RESEARCH ARTICLE

Molecular dissection of an intronic enhancer governing cold-induced expression of the vacuolar invertase gene in potato

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Abstract

Potato (*Solanum tuberosum*) is the third most important food crop in the world. Potato tubers must be stored at cold temperatures to minimize sprouting and losses due to disease. However, cold temperatures strongly induce the expression of the potato vacuolar invertase gene (*VInv*) and cause reducing sugar accumulation. This process, referred to as "cold-induced sweetening", is a major postharvest problem for the potato industry. We discovered that the cold-induced expression of *VInv* is controlled by a 200-bp enhancer, *VInv*In2En, located in its second intron. We identified several DNA motifs in *VInv*In2En that bind transcription factors involved in the plant cold stress response. Mutation of these DNA motifs abolished *VInv*In2En function as a transcriptional enhancer. We developed *VInv*In2En deletion lines in both diploid and tetraploid potato using clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated nuclease 9 (Cas9)-mediated gene editing. *VInv* transcription in cold-stored tubers was significantly reduced in the deletion lines. Interestingly, the *VInv*In2En sequence is highly conserved among distantly related *Solanum* species, including tomato (*Solanum lycopersicum*) and other non-tuber-bearing species. We conclude that the *VInv* gene as well as the *VInv*In2En enhancer have adopted distinct roles in the cold stress response in tubers of tuber-bearing *Solanum* species.

Keywords: enhancer, intron, vacuolar invertase, cold-induced sweetening

Introduction

Potato (*Solanum tuberosum*) is the third most important food crop in the world in terms of human consumption (Devaux et al., 2020). In addition, French fries and potato chips are among the most consumed snacks, especially in developed countries. Unlike the grain crops, storage is one of the most important issues related to the potato industry because tubers must be stored at cold temperatures to prevent sprouting and diseases. Unfortunately, cold storage triggers the breakdown of starch and accumulation of reducing sugars, which is referred to as "cold-induced sweetening" (CIS) (Dale and Bradshaw, 2003), a costly and nagging problem for the potato processing industry (Sowokinos, 2001). The reducing sugars in tubers will react with free amino acids via a nonenzymatic, Maillard-type reaction during high-temperature processing. This reaction results in products with dark color and bitter taste and produces acrylamide, a potential carcinogen (Mottram et al., 2002; Stadler et al., 2002). Reducing sugars are the primary determinants for the acrylamide content in fried potato products (Amrein et al., 2003; Becalski et al., 2004; Zhu et al., 2016). Thus, developing methods to minimize reducing sugars in cold-stored tubers has been an important research focus to reduce acrylamide in fried potato products.

CIS was reported to be associated with numerous genetic loci based on genetic mapping (Menendez et al., 2002; Li et al., 2008; Braun et al., 2017), genome-wide association studies (GWAS) (Byrne et al., 2020), and comparative proteomics studies between CIS-resistant and CIS-susceptible potato cultivars (Fischer et al., 2013). This can be explained by the fact that CIS is likely linked to numerous enzymes that function in central carbohydrate metabolism in potato tubers (Sowokinos, 2001). The vacuolar invertase gene (*VInv*) received a major attention on its potential role in CIS. Partial control of CIS was accomplished by manipulating the activity of the VINV protein (Greiner et al., 1999; Agarwal et al., 2003) or the transcription of the *VInv* gene (Zrenner et al., 1996; Zhang et al., 2008). Silencing of *VInv* using RNAi resulted in nearly full control of CIS in at least some potato cultivars (Bhaskar et al., 2010; Ye et al., 2010). Interestingly, *VInv* gene transcription in tubers is maintained at a minimal level under room temperature. *VInv* is dramatically upregulated during cold storage in CIS-susceptible potato cultivars (Zrenner et al., 1996; Bagnaresi et al., 2008; Bhaskar et al., 2010), causing rapid accumulation of reducing sugars. Silencing of the *VInv* gene has been proven to be an effective approach to control CIS in many potato cultivars (Bhaskar et al., 2010; Ye et al., 2010; Liu et al., 2011; Wu et al., 2011; Clasen et al., 2016; Ly et al., 2023). Concordantly, overexpression of

StInvInh2, which encodes a vacuolar invertase inhibitor, can also reduce potato CIS (Liu et al., 2013; Mckenzie et al., 2013).

Interestingly, the upregulation of *VInv* in cold-stored tubers is not controlled by its promoter (Ou et al., 2013). The *VInv* promoter is required to respond to sugars, indole-3-acetic acid (IAA), and gibberellic acid (GA_3), but not to cold temperatures (Ou et al., 2013). Here we report discovery of a 200-bp transcriptional enhancer, *VInv*In2En, located in the second intron of *VInv*. This enhancer is responsible for the cold-induced expression of the *VInv* gene. We identified several DNA motifs that bind transcription factors (TFs) involved in plant response to cold stress. Mutation of these motifs abolished the function of *VInv*In2En. We developed *VInv*In2En deletion lines in both diploid and tetraploid potato lines using CRISPR/Cas9-mediated genome editing. *VInv* transcription was significantly reduced in the deletion lines during cold storage. Interestingly, the *VInv*In2En sequence was found to be highly conserved among distantly related plant species, revealing an evolutionary trajectory of the *VInv* gene in response to cold stress in the tuber-bearing *Solanum* species.

Results

Discovery of a cold-responsive intronic enhancer within *VInv* gene

Genomic regions containing active *cis*-regulatory elements (CREs), such as promoters and transcriptional enhancers, can be identified as DNase I hypersensitive sites (DHSs) (Zhang et al., 2012; Jiang, 2015; Zhao et al., 2018). We previously developed genome-wide DHS maps in DM1-3 potato using chromatin isolated from tuber tissue (Zeng et al., 2019). DM1-3 is a homozygous diploid clone and was developed from chromosome doubling of a monoploid derived from an *S. tuberosum* Phureja Group clone (Mribu and Veilleux, 1990; Paz and Veilleux, 1999) and has been fully sequenced ($2n = 2x = 24$) (The Potato Genome Sequencing Consortium, 2011; Pham et al., 2020). We detected a 475-bp DHS within the second intron of *VInv* (**Figure 1A**), suggesting that this intron may play a role in regulation of the expression of *VInv*. We have recently demonstrated the enhancer function of several intronic DHSs in Arabidopsis (*Arabidopsis thaliana*) (Meng et al., 2021).

To confirm its *cis*-regulatory function, we cloned the entire *VInv* second intron (1,327 bp) from RH potato, which is a heterozygous diploid clone (van Os et al., 2006) and has recently been fully sequenced (Zhou et al., 2020). RH is susceptible to CIS (**Supplemental Figure S1**).

The intron was cloned into the pKGWFS 7.0 vector containing a minimal 35S promoter (-50 to -2 bp) (m35S) and the β-glucuronidase (GUS) reporter gene (Zhu et al., 2015). Katahdin, a CIS-susceptible tetraploid cultivar (Bhaskar et al., 2010), was used for transformation. We developed 20 transgenic Katahdin lines from the intron construct (*VInv*In2) and 20 lines from a reverse construct (*VInv*In2R) in which the sequence orientation of the cloned intron is reversed. We detected minimal GUS signals in transgenic tubers stored at room temperature (22°C). In contrast, substantially enhanced GUS signals were detected in the transgenic tubers after 4 weeks of cold storage (4°C) (**Figure 1B**). These results indicate that intron 2 of *VInv* contains an enhancer that is responsible for its cold-induced expression.

Dissection of intronic enhancer via reporter gene assays in *A. thaliana*

Since the entire intron 2 from RH potato was used for GUS reporter assays, the precise size and position of the predicted enhancer within intron 2 could not be determined. We attempted to fine-map the enhancer using reporter gene assay in *A. thaliana*. We first examined the GUS signal profiles of transgenic *A. thaliana* plants using the *VInv*In2 and *VInv*In2R constructs. Consistent and strong GUS signals were detected in stems and petioles in transgenic plants derived from both constructs. In addition, relatively weak and sporadic GUS signals were also detected in roots (**Figure 2B**). The *VInv* gene is expressed at relatively high levels in several non-tuber tissues of potato, including both petiole and stem (The Potato Genome Sequencing Consortium, 2011; Zhou et al., 2020). Thus, the GUS signal patterns observed in the transgenic *A. thaliana* plants correspond well with the *VInv* expression patterns in potato tissues.

We next divided the 1327-bp intron 2 into ten DNA fragments (#1 to #10) using five breaks (b1 to b5, **Figure 2A**). Each fragment was ligated to the m35S promoter and cloned into the pKGWFS 7.0 vector. Transgenic plants derived from DNA fragments #1, #2, #9, and #10 showed strong GUS signals in stems and petioles, which were similar to the transgenic plants developed from the *VInv*In2 and *VInv*In2R constructs. Similar but weaker signals were detected from transgenic plants derived from fragment #8 (**Figure 2C**). These results indicated that the enhancer driving *GUS* expression in stems and petioles is located between b2 and b4, which was named fragment #11 (**Figure 2D**).

We next further divided the 600-bp fragment #11 into 13 sub-fragments (#12 to #24, **Figure 2D**) for GUS reporter assays. Transgenic plants derived from construct #21 (200 bp)

showed strong GUS signals in stems and petioles. In contrast, transgenic plants derived from constructs #17 and #19 did not show GUS signals (**Figure 2E**). Thus, the core enhancer in intron 2 was mapped within the 200-bp #21 sequence and was named as *VInv*In2En thereafter. *VInv*In2En spans 678-877 bp in the intron and is located within the 475-bp DHS, which spans 597-1,071 bp in the intron (**Figure 1A**).

To confirm the function of *VInv*In2En in potato, we developed transgenic lines using a *VInv*In2En-m35S-GUS construct in Katahdin potato. We detected minimal GUS signals in tubers stored at room temperature (22°C) but strong GUS signals in cold-stored tubers (4°C) from three independent transgenic lines (**Figure 2F**). Thus, the *VInv*In2En sequence retains the same function as the entire intron 2 in potato (**Figure 1B**).

Identification of DNA motifs related to *VInv*In2En function

We speculated that *VInv*In2En contains DNA motifs bound by TFs involved in plant response to cold stress. We identified putative DNA motifs related to a total of 15 TFs in the intron 2 sequence of RH (Zhou et al., 2020). These motifs were consistently detected by two independent programs using both CIS-BP (Weirauch et al., 2014) and PlantPAN 3.0 (Chow et al., 2019). Interestingly, 10 of these 15 TFs were previously reported to be associated with responses to cold stress in one or multiple plant species (**Figure 3A**), including AT-hook (Dahro et al., 2022), C2H2 ZF (He et al., 2019), MADS-box (Chen et al., 2019), NAC/NAM (Li et al., 2016), bHLH (Xie et al., 2012), CBF/NF-Y (Zhou et al., 2022; Zhang et al., 2023), bZIP (Liu et al., 2018; Li et al., 2022b), B3 (Verma and Bhatia, 2019), TCP (Li et al., 2022a), and GATA (Zhang et al., 2021).

Several TF motifs were enriched in the 200-bp *VInv*In2En, including bHLH and CBF/NF-Y. In addition, motifs related to TCP and GATA were found only in the 200-bp enhancer region (**Figure 3A**). We designed mutated versions of *VInv*In2En to test the function of the DNA motifs related to B3, bHLH, CBF/NF-Y, TCP and GATA. In each construct, the target motif(s) were mutated by replacing1-3 nucleotide(s) within the sequence (**Figure 3B**, **Supplemental Table S1**). Transgenic *A. thaliana* plants using *VInv*In2En with a mutated B3 motif showed similar GUS signal patterns as those from wild type *VInv*In2En. Reduced GUS signals were detected from transgenic plants using *VInv*In2En with two mutated bHLH motifs. In contrast, we did not detect any GUS signals from transgenic plants derived from the three

constructs with mutated motifs related to CBF/NF-Y, TCP, and GATA. Most strikingly, a single nucleotide mutation within the GATA motif resulted in a complete loss of function of the *VInv*In2En enhancer (**Figure 3B**). These results indicated that CBF/NF-Y, TCP and GATA all play important roles for *VInv*In2En driving *GUS* expression in stems and petioles.

To seek additional functional evidence of the three DNA motifs identified in *VInv*In2En, we conducted a yeast one-hybrid (Y1H) assay using triple copies of the *VInv*In2En sequence as a bait (see Materials and Methods). A total of 387 yeast colonies were obtained by screening a cDNA library developed from cold-treated tuber tissues from RH potato. All 387 clones were fully sequenced. The sequences were used for BLAST search in the DM1-3 potato cDNA (v6.1) database using Spud DB blastn program (http://spuddb.uga.edu/blast.shtml). All of the best matched cDNA sequences with an E-value <1e-5 and a minimum sequence identity of 82% were kept for further analyses. A total of 33 unique cDNA sequences were obtained after filtering out repeated cDNA sequences. The candidate proteins related to these 33 cDNAs were used for further validation using point-to-point Y1H assay (see Materials and Methods). Five proteins, including StNF-YC1 and StNF-YC9 (**Figure 4**), were validated as positive interacting proteins binding to *VInv*In2En. The StNF-YC1 and StNF-YC9 proteins share 86% and 73% sequence similarity with AtNF-YC1 and AtNF-YC9 of *A. thaliana*, respectively. These results validated the predicted role of CBF/NF-Y family TFs in regulation of CIS mediated by the *VInv*In2En enhancer.

Genome editing of *VInv*In2En in diploid potato

The *in vivo* function of a predicted enhancer can be validated by mutation or deletion using genome editing (Meng et al., 2021; Zhao et al., 2022; Fang et al., 2023). We attempted to develop *VInv*In2En deletion lines in potato to validate its *in vivo* function. We first conducted CRISPR/Cas experiments using a self-compatible diploid clone DMF5-73-1. This clone was self-pollinated for five generations from a self-compatible diploid hybrid DM1-3 × M6 (Endelman and Jansky, 2016). DMF5-73-1 is amenable to *Agrobacterium*-mediated transformation (Butler et al., 2020). Five sgRNAs flanking *VInv*In2En (1a, 2a, 3a, 1b and 2b) and a single sgRNA (3b) targeting *VInv*In2En (**Figure 5A, Supplemental Table S2**) were designed and assembled into a single construct (**Supplemental Figure S2**). Primary transformants were generated using a hairy root-based procedure to create stable CRISPR/Cas mutants in the first

generation (T0) (Butler et al., 2020). T0 events carrying targeted deletions were self-pollinated and the progeny were screened for homozygous mutations (T1). We identified three homozygous T1 deletion lines (**Figure 5B**). Two lines, 13-1-3 and 13-2-1, were derived from the same hairy root culture. Sequencing analysis showed that deletion line 2-2-8 lost 369 bp, including the entire 200-bp *VInv*In2En. Lines 13-1-3 and 13-2-1 lost 394 bp, including the first 122 bp of *VInv*In2En (**Supplemental Figure S3**), which spans the GATA and the three CBF/NF-Y motifs.

DMF5-73-1 is not susceptible to CIS and expresses a weak CIS phenotype. Tubers harvested from the three deletion lines were stored at 12.8°C for 6 weeks followed by at 6.7°C for nine additional weeks, a storage procedure used to maximize the CIS phenotype. Tuber tissues were then sampled for RNA extraction and RT-qPCR analysis. We found that the expression of *VInv* gene was reduced by 54% for 2-2-8, 45% for 13-1-3, and 41% for 13-2-1, respectively, compared to the wild-type DMF5-73-1 (**Figure 5C**). However, it was challenging to perform chipping analysis from the deletion lines because all three lines have small tubers and are associated with the "jelly end" defect derived from the parental clone DM1-3 (Endelman and Jansky, 2016) (**Figure 5D**). Nevertheless, potato chips processed from cold-stored tubers of line 13-1-3 showed a lighter color compared to those processed from wild-type DMF5-73-1 (**Figure 5D**).

Genome editing of *VInv*In2En in tetraploid potato

DMF5-73-1 is ideal for CRISPR/Cas experiments due to its self-compatibility that allows for identification of homozygous deletions. However, DMF5-73-1 is not an ideal line to accurately evaluate the impact of *VInv*In2En on CIS since it is resistant to CIS and has poor tuber traits. In addition, DMF5-73-1 retains a significant level of heterozygosity. Hence, the homozygous deletion lines developed from this clone are phenotypically different from the parental DMF5-73-1 (**Figure 5D**). We next attempted to conduct CRISPR/Cas experiments in Katahdin, a tetraploid potato cultivar that is highly susceptible to CIS (Bhaskar et al., 2010). We first amplified and sequenced the intron 2 of *VInv* from Katahdin. We identified three haplotypes: A (2 copies), B, and C. These haplotypes are differentiated by SNPs and small indels, including those within the *VInv*In2En region (**Supplemental Figure S4**). We designed four sgRNAs, including R1 outside of *VInv*In2En, and R2, R3, and R4 inside the *VInv*In2En boundary (**Figure**

5A, Supplemental Figure S4, Supplemental Table S2). The four sgRNAs were assembled into a single construct for CRISPR/Cas experiments (**Supplemental Figure S5**).

We identified three different T0 CRISPR/Cas9 lines, KV78, KV87, and KV108. PCR amplifications using primers *VInv*-Edit-F/R that span the four sgRNAs (**Supplemental Figure S4**, **Supplemental Table S3**) produced additional smaller bands as well as the wild-type band (**Figure 5E**), suggesting that all three T0 lines contain both intact and deleted intron 2, possibly derived from different lineages of cells. We then isolated and mixed all DNA fragments visible on the agarose gel, including the wild-type band, from all three lines. The mixed DNA fragments were cloned and a minimum of 60 randomly selected clones from each line were fully sequenced. Sequence analysis confirmed that each of the three T0 lines contained different types of deletions within *VInv*In2En, ranging from 3 to 124 bp deletions within *VInv*In2En associated with haplotype A, and 1 to 15 bp deletions within *VInv*In2En associated with haplotype B (**Supplemental Figure S6**). However, no deletions were detected in *VInv*In2En associated with haplotype C, probably due to the SNPs located in the PAM sequences downstream of sgRNAs R2 and R3. Based on the number of individual sequences related to *VInv*In2En, 67.7% of the haplotype A sequences from KV78 contained a deletion ranging from 4 to 97 bp; 64.7% of the haplotype A sequences from KV87 contained a deletion of 3 to 64 bp; 59.3% of the haplotype A sequences from KV108 contained a 4 to 124 bp deletion (**Supplemental Figure S6**).

We amplified the cDNAs of *VInv* from the three CRISPR/Cas lines using primers Splicing-F/R spanning exons 1 to 3 (**Supplemental Table S3**). Sequencing of the PCR products showed that the transcripts from the three CRISPR/Cas lines were identical to those from wild-type Katahdin (**Figure 5F**). Thus, the deletions occurred in *VInv*In2En did not affect the splicing of the *VInv* gene. We next analyzed the expression of the *VInv* gene in the three CRISPR/Cas lines using RNAs isolated from tubers stored for two weeks under 22°C and 4°C, respectively. A similar and minimal level of *VInv* expression was observed in 22°C-stored tubers from wild type Katahdin and all three CRISPR/Case lines. In contrast, the expression level of *VInv* in 4°C-stored tubers of the three CRISPR/Cas lines was only 6.6%, 16.4%, and 27.3%, respectively, of the wild-type Katahdin (**Figure 5G, Supplemental Table S4**). Potato chipping was performed using tubers stored under 22°C and 4°C, respectively. Potato chips processed from tubers stored under 22°C showed a similar color from all three lines as well as wild type Katahdin (**Supplemental**

Figure S7). After the 4 weeks of storage of the tubers under 4°C, chips from KV78, KV87, and KV108 all showed a lighter color than those from Katahdin (**Supplemental Figure S7**).

Collectively, these results showed that although the deletions associated with *VInv*In2En of Katahdin are in heterozygous and mosaic conditions in the three T0 CRISPR/Cas lines, the deletions resulted in a significant reduction of *VInv* expression under cold storage condition, confirming the cold-responsive function of *VInv*In2En in Katahdin.

Evolution of *VInv* gene and *VInv*In2En enhancer

We computationally extracted the DNA sequence of *VInv* gene from a total of 28 sequenced Solanaceous species. Sequences from several distantly related species, including *A. thaliana*, cucumber (*Cucumis sativus*) and soybean (*Glycine max*), were used as outgroups in evolutionary analysis. The VINV protein of potato shared 92-99% sequence similarity with those from tomato and wild *Solanum* species (**Supplemental Figure S8**). In addition, the structure of the *VInv* genes is also highly conserved among different species (**Figure 6**). The distinct small exon 2 (9 bp) was detected in all Solanaceous species, as well as in several distantly related plant species. In addition, a large intron 2 was identified following the small exon 2 in all species (**Figure 6**), with sizes ranging from 780 bp to 2,997 bp (**Supplemental Table S5**).

The 1,327-bp intron 2 sequence from RH (Zhou et al., 2020) was used to align the intron 2 sequences from other Solanaceous species. Homologous sequences were detected in the same intron of the *VInv* gene from all *Solanum* species, as well as from several distantly related species, including eggplant (*Solanum melongena*) and pepper (*Capsicum annuum*) (**Supplemental Table S5**). We next aligned the 200-bp *VInv*In2En sequence from RH to the intron 2 sequences from different species. Surprisingly, the *VInv*In2En sequences were more conserved than the intron 2 sequences among the species analyzed (**Supplemental Table S5**). Furthermore, the DNA motifs related to CBF/NF-Y, TCP and GATA were detected in the *VInv*In2En sequences from distantly related *Solanum* species (**Supplemental Table S5**). Therefore, *VInv*In2En represents a conserved enhancer sequence in *Solanum* species.

We extracted the *VInv*In2En sequence from several different potato genotypes to further exploit its sequence polymorphism (**Supplemental Figure S9**), including diploid potato clones M6 (Jansky et al., 2014) and H28-7 (Bhaskar et al., 2010), which are resistant to CIS. SNPs and small indels were observed throughout the *VInv*In2En sequence in comparison between CIS

resistant (H28-7 and M6) and CIS susceptible (RH) genotypes, including SNPs located in the CBF/NF-Y, GATA and TCP motifs (**Supplemental Figure S9**). Thus, sequence polymorphism of *VInv*In2En may contribute to the level of CIS resistance of different potato genotypes.

Discussion

Invertases hydrolyze sucrose into glucose and fructose, thereby playing important roles in metabolism and development in plants (Ruan et al., 2010). Different plant invertases have been found to be specific to the cell wall, vacuole, or cytosol, respectively. Both cell wall and vacuolar invertases are also known to contribute to defense responses to abiotic and biotic stresses (Wan et al., 2018). Vacuolar invertases play essential roles in cell expansion and sugar accumulation, which are related to plant growth and development (Ruan et al., 2010; Wan et al., 2018). Therefore, silencing of the vacuolar invertase gene can cause major developmental defects in plants. For example, silencing of the vacuolar invertase gene in tomato (*Solanum lycopersicum*) resulted in substantially smaller fruits (Klann et al., 1996). Major developmental defects were also reported in silencing of the vacuolar invertase gene in several other species, including carrot (*Daucus carota*) (Tang et al., 1999), muskmelon (*Cucumis melo*) (Yu et al., 2008), cotton (*Gossypium hirsutum*) (Wang et al., 2014; Wang and Ruan, 2016), and rice (*Oryza sativa*) (Lee et al., 2019; Deng et al., 2020).

VInv (*Pain-1*) is the only vacuolar invertase gene identified in the potato genome (Bhaskar et al., 2010; Draffehn et al., 2010). Interestingly, silencing of the *VInv* gene by RNAi in potato did not cause unambiguous defects in growth and development (Bhaskar et al., 2010). The potato RNAi lines did not show yield loss in field-based yield trials (Bhaskar et al., 2010). These results suggest that the *VInv* gene may not play a similar developmental role in potato as compared to other plant species. Although *VInv* is expressed in non-tuber tissues, the expression of *VInv* is not upregulated by cold stress in several non-tuber tissues, including petiole, stem, and root (X.B. Zhu, unpublished data). Similarly, the GUS signals in the transgenic *A. thaliana* plants derived from *VInv*In2 and *VInv*In2En constructs were not enhanced by cold stress. We hypothesize that the *VInv* gene has adapted for a distinct role in the tuber-bearing species in response to cold stress. A high level of *VInv* expression at cold temperatures would generate more sugars in tuber cells, which in turn would affect the osmotic pressure and increase the freezing tolerance of tuber cells that contain a high percentage of water.

The *VInv*In2En sequence is conserved among distantly related *Solanum* species, including tomato and several other non-tuber-bearing species (**Supplemental Table S5, Supplemental Figure S9**). Thus, *VInv*In2En emerged before the divergence between tuber-bearing and non-tuber-bearing species. We speculate that *VInv*In2En contains unidentified sequence motif(s) that are responsible for its tuber-specific function. We previously showed that the CIS-resistant diploid potato germplasm line H28-7 exhibits a very low level of *VInv* expression in cold-stored tubers (Bhaskar et al., 2010). Interestingly, we detected a SNP in each of the two CBF/NF-Y motifs in *VInv*In2E between H28-7 and RH (**Supplemental Figure S9**). These results suggest that variation of the *VInv*In2En sequence is likely the key factor for the resistance of the CIS-resistant germplasm. By contrast, an identical *VInv*In2En sequence was observed in DM1-3 and RH potatoes (**Supplemental Figure S9**), which have different levels of resistance to CIS. Thus, the *VInv*-mediated cold tolerance is likely associated with additional factors depending on species or genotypes within a species. This hypothesis is supported by previous reports demonstrating an invertase inhibitor, StInvInh2, which specifically suppresses the activity of the VINV protein (Liu et al., 2010; Brummell et al., 2011). A combination of *VInv*In2En-mediated cold-induced expression of *VInv* and post-transcriptional regulation of VINV protein provide a multilayer of defense system for potato to adapt to different environments and/or stress conditions.

Several TFs, including CBF/NF-Y, TCP, and GATA, may play a role in *VInv*In2En-mediated regulation of *VInv* under cold conditions, since mutations of the predicted binding sites of these TFs abolished the function of *VInv*In2En as a transcriptional enhancer in *A. thaliana* (**Figure 3**). CBF/NF-Y, TCP, and GATA are large TF families in plants and include 41, 31, and 49 genes, respectively, in the potato genome (Wang et al., 2019; Li et al., 2021; Yu et al., 2022). Although there are no reports yet on cold response associated with these TFs in potato, specific members from the CBF/NF-Y, TCP, and GATA families have been documented for playing a role in cold temperature response in other plant species. For example, a GATA-family TF in rice, OsGATA16, was induced by cold treatment, and can improve cold tolerance by repressing some cold-related genes (Zhang et al., 2021). A TCP1 TF in *Chrysanthemum morifolium*, DgTCP1, was induced by cold temperature and can regulate peroxidase activity and reduce ROS accumulation (Li et al., 2022a). It is interesting to note the presence of three CBF/NF-Y binding sites in close vicinity within *VInv*In2En. The NF-Y TFs have been documented to confer

response to various types of abiotic stresses, including drought, salt, nutrient and temperature (Zhang et al., 2023). Thus, it will be essential to validate the functions of these TF-binding sites in potato and to identify a specific member(s) from these TF families that are responsible for the function of *VInv*In2En.

Materials and Methods

Enhancer validation using transgenic assays in potato

An intronic DHS within intron 2 of *VInv* gene was identified from the DHS data published previously (Zeng et al., 2019). The entire intron 2 from the *VInv* gene of RH potato (*Solanum tuberosum*) was used for enhancer validation using a GUS reporter system (Zhu et al., 2015). The forward (*VInv*In2) and reverse (*VInv*In2R) sequences of intron 2 were amplified from genomic DNA of RH potato using PCR with primers VIT-F6/R6 and VIT-F8/R8 (**Supplemental Table S3**), respectively, and were ligated to a minimal 35S promoter (-50 to -2 bp) (m35S) through the *Eco*RI cloning site. The ligated PCR products were cloned into the pENTR/D directional TOPO cloning vector (Invitrogen) and then transferred into the pKGWFS 7.0 vector containing the GUS reporter using the LR Clonase recombination method (Zhu et al., 2015). Constructs were transferred into *Agrobacterium tumefaciens* strain GV3101 (pMP90), followed by transformation to potato variety Katahdin using methods described previously (Bhaskar et al., 2008; Bhaskar et al., 2010).

Transgenic Katahdin lines derived from the forward or reverse construct were obtained and screened using PCR with the kanamycin gene-specific primers Kan-F/R and the construct-specific primers (**Supplemental Table S3**). All transgenic lines with three replicates for each lin were grown in greenhouses using photoperiod of 16-h daylight at 22°C and 8-h darkness at 16°C (50%-70% humidity), and light intensity of 500 μmol m^{-2} s^{-1} (natural light combined with light of high-pressure sodium lamps) until leaves became senesced naturally. Tubers harvested from each line were divided into two groups: stored in dark at 22°C (50%-70% humidity) or 4°C (60%-70% humidity) for 4 weeks, respectively. Tuber slices prepared by slicing longitudinal sections 2-mm thick from the center of individual tubers were examined for GUS activity. Tuber slices were placed in a plastic plate (70 x 15 mm) and soaked in GUS-staining solution (100 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1% [v/v] Triton X-100, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and 0.05% [w/v] X-Gluc), with vacuum infiltration for 30 min and

incubation in dark at 37°C overnight. Tuber slices were washed in 80% (v/v) ethanol several times. Images of tuber slices were captured using an EPSON Perfection 4180 scanner.

Enhancer dissection using transgenic assays in *A. thaliana*

Seeds of Arabidopsis (*Arabidopsis thaliana*) accession Col-0 were germinated in one-half-strength Murashige and Skoog (0.5 x MS) medium, and the seedlings were transplanted in potting soil and grown in plant growth chambers with 16/8 h light/dark cycles at 23°C and light intensity of 150 μmol m^{-2} s^{-1} (white fluorescent lamps) until flowering. The *VInv*In2 and *VInv*In2R constructs were initially used to transform *A. thaliana* accession Col-0 using the floral dip method (Clough and Bent, 1998). Transgenic seedlings were screened on solid 0.5 x MS medium containing kanamycin (50 μg mL^{-1}) and were grown in an illumination incubator with the same light-dark condition described above and were examined for GUS activity according to published protocols (Zhu et al., 2015).

To map the position of the enhancer within the intron 2 of *VInv*, we divided the intron 2 into ten DNA fragments (#1 to #10) using five breaks (b1 to b5) for transgenic assays. The stem/petiole-specific enhancer (within DNA fragment #11) was further divided into 14 (#11 to #24) sub-fragments. All target DNA fragments together with the m35S were synthesized from GenScript Inc. and cloned into the pKGWFS 7.0 vector containing the m35S and the *GUS* reporter gene (Zhu et al., 2015). Images of transgenic *A. thaliana* seedlings were captured using the EPSON Perfection 4180 scanner to record the GUS signals.

Analysis of TF-binding motifs

TF-binding motifs and their corresponding TFs within intron 2 of *VInv* were identified using two independent programs of CIS-BP (Weirauch et al., 2014) and PlantPAN 3.0 (Chow et al., 2019) with default parameters. DNA motifs consistently detected by both programs were used for further analysis. Motifs reported to be associated with cold response in one or multiple plant species were mapped to the intron 2 of *VInv* using TBtools (Chen et al., 2020).

Development of CRISPR/Cas deletion lines

A self-compatible diploid potato clone DMF5-73-1 was developed from a cross between *S. tuberosum* Gp. Phureja DM 1-3 516 R44 (DM1-3) and *Solanum chacoense* (M6) (Endelman

and Jansky, 2016) and has been self-pollinated for five generations. Wild type (WT) and CRISPR/Cas lines were propagated *in vitro* on Murashige and Skoog (MS) medium (MS basal salts plus vitamins, 3% sucrose, 0.7% plant agar, pH 5.8) (Murashige and Skoog, 1962). *In vitro* plants were maintained in growth chambers with 16-h-light/8-h-dark photoperiod at 22°C and average light intensity of 200 μmol m^{-2} s^{-1} (white fluorescent lamps) *Pro*.

The Csy4-based CRISPR/Cas9 system (Cermak et al., 2017) was used to develop *VInv*In2En deletion lines in DMF5-73-1. In brief, five sgRNAs flanking *VInv*In2En (1a, 2a, 3a, 1b and 2b) and a single sgRNA (3b) targeting *VInv*In2En (**Supplemental Table S2**) were designed using program of CRISPR-P v2.0 (Liu et al., 2017). The six gRNAs were linked by Csy4 binding sites and then cloned into the Csy4 multiplexing vector (**Supplemental Figure S3**) based on published methods (Cermak et al., 2017). The construct was delivered into *A. tumefaciens* GV3101 (pMP90) and was used to conduct hairy root-based *Agrobacterium* transformation (Butler et al., 2020). T0 CRISPR/Cas lines showing the expected smaller PCR products were further confirmed by Sanger sequencing using *VInv*-mut-F1/R1 primers (**Supplemental Table S3**). Several T0 lines with large deletion of *VInv*In2En were grown under greenhouse conditions as described above, followed by subsequent self-pollination to obtain homozygous T1 deletion lines.

A tetraploid potato cultivar Katahdin was used to develop deletion lines using the U3/U6-based CRISPR/Cas9 system (Hu et al., 2019). Four sgRNAs, including R1 outside of *VInv*In2En, and R2, R3, and R4 inside the *VInv*In2En (**Supplemental Table S2**), were designed using CRISPR-P v2.0 (Liu et al., 2017). The sgRNAs were assembled into four expression cassettes (*ProAtU3b:gRNA1*, *ProAtU3d:gRNA2*, *ProAtU6-29:gRNA3*, and *ProAtU6-29:gRNA4*), which were cloned into the pHNCas9 vector by using the Golden Gate cloning strategy (Ma et al., 2015; Xie et al., 2015; Ma et al., 2016; Hu et al., 2019). The construct pHNCas9::*VInv*In2En was introduced into *A. tumefaciens* GV3101 (pMP90) and was used to transform Katahdin according to published protocols (Bhaskar et al., 2008). Positive transformants were screened using PCR with primers *Kan*-F3/R3, *Cas*-F1/R1, and *VInv*-Edit-F/R (**Supplemental Table S3**). Transgenic lines containing additional smaller bands (2% agarose gel) were further confirmed by Sanger sequencing. PCR products were purified by using QIAquick PCR Purification Kit (Qiagen) and were cloned into *Escherichia coli* using pMD^{TM}19-T vector (TaKaRa). A minimum of 60 randomly selected positive colonies derived from each deletion line were fully sequenced.

Statistical analysis of different types of deletions was conducted on each of the Katahdin CRISPR/Cas deletion lines containing three haplotypes, A (2 copies), B, and C.

Greenhouse trials, tuber sample preparation, and chipping analysis

Each of 10 seed tubers of RH potato was planted in potting soil under normal greenhouse conditions as described above. Standard cultivation and management practices were followed throughout the growing period. Tubers were harvested 120 days after seedling emergence when leaves senesced naturally. Tubers harvested from two pots were combined together as one biological replicate. Tubers of five biological replicates were stored in dark at 22°C (50%-70% humidity) for 10 days and then divided into two groups. Each group was stored in dark at 22°C (50%-70% humidity) or 4°C (60%-70% humidity) for 0, 2, 4, 8, and 16 weeks, respectively.

Three T0 CRISPR/Cas deletion lines (three plants for each line) developed from Katahdin were grown under normal greenhouse conditions as described above. Tubers harvested from the same line were combined together and stored under dark at 22°C for 10 days, and then divided into two groups for 22°C (50%-70% humidity) or 4°C (60%-70% humidity) treatments, and each group of tubers with three replicates were treated for 2 and 4 weeks, respectively.

Tuber samples of 1.5-mm thick slices (1-3 slices for each tuber) prepared from apical to basal end of the tuber were taken for chipping analysis. The remaining tuber samples were frozen in liquid nitrogen and used for analysis of *VInv* expression. Tuber slices were fried in cottonseed oil at 191°C for 2 min or until the cessation of bubbles. Chip color of cold-stored tubers is compared to that of the corresponding controls.

***VInv* transcription and splicing assays**

RNAs were extracted from tuber tissues using Plant RNA Isolation Mini Kit (Agilent) following the manufacturer's instructions and were reverse transcribed to cDNAs using Invitrogen SuperScript™ III Reverse Transcriptase Kit (Invitrogen) with oligo$(dT)_{20}$ primer. *VInv* transcripts were quantified by reverse transcription quantitative PCR (RT-qPCR) using the SYBR Advantage qPCR Premix (Clontech) with the specific primers for *VInv* and the reference gene *Actin97* described previously (Zhu et al., 2014; Zhu et al., 2016). RT-qPCR was performed on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) with a program of 30 s at 95°C, 40 cycles of 10 s at 95°C, 20 s at 60°C for *VInv* and *Actin97*, and 30 s at 72°C, followed

by a plate read. Then 2 s at 50°C to 95°C with 0.2°C steps for melting curve, followed by a final extension step of 10 min at 72°C. Relative expression levels of *VInv* gene were calculated using Gene Expression Macro software version 1.1 (Bio-Rad Laboratories). Data for each treatment are presented as standard error (SE) of means of the three biological replicates. For RT-qPCR data of three Katahdin CRISPR/Cas9 lines and the wild type, analyses of variance (ANOVA) were carried out using PROC GLM in the Statistical Analysis System version 9.1 (SAS v9.1) (SAS Institute Inc, Cary, NC) (**Supplemental Table S4**).

To examine whether *VInv*In2En deletions affect *VInv* gene splicing, we prepared cDNAs from tuber tissues of the three Katahdin CRISPR/Cas lines. Exon 1 to exon 3 of *VInv* was amplified using primers Splicing-F/R (**Supplemental Table S3**, amplicon size: 612 bp). The RT-PCR products were purified by using QIAquick PCR Purification Kit (Qiagen) and then used for Sanger sequencing.

Yeast one-hybrid (Y1H) assay

Triple copies of the *VInv*In2En sequence (*VInv*In2En*3) were synthesized and used to develop a bait plasmid p*VInv*In2En*3-AbAi. The bait plasmid was used to screen the cDNA library, developed from cold-treated tuber tissues of RH potato, according to methods described in the Matchmaker® Gold Yeast One-Hybrid Library Screening System User Manual (Clontech, http://www.takarabio.com/). Yeast (*Saccharomyces cerevisiae*) colonies were cultured on plates containing SD/-Leu/AbA$^{200\ ng/mL}$ medium at 30°C for 3-5 days, and those greater than 2 mm in diameter were analyzed by PCR amplification and Sanger sequencing using primers pGADT7-F/R (**Supplemental Table S3**). The resulted sequences from the 387 yeast colonies were used for BLAST search in the DM1-3 potato cDNA (v6.1) database by using Spud DB blastn program with default parameters (http://spuddb.uga.edu/blast.shtml). We identified the best matched cDNA sequence for each of the 387 sequences. The cDNA sequences with an E-value <1e-5 and a minimum sequence identity of 82% were kept for further analyses. We identified a total of 33 unique cDNA sequences after filtering out repetitive cDNA sequences.

To further validate the interactions between the candidate proteins and the *VInv*In2En enhancer, point-to-point Y1H assay was performed. Full-length CDSs of the candidate proteins related to the 33 identified cDNAs were inserted into the prey vector pGADT7 by using HB-infusion™ Cloning Kit (HANBIO, https://www.hanbio.net/en/company.shtml/). The prey

plasmids were subsequently transformed into the bait yeast strain Y1HGold[p*VInv*In2En*3-AbAi] by using the Yeastmaker™ Yeast Transformation System 2 (Clontech, http://www.takarabio.com/). The yeast colonies were transferred to plates containing SD/-Leu/AbA$^{200\ ng/mL}$ medium and then allowed to grow at 30°C for 3 days.

Analysis of *VInv* evolution

A total of 28 Solanaceous species (https://solgenomics.net/) (Tang et al., 2022) and several other dicot species (**Supplemental Table S5**), including *A. thaliana*, cucumber (*Cucumis sativus*), and soybean (*Glycine max*), were selected for evolutionary analysis of the *VInv* gene. Information on evolutionary timescale of life for all 31 species were collected from the TimeTree 5 database (http://www.timetree.org/) (Kumar et al., 2022) and visualized in MEGA X software (Kumar et al., 2018).

Protein sequences of *VInv* gene from the 31 different plant species were extracted and aligned to that from RH potato using the NCBI BLASTp program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The intron and exon composition of the *VInv* gene from 31 plant species were analyzed using the online tool GSDS 2.0 (Hu et al., 2015). The 1327-bp intron 2 and the 200-bp *VInv*In2En sequences from RH were used to align the intron 2 sequences from other 30 species to identify homologous sequences using program of NCBI BLASTn.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL libraries under the following accession numbers: Soltu.DM.03G015280 (*VInv*), Soltu.DM.01G024340 (StNF-YC9), and Soltu.DM.06G027230 (StNF-YC1). All mutants and transgenic lines are described in **Supplemental Table S6**.

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Availability of data and materials

All materials, including constructs, are available upon request.

Author contributions

J.J. conceived the research. X.Z., A.C., N.M.B. and J.J. designed the experiments. X.Z., A.C., N.M.B., Z.Z., H.X., L.W., and Z.L. conducted the experiments. X.Z., D.E., D.S.D., and J.J. analyzed the data. X.Z. and J.J. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Figure legends

Figure 1. Discovery of a cold-responsive intronic enhancer in *VInv* gene. (**A**) DNase-hypersensitive sites (DHSs) associated with *VInv* gene. DHS map was developed from tuber tissue of DM1-3 potato. Two DHSs (red bars), one at the 5′ of the gene and one in the second intron, were detected. (**B**) β-glucuronidase (GUS) reporter gene assays of the second intron of *VInv* gene in Katahdin potato. Constructs using a minimal 35S promoter (m35S) and a full-length 35S promoters were used as negative and positive controls. Tubers from transgenic Katahdin lines developed using the intronic construct (*VInv*In2) and a reverse construct (*VInv*In2R) showed minimal GUS signals under room temperature (22°C). Strong GUS signals were detected from tubers after 4 weeks of cold storage under 4°C. The scale bar represent 2 cm.

Figure 2. Identification of transcriptional enhancers in intron 2 of *VInv* gene. (**A**) A diagram illustrating the sizes and positions of 10 sub-fragments derived from intron 2 of the *VInv* gene.

The 1327-bp intron was divided into ten fragments (#1 to #10) using five breaks (b1 to b5). (**B**) β-glucuronidase (GUS) reporter gene assays of the intron 2 in *A. thaliana*. Constructs with minimal 35S promoter (m35S) and a full-length 35S promoter (35S) were used as negative and positive controls. (**C**) *GUS* expression patterns of representative *A. thaliana* transgenic seedlings derived from each of the ten constructs consisting of a fragment ligated with the m35S promoter and the GUS reporter gene. (**D**) A diagram illustrating the sizes and positions of the 13 fragments derived from the DNA fragment #11. A dashed red line marks the middle point of the 600-bp segment #11. "+" and "-" indicate the derived transgenic seedlings showing positive and negative GUS signals, respectively. (**E**) GUS staining of 20 *A. thaliana* transgenic seedlings derived from constructs #11, #17, #19, and #21, respectively. (**F**) GUS reporter gene assay of the 200-bp *VInv*In2En enhancer in Katahdin potato. Tubers from three independent transgenic lines showed minimal GUS signals under 22°C but strong signals from tubers after 4 weeks of cold storage under 4°C. All numbers above bars/lines in (A) and (D) indicate base pairs. The scale bar represents 2 mm in (B, C) and 1 cm in (E, F). Fragment numbers highlighted in red color in (A, C, D, E) indicate representitive constructs with full enhancer function.

Figure 3. Distribution and function of DNA motifs in intron 2 and the *VInv*In2En enhancer. (**A**) Distribution of DNA motifs related to transcription factors (TFs) involved in response to cold stress. Each vertical bar represents a potential TF-binding site. A red horizonal bar marks the position of the 200-bp enhancer. Vertical blue bars indicate that the binding sites of a relevant TF are enriched or exclusively located within the 200-bp enhancer. Vertical green bars indicate that the binding sites of a relevant TF are not enriched within the enhancer (**B**) Transgenic assays of *VInv*In2En with mutated DNA motifs related to five different TFs. Red colored nucleotides indicate the replaced sequence(s) in each construct. No β-glucuronidase (GUS) signals were detected in any transgenic *A. thaliana* plants derived from the three constructs with mutated motifs related to CBF/NF-Y, TCP, and GATA.

Figure 4. Identification of StNF-YC1 and StNF-YC9 proteins that bind to *VInv*In2En using yeast one-hybrid assay. Triple copies of the *VInv*In2En sequence (*VInv*In2En*3) were synthesized to develop the bait plasmid p*VInv*In2En*3-AbAi. The pGADT7 vector was used as negative control,

and a combination of two constructs (p53-AbAi and pGADT7-Rec-p53) was used as positive control.

Figure 5. Functional validation of the *VInv*In2En enhancer using genome editing. (**A**) A diagram illustrating the positions of all sgRNAs within and outside of intron 2 of *VInv* gene. The red bar marks the 200-bp enhancer *VInv*In2En. Red arrows indicate the position of sgRNAs R1, R2, R3, and R4. Blue arrows indicate the position of sgRNAs 1a, 2a, 3a, 1b, 2b, and 3b. (**B**) Gel electrophoresis of PCR products amplified from the three homozygous CRISPR/Cas9 deletion lines (2-2-8, 13-1-3 and 13-2-1) developed from the wild-type (WT) DMF5-73-1. (**C**) Reverse transcription quantitative PCR (RT-qPCR)-based transcription analysis of *VInv* gene in cold-stored potato tissues from the three homozygous deletion lines (2-2-8, 13-1-3 and 13-2-1). All three lines showed significant reduction of *VInv* expression relative to the *Actin97* reference gene. The *y* axis represents the relative expression level normalized by setting *VInv* expression in cold-stored tubers of the wild-type (WT) DMF5-73-1 to 1. Data is presented as mean ± standard deviation (SD) from three biological replicates and was tested by the Student *t*-test (*$P < 0.05$). (**D**) Chipping of tubers from deletion line 13-1-3 and from the WT DMF5-73-1. Note: (1) the dark color toward one end of each chip is caused by the "jelly end" problem (two examples are indicated by arrows) associated with both 13-1-3 and WT. (2) 13-1-3 is a selfed progeny of a T0 DMF5-73-1 (heterozygous) transgenic line. Thus, the tubers from the two lines show different shapes. Three tubers from each line were used for chipping, and two chips from each tuber were included in the illustration. (**E**) Gel electrophoresis of PCR products amplified from the genomic DNA of three T0 CRISPR/Cas9 lines (KV78, KV87 and KV108) developed from tetraploid potato cultivar Katahdin. Red arrows indicate fragments resulted from deletions within *VInv*In2En. (**F**) Sequencing of PCR products amplified from cDNAs of the three CRISPR/Cas9 lines. Normal splicing between exon 1 and exon 3 was detected in all three lines. (**G**) Reverse transcription quantitative (RT-qPCR)-based analysis of *VInv* expression relative to the *Actin97* gene of the three CRISPR/Cas lines. Expression was analyzed using tubers after 2 weeks of storage at 22°C and 4°C, respectively. The *y* axis represents the relative expression level normalized by setting *VInv* expression in 22°C-stored tubers of the wild-type Katahdin to 1. Data is presented as mean ± standard deviation (SD) from three biological replicates and were tested

by using PROC GLM analyses of variance (ANOVA). Different lower case letters represent statistically significant differences at P = 0.05.

Figure 6. Composition of introns and exons of *VInv* genes from different plant species. A total of 28 Solanaceous species and eight distantly related dicot species were selected for the analysis. The distinctly small exon 2 (9 bp) was detected in all Solanaceous species, as well as in five distantly related plant species. In addition, a large intron 2 (ranging from 780 bp to 2,997 bp) following the small exon 2, was identified in most plant species

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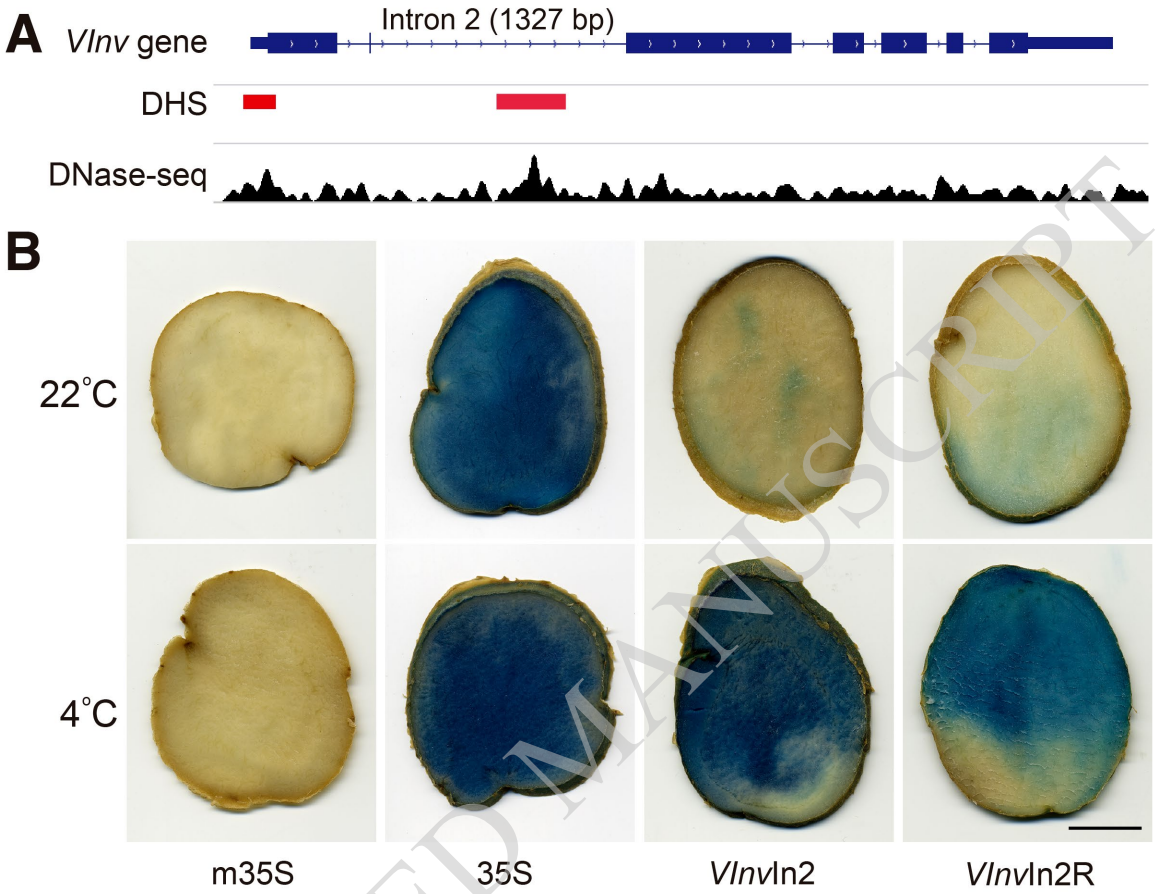

Figure 1. Discovery of a cold-responsive intronic enhancer in *VInv* gene.
(**A**) DNase-hypersentitive sites (DHSs) associated with *VInv* gene. DHS map was developed from tuber tissue of DM1-3 potato. Two DHSs (red bars), one at the 5′ of the gene and one in the second intron, were detected.
(**B**) β-glucuronidase (GUS) reporter gene assays of the second intron of *VInv* gene in Katahdin potato. Constructs using a minimal 35S promoter (m35S) and a full-length 35S promoters were used as negative and positive controls. Tubers from transgenic Katahdin lines developed using the intronic construct (*VInv*In2) and a reverse construct (*VInv*In2R) showed minimal GUS signals under room temperature (22°C). Strong GUS signals were detected from tubers after 4 weeks of cold storage under 4°C. The scale bar represents 2 cm.

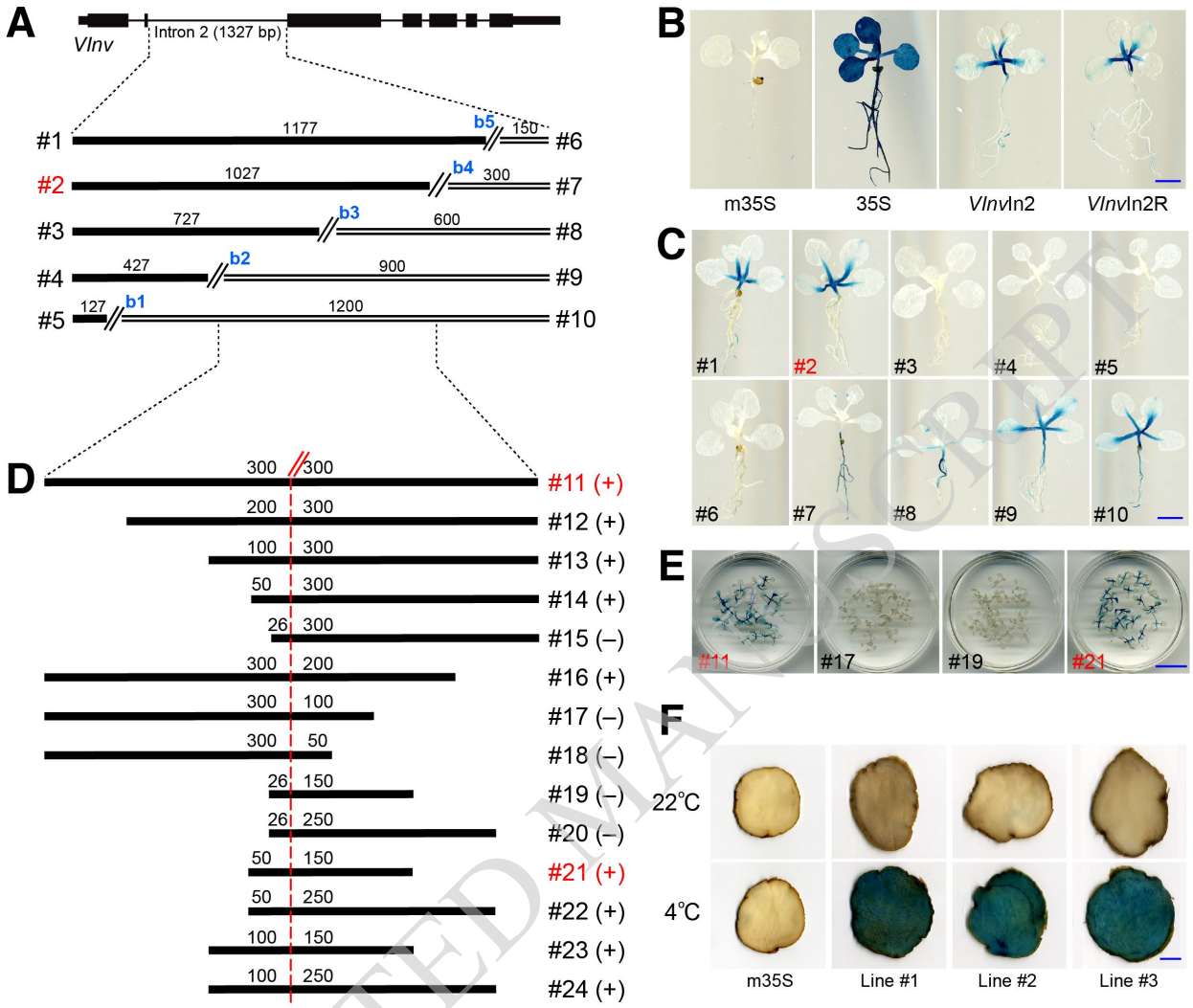

Figure 2. Identification of transcriptional enhancers in intron 2 of *VInv* gene.
(**A**) A diagram illustrating the sizes and positions of 10 sub-fragments derived from intron 2 of the *VInv* gene. The 1327-bp intron was divided into ten fragments (#1 to #10) using five breaks (b1 to b5).
(**B**) β-glucuronidase (GUS) reporter gene assays of the intron 2 in *A. thaliana*. Constructs with minimal 35S promoter (m35S) and a full-length 35S promoter (35S) were used as negative and positive controls.
(**C**) *GUS* expression patterns of representative *A. thaliana* transgenic seedlings derived from each of the ten constructs consisting of a fragment ligated with the m35S promoter and the GUS reporter gene.
(**D**) A diagram illustrating the sizes and positions of the 13 fragments derived from the DNA fragment #11. A dashed red line marks the middle point of the 600-bp segment #11. "+" and "-" indicate the derived transgenic seedlings showing positive and negative GUS signals, respectively.
(**E**) GUS staining of 20 *A. thaliana* transgenic seedlings derived from constructs #11, #17, #19, and #21, respectively.
(**F**) GUS reporter gene assay of the 200-bp *VInv*In2En enhancer in Katahdin potato. Tubers from three independent transgenic lines showed minimal GUS signals under 22°C but strong signals from tubers after 4 weeks of cold storage under 4°C.
All numbers above bars/lines in (A) and (D) indicate base pairs. The scale bar represents 2 mm in (B, C) and 1 cm in (E, F). Fragment numbers highlighted in red color in (A, C, D, E) indicate representitive constructs with full enhancer function.

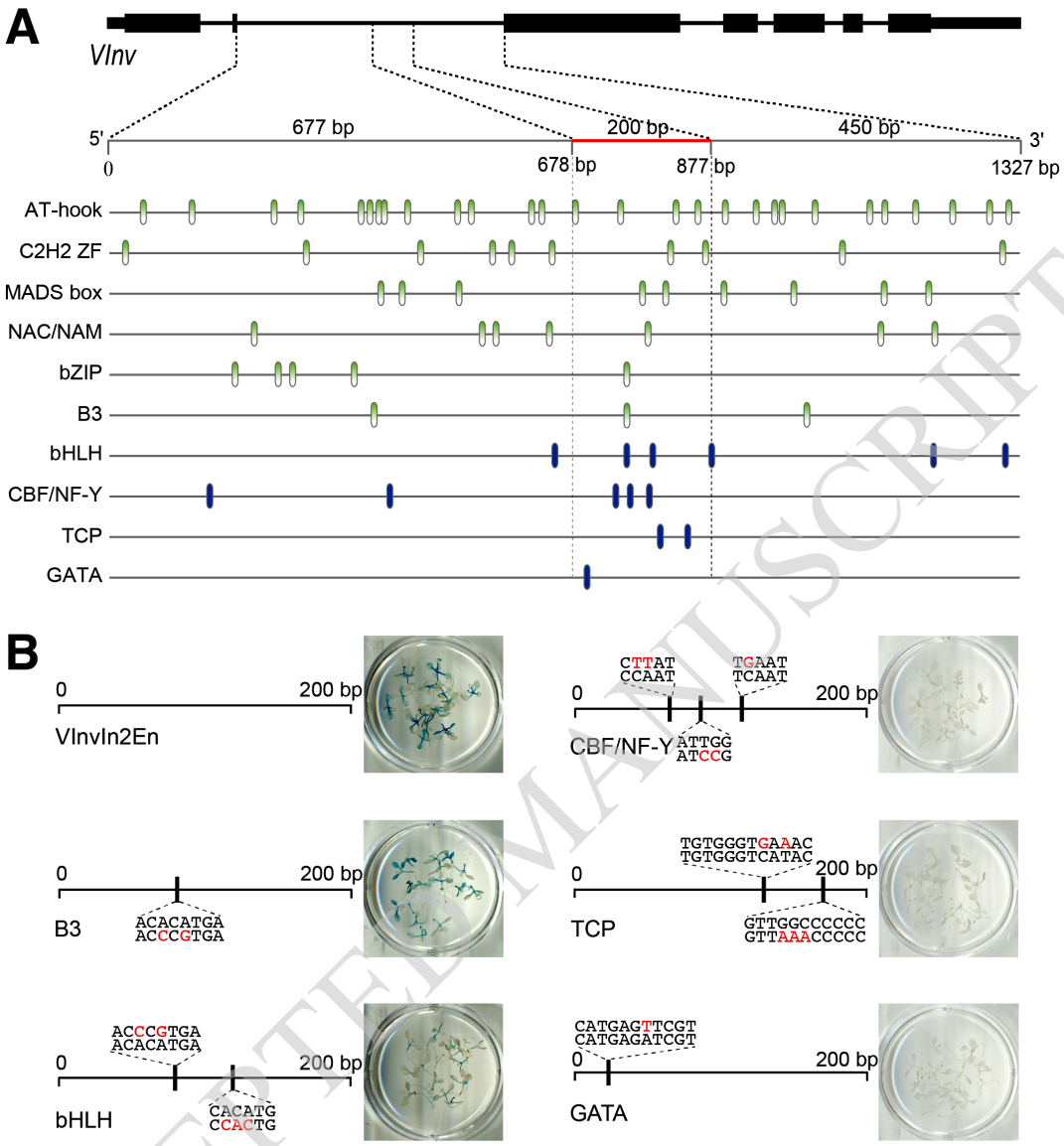

Figure 3. Distribution and function of DNA motifs in intron 2 and the *VInv*In2En enhancer.
(**A**) Distribution of DNA motifs related to transcription factors (TFs) involved in response to cold stress. Each vertical bar represents a potential TF-binding site. A red horizonal bar marks the position of the 200-bp enhancer. Vertical blue bars indicate that the binding sites of a relevant TF are enriched or exclusively located within the 200-bp enhancer. Vertical green bars indicate that the binding sites of a relevant TF are not enriched within the enhancer
(**B**) Transgenic assays of *VInv*In2En with mutated DNA motifs related to five different TFs. Red colored nucleotides indicate the replaced sequence(s) in each construct. No β-glucuronidase (GUS) signals were detected in any transgenic *A. thaliana* plants derived from the three constructs with mutated motifs related to CBF/NF-Y, TCP, and GATA.

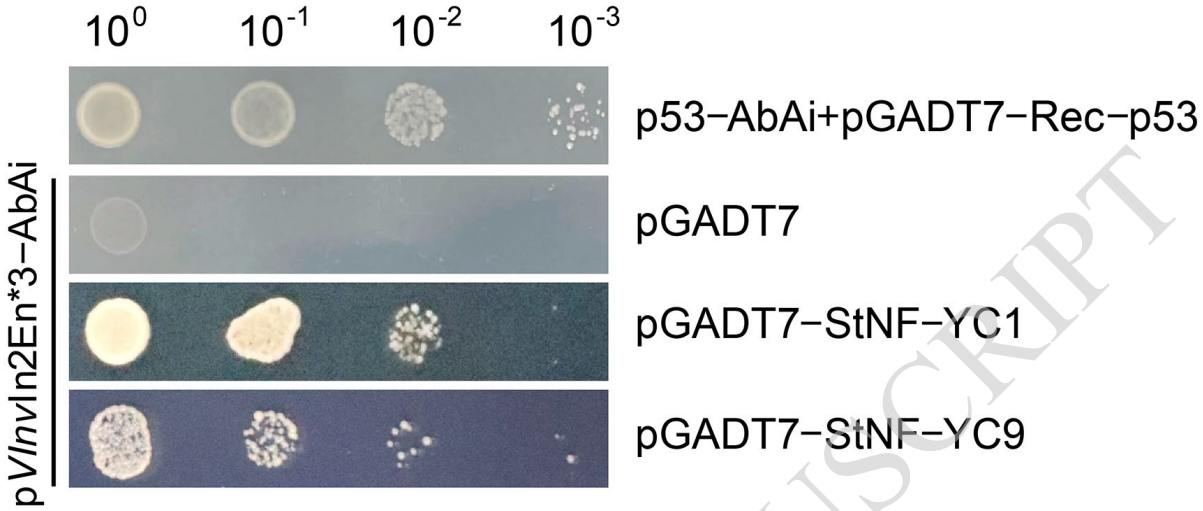

Figure 4. Identification of StNF-YC1 and StNF-YC9 proteins that bind to *VInv*In2En using yeast one-hybrid assay. Triple copies of the *VInv*In2En sequence (*VInv*In2En*3) were synthesized to develop the bait plasmid p*VInv*In2En*3-AbAi. The pGADT7 vector was used as negative control, and a combination of two constructs (p53-AbAi and pGADT7-Rec-p53) was used as positive control.

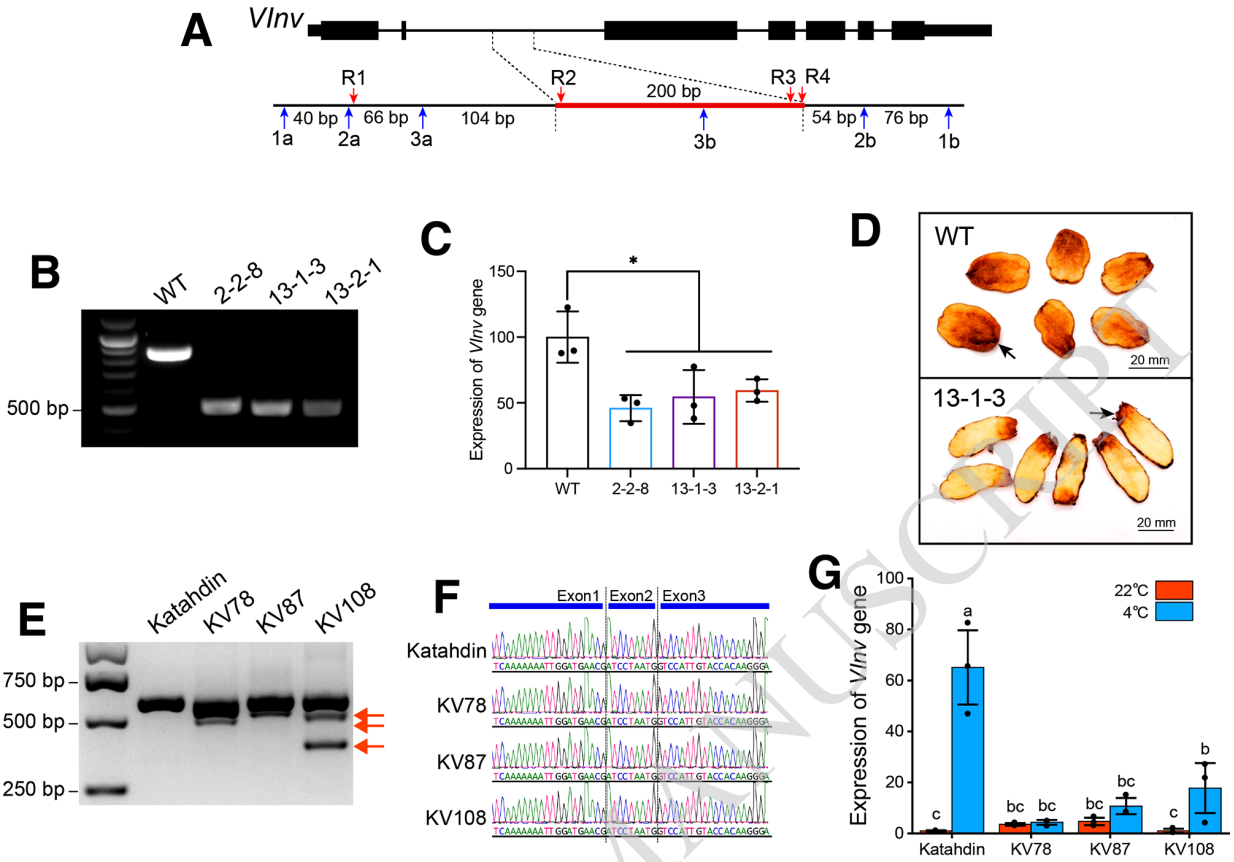

Figure 5. Functional validation of the *VInv*In2En enhancer using genome editing.
(**A**) A diagram illustrating the positions of all sgRNAs within and outside of intron 2 of *VInv* gene. The red bar marks the 200-bp enhancer *VInv*In2En. Red arrows indicate the position of sgRNAs R1, R2, R3, and R4. Blue arrows indicate the position of sgRNAs 1a, 2a, 3a, 1b, 2b, and 3b.
(**B**) Gel electrophoresis of PCR products amplified from the three homozygous CRISPR/Cas9 deletion lines (2-2-8, 13-1-3 and 13-2-1) developed from the wild-type (WT) DMF5-73-1.
(**C**) Reverse transcription quantitative PCR (RT-qPCR)-based transcription analysis of *VInv* gene in cold-stored potato tissues from the three homozygous deletion lines (2-2-8, 13-1-3 and 13-2-1). All three lines showed significant reduction of *VInv* expression relative to the *Actin97* reference gene. The *y* axis represents the relative expression level normalized by setting *VInv* expression in cold-stored tubers of the wild-type (WT) DMF5-73-1 to 1. Data is presented as mean ± standard deviation (SD) from three biological replicates and was tested by the Student *t*-test (*P< 0.05).
(**D**) Chipping of tubers from deletion line 13-1-3 and from the WT DMF5-73-1. Note: (1) the dark color toward one end of each chip is caused by the "jelly end" problem (two examples are indicated by arrows) associated with both 13-1-3 and WT. (2) 13-1-3 is a selfed progeny of a T0 DMF5-73-1 (heterozygous) transgenic line. Thus, the tubers from the two lines show different shapes. Three tubers from each line were used for chipping, and two chips from each tuber were included in the illustration.
(**E**) Gel electrophoresis of PCR products amplified from the genomic DNA of three T0 CRISPR/Cas9 lines (KV78, KV87 and KV108) developed from tetraploid potato cultivar Katahdin. Red arrows indicate fragments resulted from deletions within *VInv*In2En.
(**F**) Sequencing of PCR products amplified from cDNAs of the three CRISPR/Cas9 lines. Normal splicing between exon 1 and exon 3 was detected in all three lines.
(**G**) Reverse transcription quantitative (RT-qPCR)-based analysis of *VInv* expression relative to the *Actin97* gene of the three CRISPR/Cas lines. Expression was analyzed using tubers after 2 weeks of storage at 22°C and 4°C, respectively. The *y* axis represents the relative expression level normalized by setting *VInv* expression in 22°C-stored tubers of the wild-type Katahdin to 1. Data is presented as mean ± standard deviation (SD) from three biological replicates and were tested by using PROC GLM analyses of variance (ANOVA). Different lower case letters represent statistically significant differences at P = 0.05.

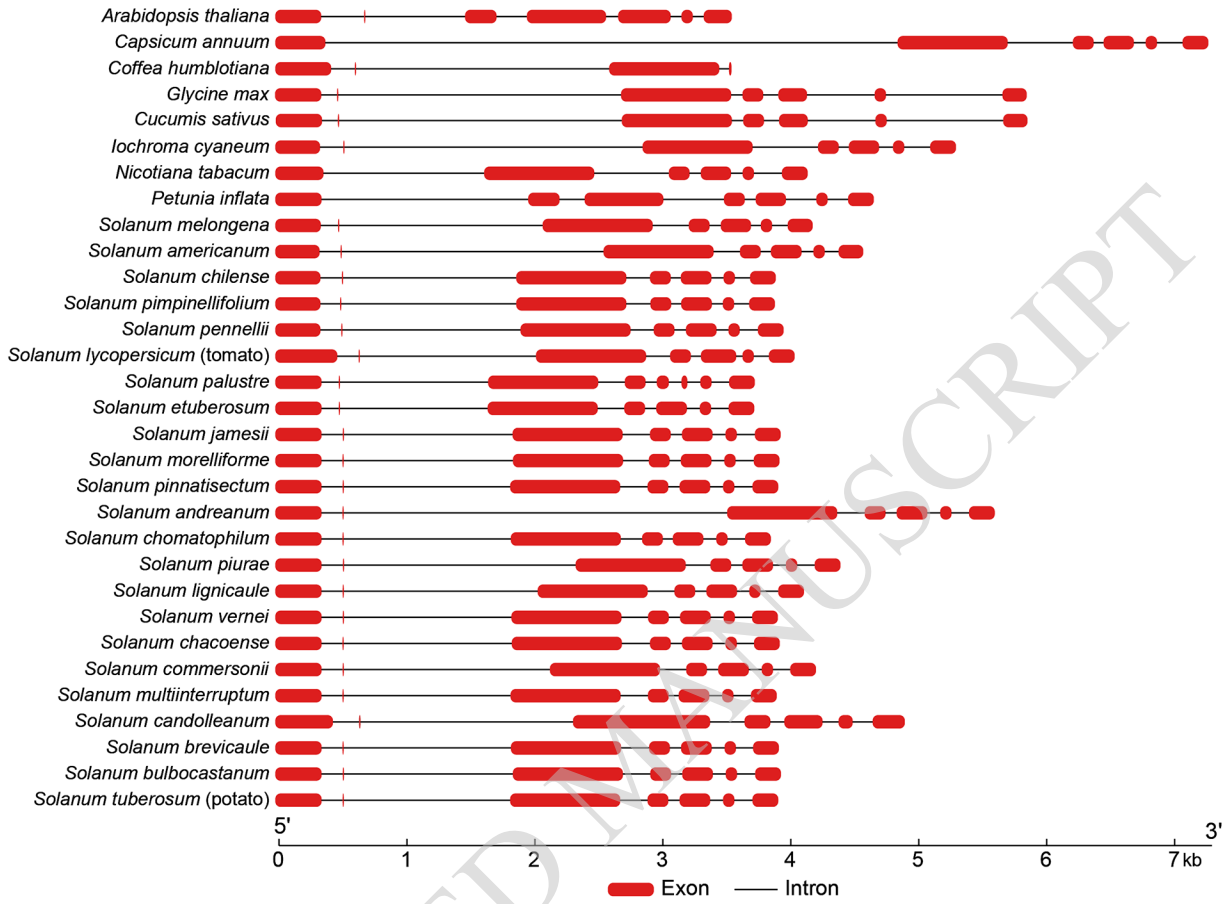

Figure 6. Composition of introns and exons of *VInv* genes from different plant species. A total of 28 Solanaceous species and eight distantly related dicot species were selected for the analysis. The distinctly small exon 2 (9 bp) was detected in all Solanaceous species, as well as in five distantly related plant species. In addition, a large intron 2 (ranging from 780 bp to 2,997 bp) following the small exon 2, was identified in most plant species.

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