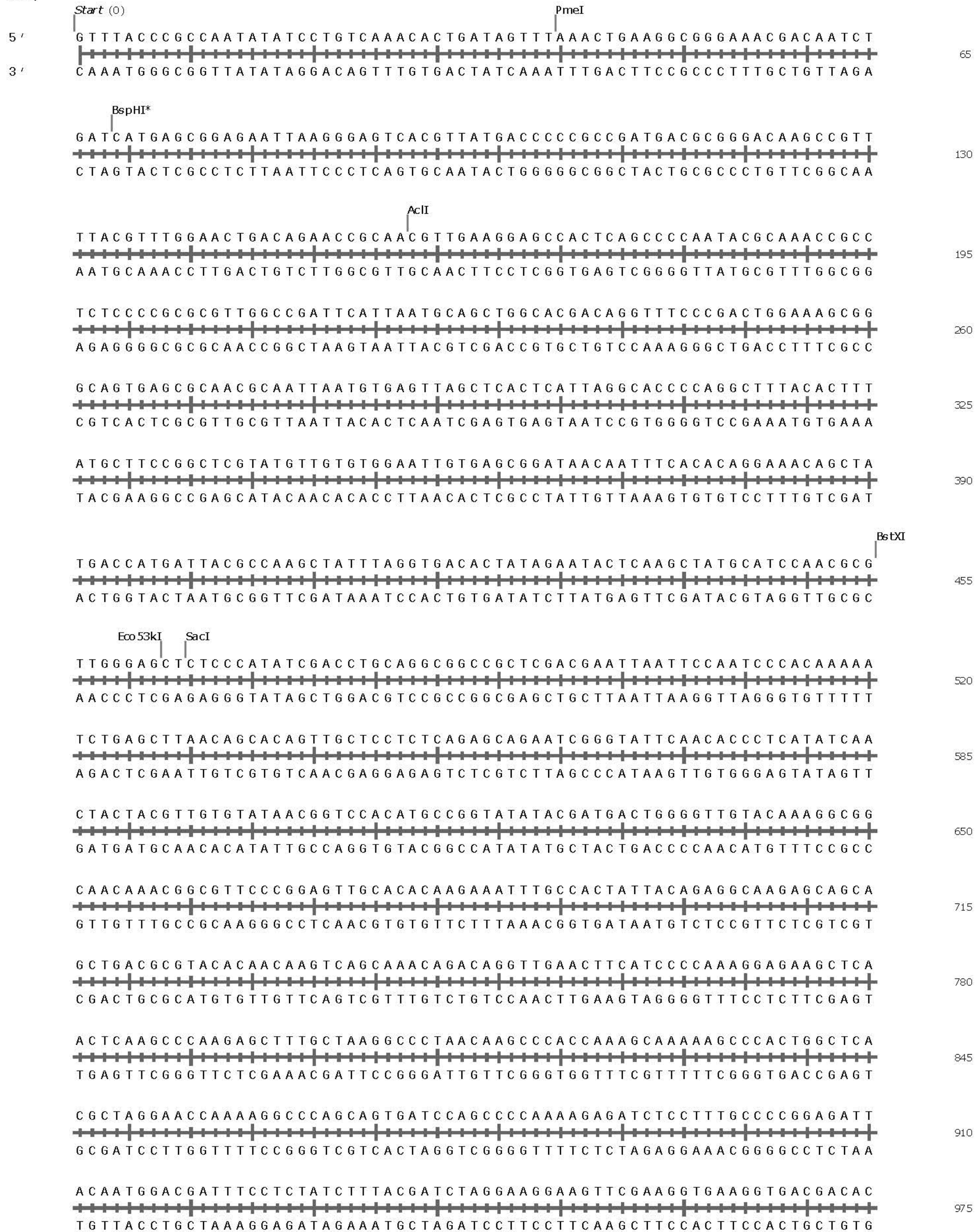
Potato Genome Sequencing Consortium, 2011. Genome sequence and analysis of the tuber crop potato. Nature 475, 189–95. https://doi.org/10.1038/nature10158

Sharma, S.K., Bolser, D., de Boer, J., Sønderkær, M., Amoros, W., 2013. Construction of reference chromosome-scale pseudomolecules for potato: integrating the potato genome with genetic and physical maps. Genes Genomes Genet. 3, 2031–47. https://doi.org/10.1534/g3.113.007153

Wright, G.D, and Thompson, P.R. (1999) Aminoglycoside phosphotransferases: proteins, structure, and mechanism. Front Biosci. 4:1-3.

8. Supplementary Information

Figure 2. Sequence of the pINVBP1 T-DNA insert:



```
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            StuI
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                                    ScaI
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           AccI
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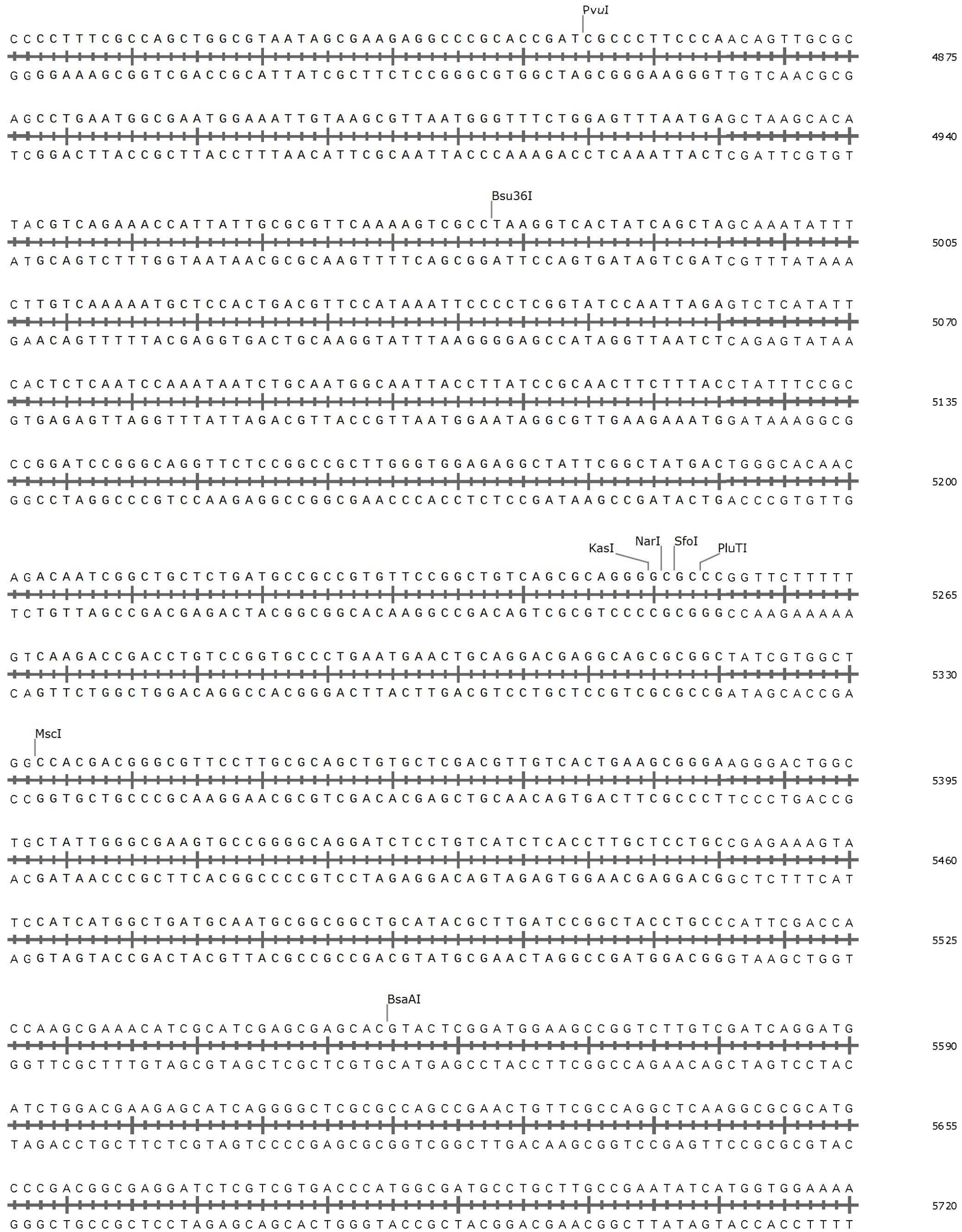
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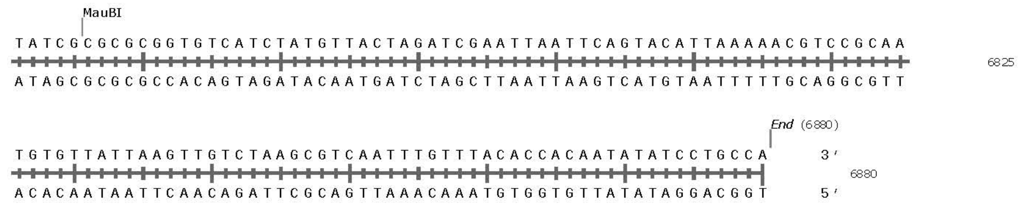
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