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RESEARCH PAPER

# Large-scale analysis of the cassava transcriptome reveals the impact of cold stress on alternative splicing

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## Abstract

**Alternative splicing is an essential post-transcriptional regulatory mechanism that can impact mRNA stability and protein diversity of eukaryotic genomes. Although numerous forms of stress-responsive alternative splicing have been identified in model plants, a large-scale study of alternative splicing dynamics under abiotic stress conditions in cassava has not been conducted. Here, we report the parallel employment of isoform-Seq, ssRNA-Seq, and Degradome-Seq to investigate the diversity, abundance, and fate of alternatively spliced isoforms in response to cold and drought stress. We identified 38 164 alternative splicing events, among which 3292 and 1025 events were significantly regulated by cold and drought stress, respectively. Intron retention was the most abundant subtype of alternative splicing. Global analysis of splicing regulators revealed that the number of their alternatively spliced isoforms and the corresponding abundance were specifically modulated by cold stress. We found that 58.5% of cold-regulated alternative splicing events introduced a premature termination codon into the transcripts, and 77.6% of differential alternative splicing events were detected by Degradome-Seq. Our data reveal that cold intensely affects both quantitative and qualitative aspects of gene expression via alternative splicing pathways, and advances our understanding of the high complexity and specificity of gene regulation in response to abiotic stresses.**

**Keywords:** Alternative splicing, cassava, cold stress, drought stress, RNA-Seq, splicing factor.

## Introduction

Precursor mRNA (pre-mRNA) splicing, which includes intron excision and exon ligation, is an essential step in eukaryotic gene expression. Besides constitutive splicing in pre-mRNAs with multiple introns, alternative splicing generates different mature mRNA sequences from individual pre-mRNAs through the selection of different exons (and introns), expanding the potential informational content of eukaryotic genomes (Stamm *et al.*, 2005). Five alternatively spliced subtypes have been observed in plants, namely exon skipping (ES), intron retention (IR), mutually exclusive exon (MXE), alternative 5' splice sites (A5SS), and alternative 3' splice sites (A3SS) (Barbazuk *et al.*, 2008). Alternative splicing takes place

within the spliceosome (Chen and Moore, 2015). Components of the spliceosome include small nuclear ribonucleoproteins (snRNPs) and numerous Ser/Arg-rich (SR) proteins (Koncz *et al.*, 2012; Erkelenz *et al.*, 2013; Filichkin *et al.*, 2015). The SR proteins are highly conserved in plants (Matlin *et al.*, 2005; Syed *et al.*, 2012). They act as splicing factors in either constitutive or alternative splicing and determine the selection of splice sites in a concentration-dependent manner by forming differential spliceosome complexes (Kalyna and Barta, 2004; Zhong *et al.*, 2009; Day *et al.*, 2012).

In plants, alternative splicing expands the repertoire of protein activities through the selection of different exons and modulates gene expression by generating different mRNA variants with altered stability, localization, or translation efficiency (Leviatan *et al.*, 2013; Laloum *et al.*, 2018). Previous studies have shown that many splice variants contain premature termination codons (PTCs) that are targeted by the nonsense-mediated decay (NMD) mechanism (Chang *et al.*, 2007; Palusa and Reddy, 2010; Kalyna *et al.*, 2012; Mastrangelo *et al.*, 2012). For example, in Arabidopsis, ~18% of spliced mRNA variants are degraded by NMD (Kalyna *et al.*, 2012), indicating that alternative splicing controls mRNA stability, thus influencing the level of functional mRNA transcripts. Interestingly, some findings suggest that transcripts with IR events are resistant to the NMD pathway in plants (Kalyna *et al.*, 2012; Gohring *et al.*, 2014). Recent studies have shown that alternative splicing is associated with peptide interference, which is mediated by small interfering peptides (Yun *et al.*, 2008; Seo *et al.*, 2011, 2013; Staudt and Wenkel, 2011). It is evident that alternative selection of 5' and 3' splice sites and ES contribute to the generation of protein variants that have different combinations of functional domains and protein structures (Marquez *et al.*, 2012; Syed *et al.*, 2012; Leviatan *et al.*, 2013).

Alternative splicing has been shown to play a role in plant responses to abiotic stresses, including heat, drought, and cold stress (Leviatan *et al.*, 2013; Thatcher *et al.*, 2016; Liu *et al.*, 2018; Zhu *et al.*, 2018). With the advent of next-generation sequencing-based approaches, a large number of plant transcripts have been shown to be alternatively spliced. For example, recent studies have found that alternative splicing occurs in ~60% of Arabidopsis, ~50% of soybean, ~40% of cotton, ~40% of maize, ~33% of rice, and ~24% of wheat intron-containing genes (Filichkin *et al.*, 2010; Zhang *et al.*, 2010; Shen *et al.*, 2014; Liu *et al.*, 2018; Wang *et al.*, 2018). A recent study has shown that a massive and rapid alternative splicing response occurs under cold stress, and hundreds of genes, such as cold-responsive transcription factors and splicing factors/RNA-binding proteins, exhibit changes in expression due to rapidly occurring alternative splicing (Calixto *et al.*, 2018). Notably, a number of splicing factors involved in pre-mRNA splicing are influenced by diverse environmental conditions (Laloum *et al.*, 2018). For example, in Arabidopsis, the expression and the splicing patterns of the SR splicing factor genes are differentially regulated by low temperature (Palusa *et al.*, 2007). The pre-mRNA splicing factor STABILIZED1 (STA1), which is crucial for temperature-responsive splicing, is induced by cold temperatures and confers freezing tolerance by alternative splicing of the *cold-regulated 15A* (*COR15A*) gene (Lee *et al.*, 2006). These findings highlight the central role of splicing factors in plant responses to various stresses by diversifying transcriptome and proteome plasticity.

Cassava (*Manihot esculenta*) is the most important tropical crop for food, bio-fuel production, and animal feed due to its starch-enriched roots. It is well adapted to barren soil and drought conditions, but is sensitive to low temperatures (Zeng *et al.*, 2014). Cold stress severely influences various physiological processes of cassava and depresses its growth, development, and economic yield (An *et al.*, 2012; Li *et al.*, 2017*a*). Recently, next-generation sequencing techniques were employed to study the gene expression in cassava on a global level. A large number of cassava protein-coding and non-coding genes involved in multiple processes have been reported that play a major role in the adaptation of plants to environmental stresses (Ballen-Taborda *et al.*, 2013; Xia *et al.*, 2014; Fu *et al.*, 2016; Khatabi *et al.*, 2016; Li *et al.*, 2017*b*; Ruan *et al.*, 2017). However, limited information is available on whether alternative splicing participated in cold and/or drought stress responses. The mechanism underlying cassava cold sensitivity and drought tolerance remains unknown.

To address these unknowns, we combined isoform-Seq (Iso-Seq) and strand-specific RNA-Seq (ssRNA-Seq) data from the cassava shoot apex under cold, drought, and control conditions to identify alternatively spliced isoforms and quantify the differential alternative splicing (DAS) events under stress. This analysis uncovered >38 000 alternative splicing events of known genes and led to the identification of thousands of cold- and drought-regulated splicing changes. These changes mostly occurred during cold stress, while alternative splicing was barely changed under drought stress conditions. Analysis of splicing changes in genes associated with alternative splicing uncovered a linear relationship between the expression changes of alternatively spliced isoforms of splicing factors/regulators and the number of alternative splicing events in other genes. By integrating multi-omics data, we improved the annotation of the cassava transcriptome and addressed the complexity of alternative splicing regulation in response to abiotic stresses. These results highlight the importance of alternative splicing in the response of cassava to cold stress and provide a useful reference for other species.

## Materials and methods

### *Plant materials and growth conditions*

Cassava (*Manihot esculenta*) cultivar 60444 was used in this research. Stems of cassava seedlings were cut into 1.0–2.0 cm long pieces with one bud and planted on an MS0 (0.1% agar) plate. Then, plates were put in a greenhouse at 26±2 °C with a photoperiod of 16 h light and 8 h dark. Two-week-old plants were transferred to a chamber for cold treatment at 4 °C with a photoperiod of 16 h light and 8 h dark. The youngest leaves and shoot apices were collected after 6, 12, and 24 h of cold treatment, and were immediately frozen in liquid nitrogen and stored at −80 °C for RNA or protein extraction. The samples collected at 24 h were used for ssRNA-Seq and Degradome-Seq. For drought treatment, seedlings were transplanted into MS0 (0.1% agar) with 20% polyethylene glycol (PEG) 6000 for 6 h and harvested immediately. As described previously (Li *et al.*, 2017*a*, *b*), the protocol for ssRNA-Seq was chosen for two reasons: (i) we observed visible phenotypic changes, including stem bending and leaf wilting, in both PEG-induced drought (6 h) and cold stress (24 h) treatments; and (ii) the expression levels of the stress-responsive marker genes

(known *DREB/CBF* genes and *COR* genes/*RD29*) are maximal at 24 h after cold stress and at 6 h after PEG treatment. Samples from no fewer than 10 individual plants were pooled. Parallel and untreated plants at the same stage were used as controls. Six independent biological replicates were used, with three for sequencing and the other three for experimental verification.

### *Iso-Seq and alternatively spliced isoform analysis*

The single-molecule real-time (SMRT) sequencing libraries of full-length cDNA in cassava under control (CK) and cold conditions were constructed using the standard Iso-Seq protocol, and sequenced on the Pacbio Sequel platform at Guangzhou Genedenovo Biotechnology Co., Ltd (Guangzhou, China). One replicate was constructed for each library. The SMRT Link v5.0.1 was used for analyzing the raw data generated from the Pacbio Sequel platform (Gordon *et al.*, 2016). Briefly, the circular consensus sequences (CCSs) were extracted from raw data, and then the CCSs were classified to identify full-length non-chimeric reads containing 5' primers, 3' primers, and a poly(A) tail. Subsequently, iterative clustering for error correction was performed to obtain high-quality polished consensus reads. Finally, these high-quality reads were mapped to the cassava genome (v6.1). Next, the transcript isoforms and alternative splicing events were identified using SUPPA with the default parameter (Alamancos *et al.*, 2015). SUPPA detected seven types of alternative splicing events: ES, MXE, IR, A5SS, A3SS, alternative first exon, and alternative last exon. Due to the high error rate in the 5' and 3' ends of isoforms detected by Iso-Seq, the alternative first exon and alternative last exon types were excluded for further analysis. The isoforms and alternative splicing events of protein-coding genes in the reference genome were also identified by SUPPA as above.

### *ssRNA-Seq and differential alternative splicing event analysis*

We combined the Iso-Seq data and previously published ssRNA-Seq data to identify DAS events under normal and stress condition (Li *et al.*, 2017*a*, *b*). Briefly, we merged the annotation file from the reference genome and the newly identified isoform annotation file using cuffmerge, and extracted the full-length cDNA sequences, which contain primary and isoform transcripts annotated by the reference genome and novel isoform transcripts detected by Iso-Seq (Roberts *et al.*, 2011). Next, the raw reads from ssRNA-Seq were mapped to the full-length cDNA using Salmon tools for quantification, and the read count in each isoform was normalized to transcripts per million (Patro *et al.*, 2017). Subsequently, SUPPA was used to detect DAS events corresponding to the five major types: ES, MXE, IR, A5SS, and A3SS (Alamancos *et al.*, 2015). Briefly, the 'psiPerEvent' mode was performed to extract the proportion spliced-in (PSI) of alternative splicing events in each sample, and then 'diffSplice' was performed to calculate the DAS based on the difference in PSI values between the control and treatments. The significant DAS events were filtered to obtain those with a false discovery rate (FDR) <0.05. Gene Ontology (GO; http://geneontology.org) enrichment analyses were conducted among genes with DAS using AgriGO (Tian *et al.*, 2017).

### *Identification of isoforms containing a PTC*

The isoform transcripts detected by Iso-Seq were mapped to the cassava genome as above, and the exon information was extracted by SUPPA with gtf format. The exon sequences of isoforms were merged as whole coding DNA sequences (CDSs) in a strand-specific manner, beginning with the start codon 'AUG' of known primary transcripts annotated by the reference genome, and then the CDSs containing alternatively spliced regions were translated into proteins. If 'TAA', 'TGA', or 'TAG' occurs before the stop codon of the primary transcript, the given isoform is defined as an isoform containing a PTC.

### *Degradome-Seq and bioinformatic analysis*

Two degradome libraries (CK and Cold) were constructed as described previously, with minor modifications (German *et al.*, 2009). One replicate was constructed for each library. The total RNA extraction, construction of libraries, and deep sequencing were performed by the Guangzhou Genedenovo Biotechnology Co., Ltd (Guangzhou, China). Briefly, poly(A)$^+$ mRNA was enriched by oligo(dT) magnetic beads. The cleavage products with a free 5'-monophosphate at their 3' termini were ligated with 5' RNA adaptor; then the samples were reverse transcribed and subjected to PCR. Libraries were further sequenced on an Illumina Hiseq 2000 platform that generated single-end reads of 50 nt. After removing low-quality reads, adaptors, tRNAs, rRNAs, small nuclear RNAs (snRNAs), and small nucleolar RNAs (snoRNAs), the Salman tool was used to map the remaining reads to the cassava full-length cDNA, including primary and isoform transcripts annotated by the reference genome and novel isoform transcripts detected by Iso-Seq. Subsequently, SUPPA was performed to identify alternative splicing events using the 'psiPerEvent' mode as above (Alamancos *et al.*, 2015). Degradome reads indicating alternative splicing events need to be detected at least once in each replicate.

### *Reverse transcription–PCR analysis*

Total RNA was prepared from the same samples using an RNA Plant kit (OMEGA), and was reverse transcribed into cDNA in a 20 μl reaction solution containing 2 μg of total RNA by using a PrimeScript™ RT reagent kit (Takara), following the manufacturer's protocol. RT–PCR was performed to validate the alternatively spliced isoforms. The specific primers used for identification of alternatively spliced isoforms are listed in Supplementary Table S10 at *JXB* online. The PCR fragments were isolated and cloned onto the pGEM-T vector (Promega) for sequencing.

### *Accessions numbers*

The Iso-Seq, ssRNA-Seq, and Degradome-Seq raw data have been deposited in the NCBI Sequence Read Archive (SRA) under the BioProject accession numbers SRP198574, SRP101302, and SRP133534, respectively.

## Results

### *Overview of cassava transcripts detected by Iso-Seq*

In this study, we applied Iso-Seq to identify transcript isoforms genome-wide in cassava under CK and cold conditions. The Iso-Seq generated 8.50 million and 8.99 million reads with a read N50 (the minimum contig length required to cover 50% of the assembled genome sequence) of 2580 and 2714, respectively, in the cassava with CK and cold treatment (Supplementary Table S1). Then, CCSs were extracted. Among them, 249 598 and 217 321 CCSs were non-chimeric full-length reads in CK and cold-treated samples, respectively (Supplementary Table S1). After the iterative clustering for error correction, 93 425 and 97 159 high-quality isoforms were obtained. Approximately 96% these high-quality isoform reads can be mapped to the cassava genome. We identified 16 504 genes with 39 105 isoforms in the CK sample, among which 11 717 were known isoforms, while 25 334 were new isoforms mapped to the known genes, and 2054 were considered as novel isoforms, which mapped to unannotated genomic regions (Table 1). In cold-treated samples, we identified 16 060 genes with 43 398 isoforms comprising 10 623 known isoforms, 30 614 new isoforms, and 2161 novel isoforms. In the reference genome, there were 33 033 genes with 41 381 transcripts (Table 1). After merging with the reference-annotated primary isoform transcripts, in total we annotated 77 606 transcripts in the cassava genome. It is noteworthy that there were

**Table 1.** Overview of cassava isoforms detected by Iso-Seq

| Samples | No. of genes in reference | No. of transcripts in reference | Mapped genes | Mapped isoforms | Known isoforms | Novel isoforms | New isoforms |
|---|---|---|---|---|---|---|---|
| **CK** | 33 033 | 41 381 | 16 504 | 39 105 | 11 717 | 2054 | 25 334 |
| **Cold** | 33 033 | 41 381 | 16 060 | 43 398 | 10 623 | 2161 | 30 614 |

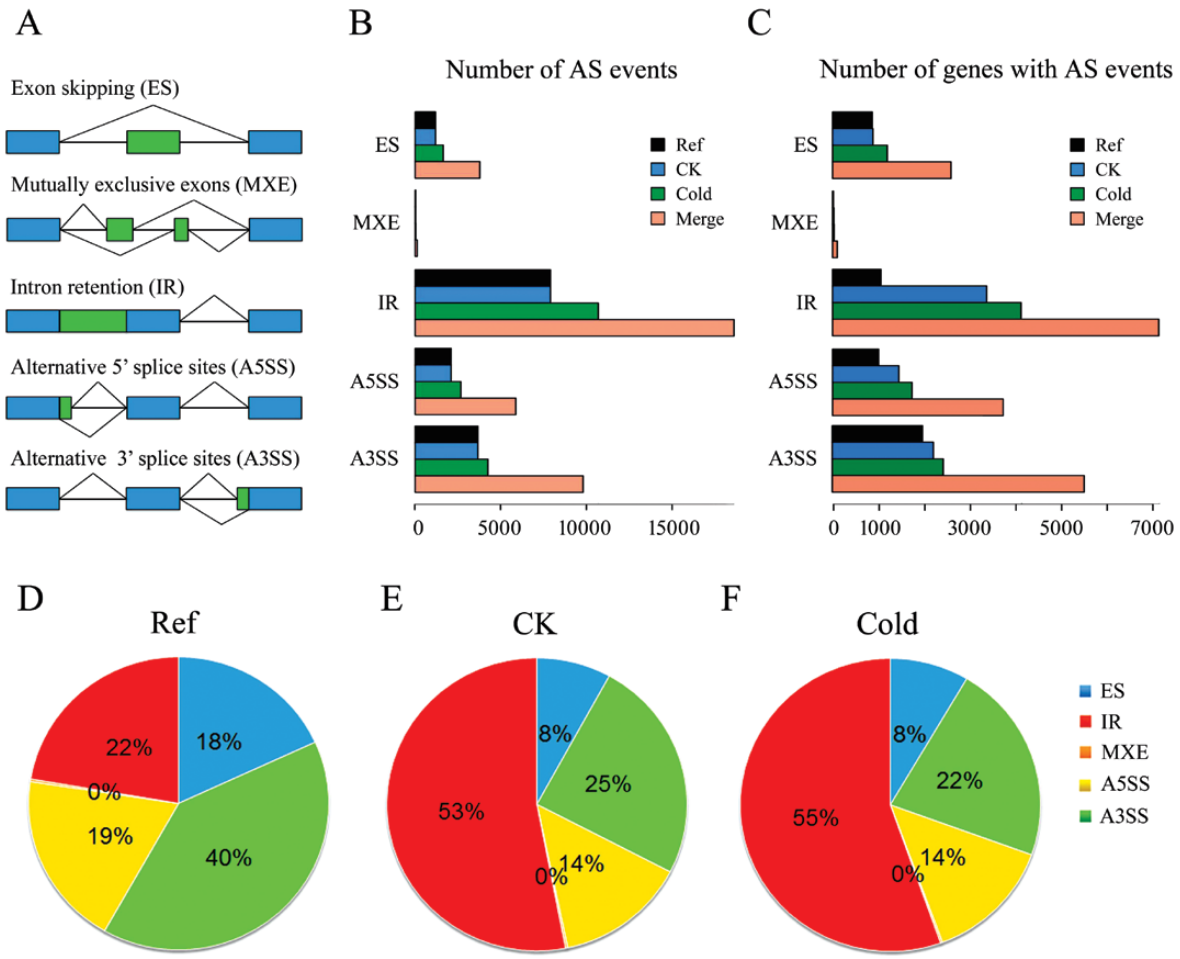


**Fig. 1.** Alternative splicing landscapes of cassava plants based on reference genome information and Iso-Seq data. (A) The representative forms of alternative splicing (AS), namely exon skipping, mutually exclusive exon, intron retention, alternative 5' splice sites, and alternative 3' splice sites. (B and C) The total number of each type of alternative splicing event (B) and corresponding genes (C) identified in the reference genome, control (CK), and cold-treated samples based on Iso-Seq data, and merged samples. (D–F) Frequency of alternatively spliced types in the reference genome (D), CK (E), and cold-treated (F) samples. (This figure is available in color at *JXB* online)

~4000 isoforms in cold-treated samples more than in the CK, indicating that cold stress may shape the landscape of alternative splicing in the cassava transcriptome.

### *Identification of known and novel alternative splicing events by Iso-Seq*

Next, we wanted to identify the alternative spacing events that occur in the isoforms relative to the constitutive splicing transcripts (primary transcripts hereafter). We focused on the five major types of alternative splicing events, ES, MXE, IR, A5SS, and A3SS (Fig. 1A). First, we examined the alternative splicing events in the current annotation gene model. We found 5724 alternative splicing events from 4028 genes (Fig. 1B, C). Using Iso-Seq, we identified a total of 14 868 and 19 275 alternative splicing events from 4708 and 5373 gene loci in CK and cold-treated samples, respectively (Fig. 1B, C). In total, we detected a 38 164 alternative splicing events from 10 431 gene loci (Supplementary Table S2), revealing that ~31.6% (10 431/33 033) of protein-coding genes exhibited alternative splicing events. Among these alternative splicing events, 15.0% had already been annotated in cassava genes, and the remaining 85.0% were identified as new alternative splicing events. In total, we detected an average of 3.65 alternative splicing events per gene, which was significantly more than in the current gene model (1.14 alternative splicing events per gene). In the reference genome, 40% of alternative splicing events were A3SS, 18% were ES, and only 22% were IR (Fig. 1D). The Iso-Seq analysis revealed that IR was the most abundant alternative splicing event (53–55%) in

cassava under CK and cold conditions. Subsequently, A3SS was the second most abundant alternatively spliced type (22–25%), and ES comprised ~8% (Figs 1E, F, S1), which is consistent with other plant species (Chamala *et al.*, 2015). This result indicates that the current genome annotation of genes with alternative splicing events overestimates the A3SS and ES events, and underestimates the IR events, in cassava (Fig. 1D–F and Supplementary Fig. S1). Under cold conditions, the number of alternative splicing events of all the five sub-types were much higher as compared with those under normal conditions (Fig. 1B, C), further indicating the potential impact of cold stress on promoting alternative splicing.

### *Analysis of DAS events and corresponding pathways in response to cold and drought stress*

In previous studies, we reported long non-coding RNA (lncRNA) and transcriptome changes in cassava in response to cold and drought stress by analyzing the ssRNA-Seq data (Li *et al.*, 2017*a*, *b*). To further understand the abiotic stress responsiveness, we examined the comprehensive profiling of the alternative splicing landscape under these stress conditions using the same ssRNA-Seq data. To examine stress-responsive splicing changes in detail, a comparative analysis was conducted on the relative expression of all alternative splicing events between stress-treated and control samples. We remapped all the ssRNAseq reads to 77 606 reference-annotated and Iso-Seq-identified isoform transcript sequences by Salmon tools. Using the SUPPA tool, 21 139, 22 471, and 22 646 alternative splicing events were identified under normal, cold, and drought conditions, respectively (Supplementary Table S3). Among them, there were a total of 3292 DAS events measured by different PSI values from 2038 gene loci in seedlings treated with cold stress and 1025 DAS events from 636 gene loci in seedlings treated with drought stress (Figs 2A, B; Supplementary Fig. S2; Supplementary Table S3). Under cold conditions, ~19.5% of genes with alternative splicing events (10 431 in total) produced DAS transcripts that contain at least one significant DAS event, as compared with CK (Supplementary Fig. S2). Cassava is known to be extremely sensitive to low temperature, and many splicing differences were observed in the cold treatment. Under drought conditions, nearly 93.9% of genes with alternative splicing events remained largely unchanged. This lack of response at the splicing level might correspond to the strong tolerance of cassava to drought stress.

In a previous study, we identified a total of 6103 and 7482 genes that were significantly differentially expressed in response to low temperature and drought stress, respectively (Li *et al.*, 2017*a*). To explore how many genes with DAS events occur in differentially expressed genes (DEGs), a comparison was made among DEGs, genes with DAS events, and known cold- or drought-related genes annotated by GO (Fig. 2C, D; Supplementary Table S4). Under cold treatment, we found that 309 genes with DAS events were also differentially expressed, of which 20 were cold-related genes according to GO annotation, but the majority of genes with DAS events (1781) were not differentially expressed (Fig. 2C). Under the drought stress treatment, nearly 18.7% of genes with DAS events were identified as DEGs. We also observed a strong overlap between the known drought-related genes and the DEGs (Fig. 2D). Subsequently, we compared all types of statistically significant alternative splicing events in response to cold and drought stress. Only 149 common DAS events from 141 genes were found to be responsive to both conditions (Fig. 2E). A large portion of alternative splicing events, especially for event types such as IR and A3SS, were uniquely responsive to cold stress. Unlike cold-induced splicing changes, fewer drought-specific alternative splicing events (1025 in total) were identified, which might be due to the lack of splicing changes under drought conditions. These newly identified stress-responsive splicing events were validated by RT–PCR (Fig. 3). As expected, the seven examined genes showed consistent alternative splicing patterns with their profiles revealed by RNA-Seq data, which further confirmed the accuracy of our bioinformatics analysis. These results indicate the overall promotion of alternative splicing by cold stress.

To investigate the influence of stress-induced alternative splicing on cellular processes, we analyzed functional categories and pathways of the genes with DAS events and DEGs under cold and drought stress. Under cold conditions, the enrichment analysis according to GO terms revealed that the genes with DAS events were involved in a range of biological processes, such as RNA processing and purine nucleotide catabolic process (Fig. 2F; Supplementary Table S5). In particular, genes with DAS events involved in RNA processing and protein modification processes were markedly increased and were only observed in the seedlings after cold treatment (Supplementary Table S5). In contrast, functional annotations of DEGs in response to cold were associated with response to chitin, hormones, and oxygen-containing compounds (Fig. 2F). Under drought conditions, negative regulation of catalytic activity was seen and protein metabolic processes were significantly enriched in the drought-responsive alternative splicing events. Some categories such as response to water stimulus and pigment metabolic processes were highly enriched in DEGs compared with genes with DAS events (Fig. 2F). These results strongly suggest that alternative splicing of genes involved in particular functions can be rapidly and differentially regulated by cold and drought stress.

### *Cold-induced alternative splicing change in splicing factors is a major reprogramming contributor of transcriptomes*

Based on the observation in Fig. 2C, a large number of cold-responsive genes produced DAS transcripts. Functional annotation of these genes showed that genes under three enriched GO terms, namely plant hormone signal transduction, protein kinases [CDPKs (calcium-dependent protein kinases) and MAPKs (mitogen-activated protein kinases)], and RNA-binding proteins, were highly enriched (Fig. 4A). RNA-binding proteins, such as heterogeneous nuclear RNPs (hnRNPs) and SR proteins, are important in splicing mRNA, polyadenylation, and stabilization (Meyer *et al.*, 2015). Different stressors may have distinct effects on splicing due to the alternative splicing-related changes to splicing-related genes. Thus, we conducted a

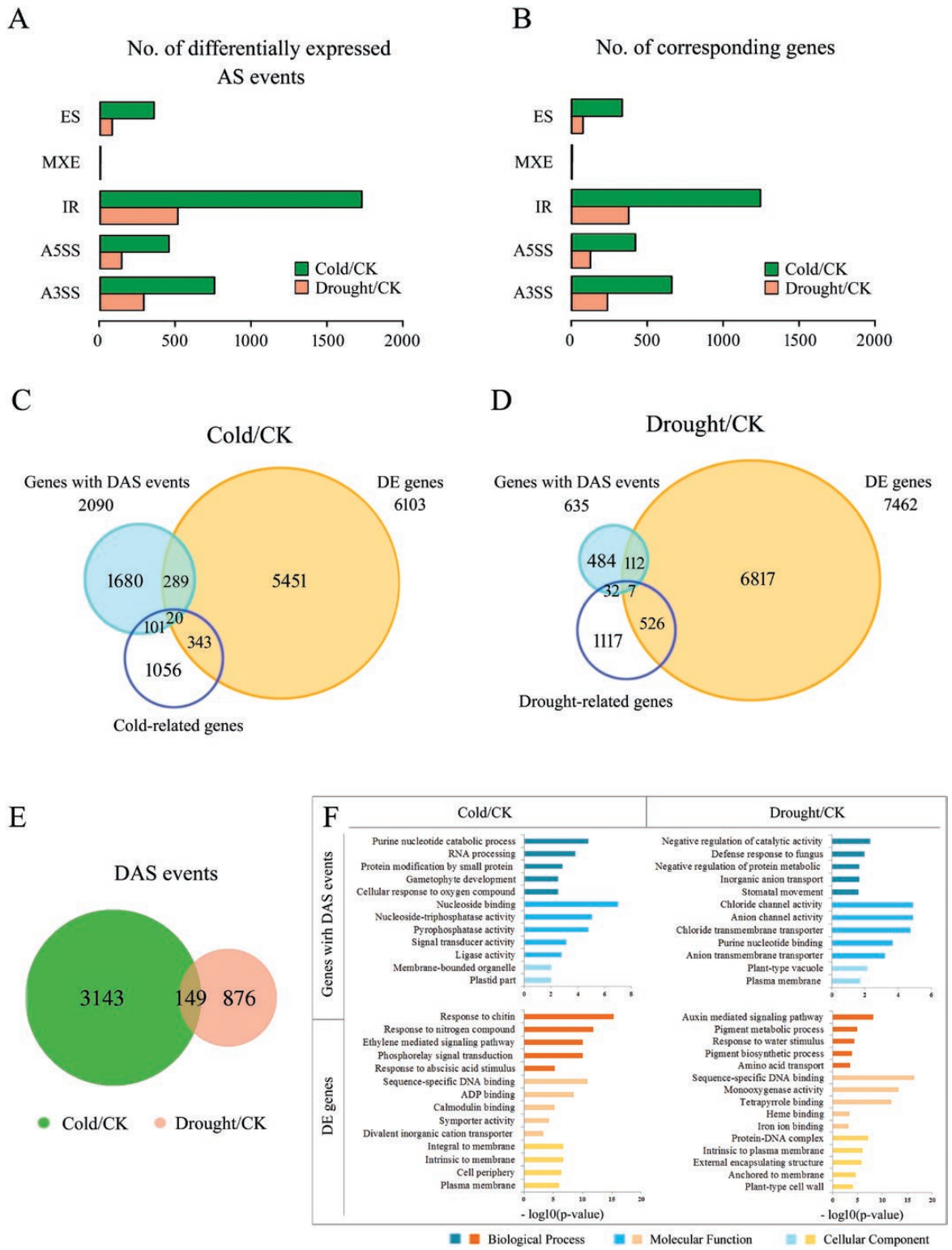


**Fig. 2.** Differential alternative splicing (DAS) and differentially expressed (DE) genes, and related pathways identified in cassava in response to cold and drought stress, respectively. (A, B) The total number of each type of DAS event (A) and corresponding genes (B) in response to cold and drought stress detected by ssRNA-Seq. (C, D) Venn diagrams show overlap of genes with DAS events, DE genes and known cold-related (C)/drought-related (D) genes annotated by GO. (E) The Venn diagram shows overlap of cold- and drought-responsive DAS events. (F) The top five enriched GO categories of genes with DAS events and DE genes under cold and drought conditions. (This figure is available in color at *JXB* online.)

more focused analysis on the expression change of genes associated with alternative splicing regulation based on GO terms. A total of 103 genes encoding splicing factors were examined (Supplementary Table S6). These genes had only minor changes in gene level expression due to stresses, with 61.9% showing a similar expression pattern; instead, a similar comparison of splicing changes yielded much more condition specificity, with 68.6% exhibiting a differential PSI (dPSI) value

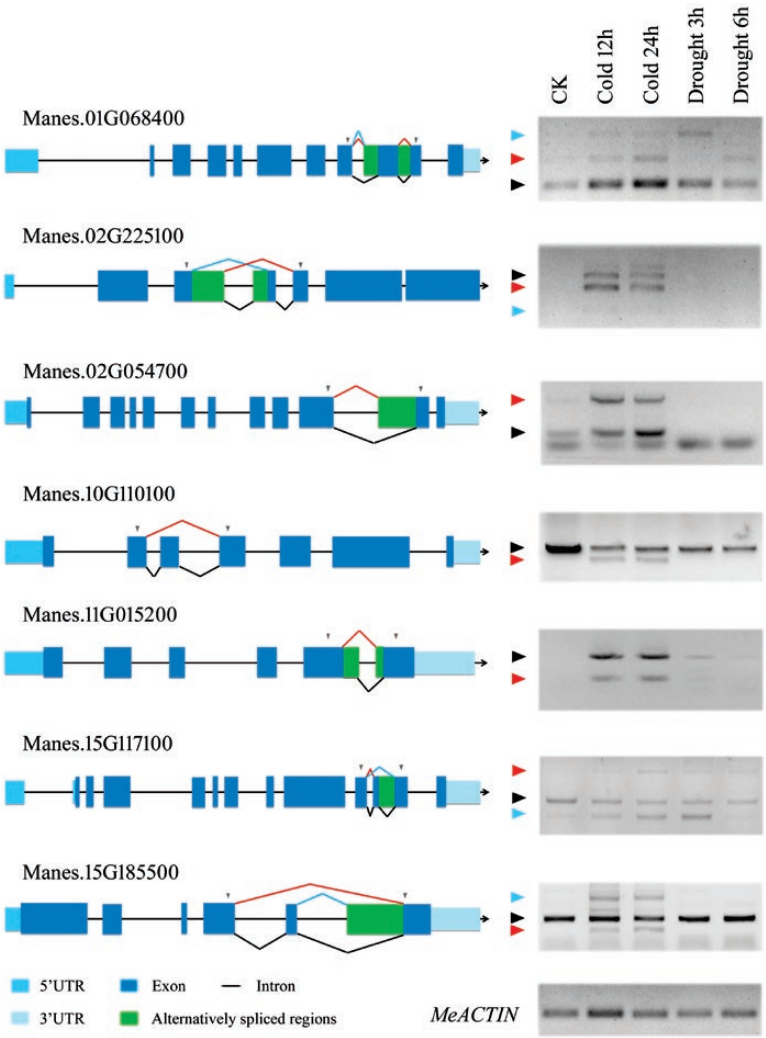


**Fig. 3.** Validation of alternative splicing events detected by ssRNA-Seq through RT–PCR. The alternative splicing events in seven randomly selected genes. In the left part of the gene model diagrams, the arrowhead indicates the position of PCR primers used for RT–PCR. The lines indicate constitutive splicing and alternativel splicing eventd. On the right hand side of the RT–PCR visualization, the black arrows indicate the bands of constitutively spliced alternatively spliced product bands. *MeACTIN* was used as an internal control. (This figure is available in color at *JXB* online.)

(Fig. 4B, C). Previous studies have shown that splicing factors are frequently alternatively spliced, often resulting in an increased or decreased number of alternative splicing events in their potential targeted genes (Zhang and Mount, 2009). Thus, the massive increase in the number of cold-responsive alternative splicing events may be due to the splicing changes of splicing factors after cold treatment.

### *Features of DAS events in transcript isoforms under cold stress*

To explore the features of DAS events under cold stress, we plot the length and dPSI distribution of each alternatively spliced. The length of skipped exons was 10–1450 nt with a median length of 96 nt, while the retained intron size was 14–7665 nt with a median length of 430 nt (Fig. 5A, B). The median extended length of A5SS events was ~67 nt, while the median extended length of A3SS was much shorter (~26 nt) (Supplementary Fig. S3A, B). We found that the dPSI of more IR events tends to be higher than 0, indicating that introns tend to be retained under cold stress, when the dPSI of ES events tends to be lower than 0, indicating that exons show a tendency to be skipped under cold stress (Fig. 5C, D). In total we found that 274 exons tend to be skipped and 1109 introns show a tendency to be retained under stress (Fig. 5E, F). Interestingly, we also found that long versions of A5SS or A3SS events tend to be enriched under stress (Supplementary Fig. S3C–F).

### *Identification of degraded transcripts with alternative splicing events by Degradome-Seq*

Alternative splicing plays an important role in modulating the abundance of productive transcripts for abiotic stress responses (Filichkin *et al.*, 2015; Laloum *et al.*, 2018). Since a large number of alternative splicing events are responsive to cold stress, we focused our attention on investigating the biological functions of cold-responsive alternative splicing events. Alternative splicing usually produces a PTC, which decreases the stability of mature RNA and serves as a trigger of nonsense-mediated decay. To predict which alternatively spliced sites or retained introns in alternatively spliced

A

| Gene ID | Name | Cold/CK Fold change | Drought/CK Fold change | AS event ID Cold | AS event ID Drought |
|---|---|---|---|---|---|
| Genes involved in plant hormone signal transduction (ABA, ETH), total number: 12 | | | | | |
| Manes.01G247300 | PYL 9 | 1.78 | 1.36 | TCONS_00003008 | -- |
| Manes.03G167400 | ETR 2 | 4.64 | 2.01 | TCONS_00016548 | -- |
| Protein kinases (CDPKs, MAPKs), total number: 18 | | | | | |
| Manes.10G059800 | CDPK1 | 1.72 | 2.02 | TCONS_00045008 | -- |
| Manes.07G012800 | MAPK1 | 1.84 | -1.62 | TCONS_00031095 | -- |
| RNA-binding proteins, total number: 13 | | | | | |
| Manes.11G164200 | CIRBP | 1.51 | 1.34 | TCONS_00048003 | -- |
| Manes.08G067200 | GRP1a | 3.62 | 1.09 | TCONS_00065639 | -- |

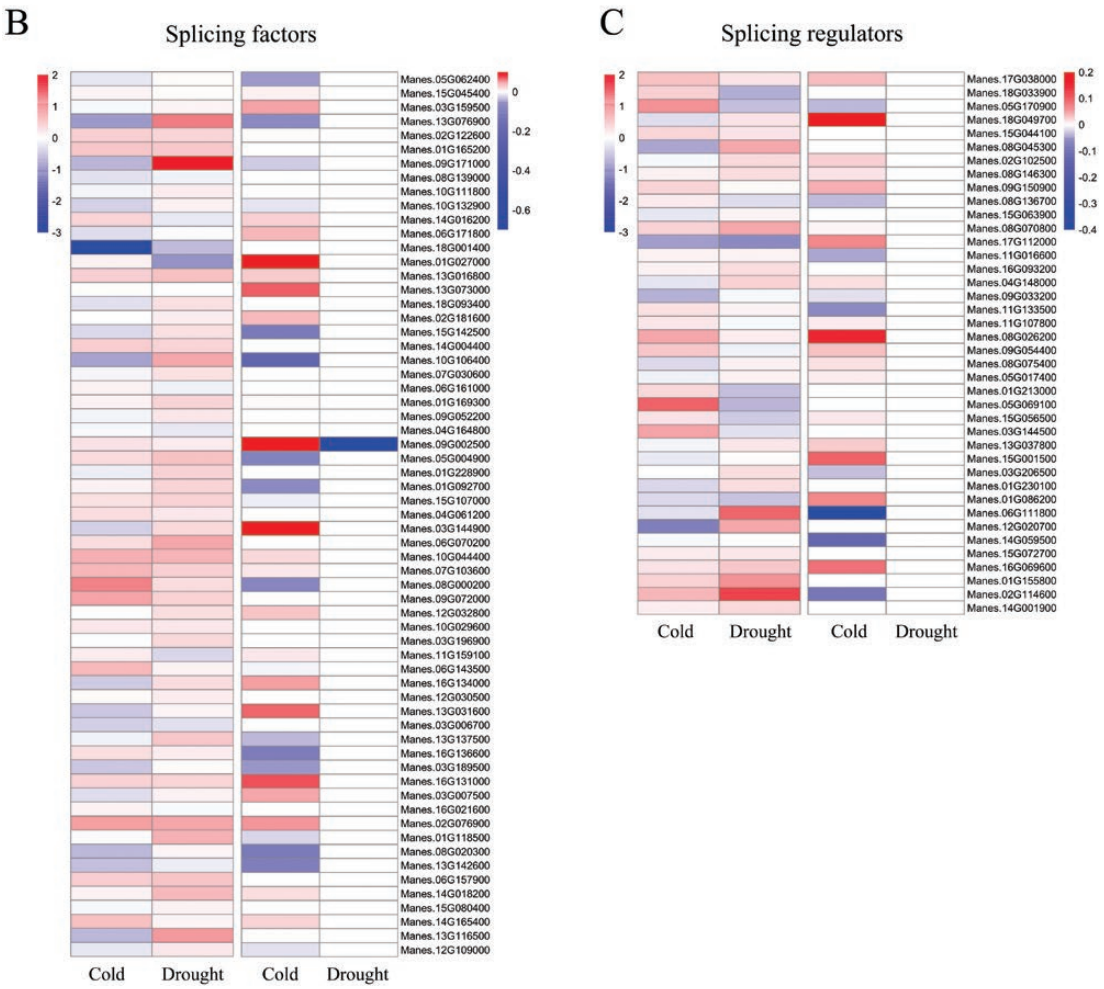


**Fig. 4.** Comparison analysis of genes with DAS events in response to cold and drought stresses. (A) Genes under three enriched GO terms, namely plant hormone signal transduction pathway, protein kinases (CDPKs and MAPKs), and RNA-binding proteins, exhibit alternative splicing under cold conditions. (B and C) Heat map of expression fold change level (left) and proportion spliced-in value difference (right) of the alternatively spliced genes encoding splicing factors (B) and regulators (C) in response to cold and drought stress. (This figure is available in color at *JXB* online.)

transcripts can generate PTCs, the CDSs of potential isoforms beginning from the start codon of known primary transcripts were extracted and translated to proteins (see the Materials and methods). Unexpectedly, among the 3292 cold-responsive alternative splicing events, 58.5% (1927 in total, i.e. 64 ES, 1233 IR, 244 A5SS, and 386 A3SS) of the events brought at least one potential PTC site to the isoforms as compared with the annotated primary transcripts (Supplementary Table S7). Focusing on these data, one essential question was raised: how many of the alternatively spliced transcripts were subjected to degradation during mRNA maturation? Given the comparatively low cost and high throughput of RNA degradome sequencing, this approach is attractive to identify degraded transcripts with alternative splicing events. Therefore, to address the question mentioned above, we continued the alternative splicing and degradome analysis.

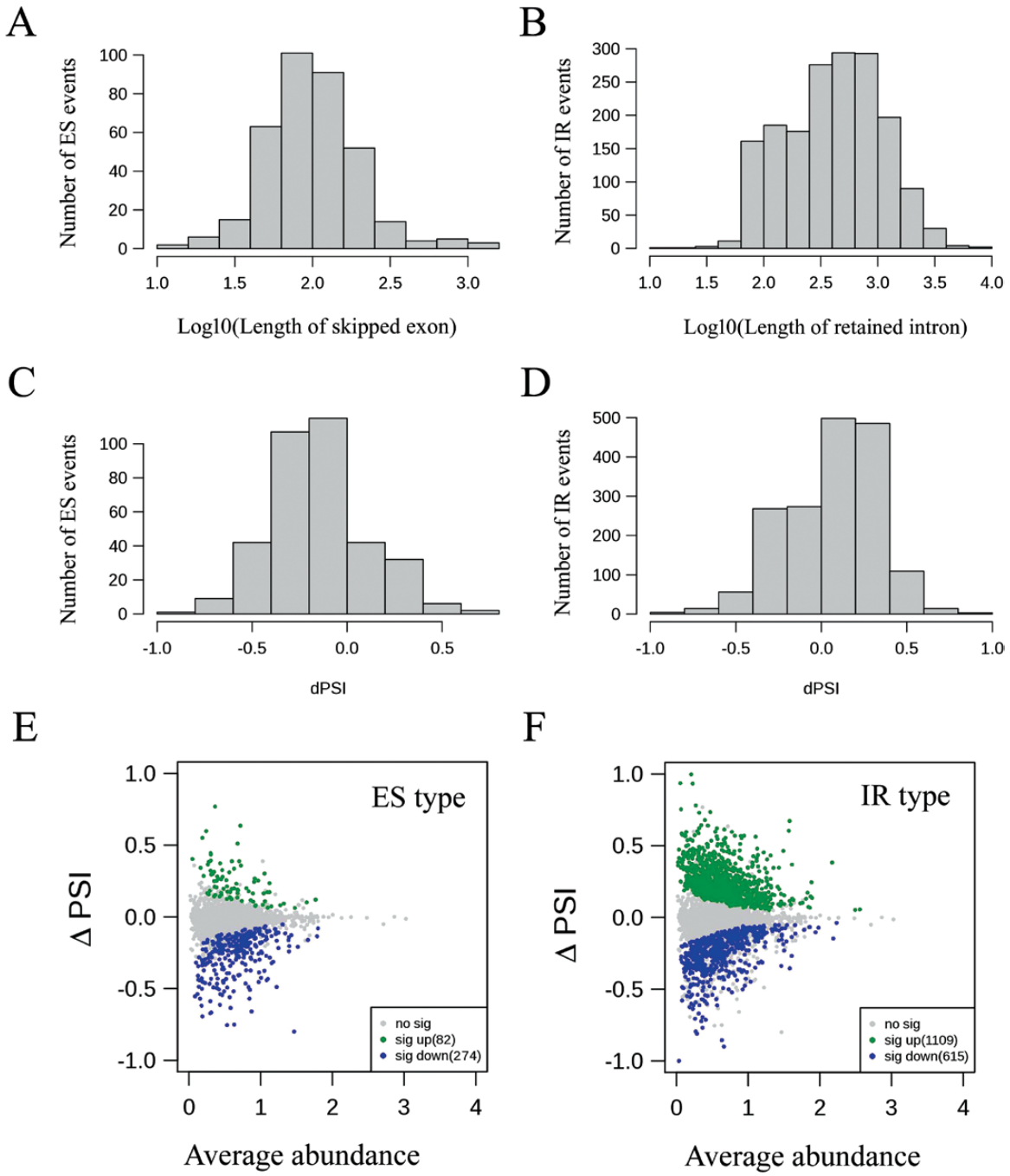


**Fig. 5.** Features of IR and ES transcripts under cold stress conditions. (A and B) Distribution of the sizes of skipped exons (A) and retained introns (B). (C, D) Distribution of the dPSI value of ES (C) and IR (D) events in response to cold stress. (E and F) MA plot showing the significance of differential PSI levels of ES (E) and IR (F) events in cold-treated samples compared with the CK. (This figure is available in color at *JXB* online.)

We generated two PARE (parallel analysis of RNA ends) libraries from cold-treated and untreated cassava seedlings and performed degradome sequencing. After discarding the low quality sequences, a total of 49 million clean tags remained. Approximately 50.6% of the total reads were mapped to primary and isoform transcripts, indicating that these transcripts have undergone degradation. Using the same SUPPA pipeline, we detected a total of 20 117 alternative splicing events from 7716 gene loci in CK samples and 21 177 from 7880 in cold-treated samples (Fig. 6A; Supplementary Table S8). Among these alternative splicing events in degraded transcripts, IR was the most abundant event under cold treatment, with a frequency of nearly 51% (Fig. 6B). By comparing all genes with alternative splicing events identified in the two sequencing data, we found that >79.3% and 80.4% of alternatively spliced genes under CK and cold conditions, respectively, in ssRNA-Seq data overlapped with those in Degradome-Seq data (Fig. 6C, D).

To measure the mRNA stability of each transcript, we initially calculated the proportion uncapped comparing the FPKM abundance of degraded mRNA with corresponding gene expression levels (Degradome-Seq FPKMs/ssRNA-Seq FPKMs) (Jiao *et al.*, 2008). We found that the proportion of uncapped

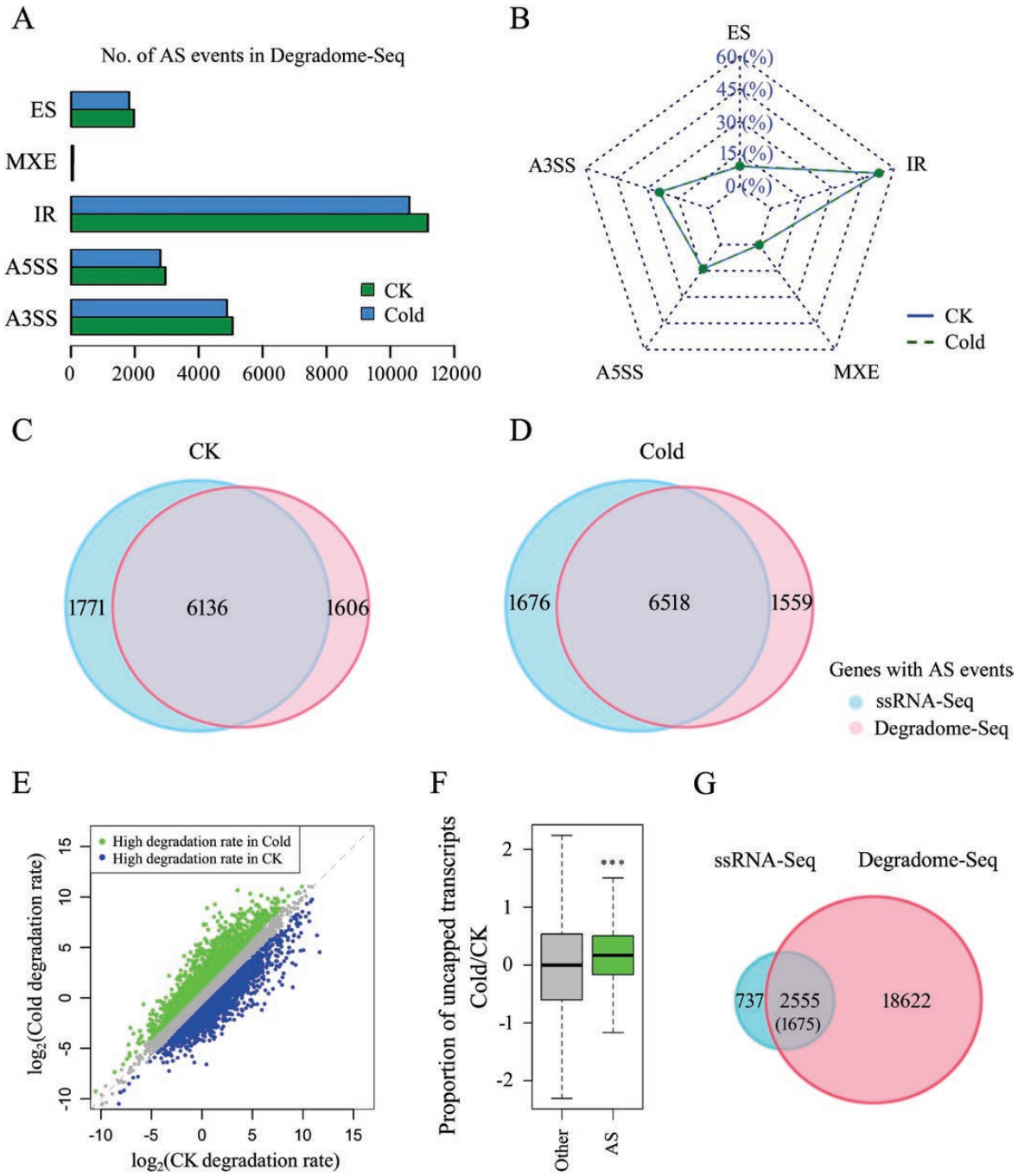


**Fig. 6.** Identification of degraded alternative splicing events using degradome sequencing data. (A) The total number of each type of alternative splicing event detected in the CK and cold-treated samples. (B) Radar plot showing the percentage of alternatively spliced types in the CK and cold-treated samples. (C and D) The Venn diagram shows the common and unique alternatively spliced genes that are detected by ssRNA-Seq and Degradome-Seq under normal (C) and cold conditions (D). (E) Scatterplot of the degradation rate of alternatively spliced transcripts between CK and cold stress samples. (F) Boxplot showing the difference in the proportion of uncapped transcripts with and without alternative splicing events in response to cold stress (*P*-value<2.2e$^{-16}$, Mann–Whitney test). (G) The Venn diagram shows DAS events identified by ssRNA-Seq and corresponding genes detected by Degradome-Seq. (This figure is available in color at *JXB* online.)

mRNA was globally changed after cold treatment (Fig. 6E). Next, we assessed the changes of the proportion uncapped between the cold and control samples for transcripts with and without alternative splicing events.This pairwise comparison revealed that no obvious bias was found toward a general decrease or increase in transcripts without an alternative splicing event during cold stress treatment. However, a significantly higher proportion of uncapped transcripts with alternative splicing events was observed when leaves were treated with cold stress than under control conditions (Fig. 6F). These results suggest that the stability of transcripts with alternative splicing events was decreased globally under cold stress. Notably, in the 3292 cold-responsive DAS events, 77.6% (2555 alternative splicing events from 1675 gene loci) were captured by Degradome-Seq (Fig. 6G).Taken together, these results demonstrated that a large proportion of alternatively spliced isoforms have undergone

degradation, possibly due to triggering NMD, while others such as IR-containing transcripts may be degraded by another pathway. To further determine the putative function of the degraded transcripts with alternative splicing events, GO analysis was performed. A total of 242 different biological processes, 81 different molecular functions, and 79 different cellular components were predicted (Supplementary Table S9). The most significantly enriched GO terms were involved in the three main categories: protein modification; cellular response to chemical stimulus; and response to temperature stimulus (Supplementary Fig. S4). Molecular function analysis found that a large number of genes with degraded alternative splicing events contained zinc ion binding activity (Supplementary Fig. S4). These results suggest that cold stress induces alternative splicing for certain genes which may generate unproductive transcripts to attenuate stress responses after low temperature exposure.

## Discussion

Plants cope with environmental changes by reorganizing their gene expression and metabolism to prepare for adverse conditions, which require strict control. Different levels of gene expression have been extensively reported and well characterized in cassava plants in response to cold and drought stress (Zhao *et al.*, 2015; Li *et al.*, 2017*a*); however, gene regulation at the post-transcriptional level is less understood. In this study, we performed an integrated multi-omics analysis for the first time to suggest that global gene regulation in response to cold and drought stress also takes place at the pre-mRNA splicing step. Iso-Seq data analysis showed that 31.6% of the protein-coding genes are alternatively spliced, which also made available complements to the cassava genome information. ssRNA-Seq data revealed a large number of alternative splicing changes at the transcript level in response to cold and drought stress, suggesting that alternative splicing can be largely regulated by abiotic stress. However, Degradome-Seq data indicated that DAS events are more likely to undergo RNA degradation.

A comparison of the types of alternative splicing present under drought conditions revealed a distinct distribution of categories compared with that seen under cold conditions. Previous studies showed that intron retention was the most prevalent splicing event in Arabidopsis (Wang and Brendel, 2006; Filichkin *et al.*, 2010; Marquez *et al.*, 2012; Calixto *et al.*, 2018); our results were consistent with this. We also observed that strong IR and A3SS induction occurred mainly in proteins with catalytic and binding activity when seedlings were exposed to cold treatment, but not to drought stress. As in our earlier study (Li *et al.*, 2017*a*), we showed that a total of 6103 genes involved in hormone signaling and metabolic pathways were significantly affected by cold stress. However, a greater number of genes (7462 in total) were differentially modulated in response to drought stress. Combined with this observation, we proposed that the abundance of cold-specific splicing events could alter the expression of corresponding genes and eventually influence cold stress response. Thus, further research should be conducted on the post-transcriptional regulation of cold-induced alternatively spliced genes.

Among cold-responsive DAS events, a large proportion of isoforms contained PTCs, which may trigger degradation of alternatively spliced transcripts during mRNA processing. For example, in Arabidopsis, RNA surveillance mechanisms, such as the NMD pathway, are considered a major determinant of the transcriptome (Kalyna *et al.*, 2012). To determine which alternatively spliced isoforms will be degraded or translated into proteins, we performed a systemic analysis of Degradome-Seq data. Our degradome study using cassava seedlings treated with cold stress showed that ~80% of the genes with alternative splicing events found in ssRNA-Seq were matched or overlapped by the Degradome-Seq analysis, and 1675 transcripts with at least 78% of DAS events were degraded. Such a large number of genes potentially generate unproductive transcripts, thus decreasing the abundance of the functional transcripts in the suppression of cellular processes including stress response and signaling processes. Inhibition of these processes could increase the negative effects of cold stress on the plant cell.

Alternative splicing events were triggered by abiotic stresses in cassava, particularly by cold stress, suggesting that the occurrence of DAS events under low temperature was not a random process. A crucial unknown raised in this study is how temperature changes regulate alternative splicing. Since the splicing factors and regulators played very important roles in both constitutive splicing and alternative splicing, we have closely examined the changes in their transcript levels and alternatively spliced variants in response to cold and drought stresses. In cassava, we obtained 156 genes annotated as encoding splicing factors or regulators; 103 genes contained splicing variation after stress treatments. Our data indicate that gene expression showed no dramatic stress-dependent changes in overall transcript levels. However, the relative levels of alternatively spliced isoforms was shown to change strikingly under cold stress, which is consistent with the results from previous studies (Duque, 2011; Palusa and Reddy, 2015). For example, in Arabidopsis, SR proteins have been shown to significantly influence alternative splicing processes, which appear to be modulated in a tissue-specific, developmentally regulated, and stress-responsive manner (Duque, 2011; Syed *et al.*, 2012). Previous work has also shown that loss of function of SR genes in Arabidopsis alters the splicing patterns of their own pre-mRNAs and those of several other genes (Yan *et al.*, 2017). Thus, in cassava, cold-induced changes in SR mRNA products could in turn alter the splicing of downstream targets, resulting in global transcriptome changes in response to cold stress. Further study of the relationship between alternative splicing and the splice patterns of splicing factor/regulator genes is necessary to explain how splicing factors/regulators affect alternative splicing.

In conclusion, we provide multi-omics data and reveal that cassava plants rapidly respond to abiotic stresses at the pre-mRNA splicing step to fine-tune the abundance of alternatively spliced transcripts. Our analysis revealed that alternative splicing can be differentially regulated in response to cold and drought stress. In particular, an mRNA alternative splicing imbalance could be responsible for decreased cold stress tolerance of cassava by increasing the degradation ratios of cold-responsive genes. Furthermore, our study also raises a central question

about how splice variants of splicing factors specifically regulate the alternative splicing under cold stress. Further investigation of this question may be valuable in dissecting mechanisms associated with alternative splicing-driven stress responses.

## Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Radar chart showing the percentage of alternative splicing types in CK, cold-treated samples of Iso-Seq data, the reference genome, and the merged result.

Fig. S2. MA plot showing significance of the differential proportion spliced-in level of alternative splicing events in stress-treated samples (cold or drought) compared with the CK.

Fig. S3. Features of splicing sites in A5SS and A3SS transcripts.

Fig. S4. GO enrichment analysis of degraded alternatively spliced genes detected by Degradome-Seq showing the top five enriched categories in biological process, molecular function, and cellular component, respectively.

Table S1. Summary of Iso-Seq raw data, classify report, cluster report, and mapped statistics.

Table S2. List of alternative splicing events identified by Iso-Seq data.

Table S3. List of the total alternative splicing events detected by ssRNA-seq and DAS events in response to cold or drought stress.

Table S4. List of the cold- and drought-related genes.

Table S5. GO terms of biological process, molecular function, and cellular component classification of genes with DAS events and DEGs under cold or drought stress.

Table S6. The 103 splicing factors/regulators showing isoform variation under cold or drought stress.

Table S7. Number of PTC-containing and non-PTC-containing alternative splicing events.

Table S8. Summary of identification of alternative splicing events using degradome sequencing data.

Table S9. GO terms of biological process, molecular function, and cellular component classification of alternatively spliced genes detected by Degradome-Seq under cold stress

Table S10. Summary of primers used in this study.

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