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Research paper

Alternative splicing coupled to nonsense-mediated mRNA decay contributes to the high-altitude adaptation of maca (*Lepidium meyenii*)

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ABSTRACT

Alpine plants remain the least studied plant communities in terrestrial ecosystems. However, how they adapt to high-altitude environments is far from clear. Here, we used RNA-seq to investigate a typical alpine plant maca (*Lepidium meyenii*) to understand its high-altitude adaptation at transcriptional and post-transcriptional level. At transcriptional level, we found that maca root significantly up-regulated plant immunity genes in day-time comparing to night-time, and up-regulated abiotic (cold/osmotic) stress response genes in Nov and Dec comparing to Oct. In addition, 17 positively selected genes were identified, which could be involved in mitochondrion. At post-transcriptional level, we found that maca had species-specific characterized alternative splicing (AS) profile which could be influenced by stress environments. For example, the alternative 3′ splice site events (A3SS, 39.62%) were predominate AS events in maca, rather than intron retention (IR, 23.17%). Interestingly, besides serine/arginine-rich (SR) proteins and long non-coding RNAs (lncRNAs), a lot of components in nonsense-mediated mRNA decay (NMD) were identified under differential alternative splicing (DAS), supporting AS coupled to NMD as essential mechanisms for maca's stress responses and high-altitude adaptation. Taken together, we first attempted to unveil maca's high-altitude adaptation mechanisms based on transcriptome and post-transcriptome evidence. Our data provided valuable insights to understand the high-altitude adaptation of alpine plants.

1. Introduction

How plants adapt to the harsh environment is one of fundamental importance in plant evolution. Alpine plants provide great resources to understand their adaptation to extreme stress environments. However, they remain the least well-studied population of terrestrial ecosystems today (Alonso-Amelot, 2008). Alpine plants are facing a shorter vegetation period, and the completion of life cycles are also in threatening (Billngs and Mooney, 1959; Bliss, 1971). Surviving the alpine landscapes, alpine plants are constantly challenged by extreme conditions and unpredictable environmental changes, such as strong wind, high ultraviolet radiation, low oxygen, low temperature or freezing, as well as fluctuating temperatures (Billngs and Mooney, 1968; Stoecklin et al., 2009). Therefore, alpine plants provide great resources to

Abbreviations: AS, alternative splicing; A3SS, the alternative 3′ splice site events; A5SS, the alternative 5′ splice site; CNCI, Coding-Non-Coding Index; CPC, Coding Potential Calculator; DAS, differential alternative splicing; DEGs, differential expressed genes; ES, exons skip; FDR, false discovery rate; FPKM, Fragments per Kilobase of transcript per Million mapped reads; GO, Gene Ontology; IR, intron retention; KEGG, Kyoto Encyclopedia of Genes and Genomes; lncRNA, long non-coding RNAs; MXE, mutually exclusive exons; NMD, nonsense-mediated mRNA decay; ORF, open reading frame; PTCs, premature termination codons; SCL, SC35-like; SEA, singular enrichment analysis; SR, serine/arginine-rich; UPF, up-frameshift

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investigate the adaptation mechanisms to alpine habitats. However, these exquisite habitats of alpine plants are threatened by the occupation of other plants under the influence of global warming (Alonso-Amelot, 2008). For example, tree lines have been advanced responding to climate warming during the last several years (Harsch et al., 2009). These invaluable resources may disappear in a foreseeable future. Therefore, a full understanding of their adaptation mechanism is urgently needed.

Alpine plants evolved various mechanisms to adapt to extreme ecological environments. Understanding how alpine plants adapt to these extreme conditions contributes significantly to evolutionary ecology. Many adaptive mechanisms have been reported in alpine plants. For example, thick glandular trichomes cover with high amounts of exudate to filter ultraviolet radiation was discovered in *Blakiella bartsiifolia*, an alpine plant in the northern Andes (Rodriguez-Hernandez et al., 2014). Alpine plants in the Páramo of San Felix had highly specialized physiological processes that had not been seen in lower altitude plants, such as accumulation of carbohydrates to anti-freeze, and synthesis of special lipids to modify cell membranes for flexibility and water permeability (Alonso-Amelot, 2008). Moreover, alpine plants have a tendency of polyploidy, because polyploids have selective advantages of heterozygous in unstable environments compared to their diploid relatives (Hagerup, 1932; Stebbins, 1966; Fawcett and Van de Peer, 2010). In short, evidence of alpine landscape adaptation accumulated in morphology, physiological processes, and genome changes. Nevertheless, the knowledge of transcriptional and post-transcriptional evidence is still relatively scarce.

Maca (*Lepidium meyenii*, 2n = 8x = 64) is a typical alpine species which provides a great example for examining the adaptation in high-altitude environments. Maca is a Brassicaceae crop which was originally cultivated in the Puna agro-ecological zones. It is an herbal plant and its root is a great medicine for human sperm health (Melnikovova et al., 2015). As a typical alpine plant, maca experienced whole-genome duplication ~6.7 M years ago and some genes evolved rapidly for adapting to the high-altitude harsh environment in a short time (Zhang et al., 2016a). With the dramatic changes in high-altitude environments, no studies, however, have investigated the transcriptome of maca in detail yet. How maca adapts to high-altitude environments in transcriptional and post-transcriptional level has not been well characterized.

The aim of this study is to investigate the high-altitude adaptation of maca on transcriptional and post-transcriptional perspectives. Based on 10 transcriptome libraries of maca root and one public transcriptome library of maca leaf (see Section 2.5 below), we analyzed the differential expressed genes (DEGs) of maca root under different time span (within a day and among three months), identified positively selected genes, investigated its characters of alternative splicing (AS) profile, and filtered serine/arginine-rich (SR) proteins and long non-coding RNAs (lncRNAs) under AS or differential alternative splicing (DAS). Our study investigated the high-altitude adaptation of maca in a detailed and thorough manner.

2. Materials and methods

2.1. Plant material collection and RNA extraction

Maca seedlings were planted in the Yungui Plateau (altitude ~3300 m). In total, 10 maca root samples were collected (see Table 1 for details), including each two samples collected in day-time (at 10 am and 2 pm, when soil temperature increased sharply) and in night-time (at 2 am and 6 am, when soil temperatures were lowest) on Oct 9, 2015, and each two samples collected on Oct 9, Nov 9 and Dec 9 in the year of 2015 (all collected at 2–4 pm). All root samples were immediately stored in liquid nitrogen and carried back to our lab for RNA extraction. Total maca root RNA was isolated using the RNA extraction Kit (Tiangen Biotech, Beijing, China), according to the manufacturer's protocol. Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and 1% agarose gel was used to check the quantity and quality of RNA samples. Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) was used to check the RNA integrity and concentration. Samples with RNA integrity number (RIN) ≥ 7, 28S:18S > 1, OD260/280 ≥ 2, and OD260/230 ≥ 2 were kept to construct cDNA libraries.

Table 1
Sampling information and data summary of maca root transcriptome.

Treatment		Replicates	Number of raw reads (million)	Number of clean reads (million)
Samples within a day	Day-time[a]	2	12,698	11,086
	Night-time[b]	2	11,745	10,285
Samples among three months	Oct[c]	2	9426	8258
	Nov[c]	2	10,246	8983
	Dec[c]	2	10,195	8971

[a] Samples were collected at 10 am and 2 pm on Oct 9, 2015.
[b] Samples were collected at 2 am and 6 am on Oct 9, 2015.
[c] Samples were collected on Oct 9, Nov 9 and Dec 9 in the year of 2015, respectively.

2.2. Library construction and Illumina sequencing

Paired-end cDNA libraries were constructed according to the standard protocol of NEBNext Ultra™ RNA Library Prep Kit for Illumina Inc. (NEB, USA). Briefly, magnetic beads attached with Oligo (dT) were used to enrich mRNA. The purified mRNA was broken into approximately 200 nt short RNA fragments. These short fragments were used as templates to synthesize the first strand of cDNA by using reverse transcriptase and random hexamer primers. The second strand of cDNA was synthesized with buffer containing DNA polymerase I and RNase H. The double-stranded cDNA fragments were blunt-ended and dA-tailed, then sequencing adaptors were ligated to these cDNA fragments. The ligation products with the insert size of 200 bp were selected and enriched by PCR amplification with specific adaptor primers. Finally, the cDNA libraries were sequenced by Illumina HiSeq™ 2500 platform with 125 bp paired-end reads. Raw reads have been deposited in the NCBI Sequence Read Archive under BioProject ID SRP142050.

2.3. Transcriptome assembly of maca root

We used a reference-based method (Bowtie/TopHat/Cufflinks/Cuffmerge pipeline (Trapnell et al., 2012)) to assemble transcriptomes of maca root. Raw reads from 10 maca root samples were collected to perform assembly. Adaptor sequences were removed and low quantity reads (ambiguous 'N' bases ≥5% or more than 20% of bases less than Q20) were filtered with custom python scripts. All reads were filtered against the human/bacteria/fungi/virus NR database to remove contaminants. Bowtie v2.2.3 (Langmead and Salzberg, 2012) and Tophat v2.0.12 (Kim et al., 2013) were used to map all the clean reads to maca reference genome [12]. The resulted BAM/SAM files which contained aligned records were further used to assemble transcriptomes by Cufflinks program v2.1.1 (Trapnell et al., 2010). Cuffmerge program (implemented in Cufflinks) merged the transcriptome assemblies from different conditions, and then Gffread program v0.9.12 (https://github.com/gpertea/gffread) was used to generate the transcripts sequences from the merged assembled transcripts. After removing transcripts caused by AS using in-house python script, unigenes with the longest sequences were kept.

2.4. Differential expression genes analysis

We identified DEGs of maca root within a day (night-time vs day-time) and among three months (Oct, Nov, and Dec). We mapped raw reads to maca genome using Bowtie/Tophat with default parameters. Expression of each unigene was normalized by Fragments per Kilobase of transcript per Million mapped reads (FPKM). To obtain the DEGs, we compared the FPKM of each unigene using DESeq2 v1.22.1 (Anders and Huber, 2010) method implemented in R package, and log2(|fold change of FPKM|) ≥ 1.5 and false discovery rate (FDR) corrected *P*-value (≤ 0.05) from Fisher's exact test were used to screen the DEGs. The plots were produced in R using the ggplot2 package (Wickham, 2016).

2.5. Identification of positively selected genes

We used gKaKs computational pipeline v1.3 (Zhang et al., 2013) to screen positively selected genes based on non-synonymous substitution (Ka) and synonymous substitution (Ks) rates of orthologues between maca and *Arabidopsis thaliana*. All calculations were performed with default parameters. Firstly, gKaKs used BLAT v360 (Kent, 2002) and bl2seq (implemented in BLAST v2.2.6 (Altschul et al., 1990)) to align *A. thaliana* CDSs to maca transcripts. Secondly, gKaKs used KaKs_calculator v2.0 (Zhang et al., 2006) to compute Ka/Ks ratios of orthologues. Thirdly, genes with Ka/Ks > 1 were considered as potential positively selected genes. After removing null values and genes with Ka/Ks < 1.5, the rest genes were considered as positively selected genes and were used for further analysis.

2.6. Alternative splicing events and differential alternative splicing events

We used ASTALAVISTA (Foissac and Sammeth, 2007) to identify AS events in maca. We analyzed maca root RNA-seq data (produced in this study) as well as maca leaf RNA-seq data (downloaded from NCBI, accession number SRR2960160 (Zhang et al., 2016a)) to get an overview of AS profile. The final merged gtf file (produced by Cuffmerge) was uploaded to ASTALAVISTA website (http://genome.crg.es/astalavista/) and run with default parameters. Five types of AS events were extracted, including alternative 3′ and 5′ splice site (A3SS and A5SS), mutually exclusive exons (MXE), intron retention (IR), and exons skip (ES). Further, the changes of 5 types of AS between different conditions (night-time vs day-time, Oct vs Nov, and Oct vs Dec) were investigated.

To identify genes under DAS, we used rMATS (Multivariate Analysis of Transcript Splicing) program v3.2.5 (Shen et al., 2014) to evaluate the difference in the exon or intron inclusion level of AS event under different conditions. DAS genes were filtered by a stringent threshold (|IncLevelDifference| ≥ 0.1, *P*-value ≤ 0.01, and FDR ≤ 0.05).

2.7. Identification of long non-coding RNAs under differential alternative splicing

We used three software to identify lncRNAs. We firstly identified non-coding RNAs by Coding-Non-Coding-Index (CNCI, v2.0) (Sun et al., 2013) with default parameters, a program distinguished coding and non-coding sequences by adjoining nucleotide triplets. The candidate non-coding sequences with FPKM > 1 were further screened through a Support Vector Machine-based classifier (Kong et al., 2007) by Coding Potential Calculator web server (CPC, http://cpc.cbi.pku.edu.cn/). Then, candidate lncRNAs were further filtered by Pfam Scan v1.3 (Bateman et al., 2000), which is a software package detecting non-coding transcripts by comparing to known protein/Pfam databases. Transcripts with protein coding potential predicted by any of the above tools were filtered out, and the rest transcripts with open reading frames (ORF) < 300 nt and length > 200 nt were further analyzed as lncRNAs. Moreover, the union sets of lncRNAs and DAS identified by rMATS were considered as lncRNAs under DAS. Finally, we used rmats2sashimiplot v2.0.3 (https://github.com/Xinglab/rmats2sashimiplot) to visualize 5 types of DAS lncRNAs between Oct and Dec. Moreover, the heatmaps were generated using the Pheatmap package, version 1.0.8 (https://cran.r-project.org/web/packages/pheatmap).

2.8. Gene annotation and enrichment

We performed gene functional enrichment analysis based on Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). The GO enrichment analysis was performed using agriGO v2.0 (Tian et al., 2017) or ClusterProfiler v3.8.1 (Yu et al., 2012) with the singular enrichment analysis (SEA) method. KEGG pathway enrichment was performed using KOBAS v3.0 (http://kobas.cbi.pku.edu.cn/) or ClusterProfiler. The GO topology and visualization of KEGG pathway were also produced by ClusterProfiler. Moreover, we also used DAVID web server (Dennis et al., 2003) for clustering enrichment analysis with default DAVID categories (UP_keywords, UP_SEQ_features, Interpro protein domains, and GO terms). All results were further screened with adjusted *P*-value of 0.05 as a threshold level of significance.

2.9. Real-time quantitative PCR verifications

To validate the reliability of our results, we used real-time quantitative PCR (RT-qPCR) to detect the expression levels of selected DEGs, lncRNAs, and SR proteins (primer information is shown in Supplemental file 1). The first cDNA was synthesized using EasyScript one-step gDNA Removal and cDNA Synthesis Supermix (TransGen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol. The qPCR was performed using Luna Universal Probe qPCR Master Mix (New England Biolabs, Ipswich, MA, USA) using a 20 μl reaction system, including 1 μl cDNA (100 ng/μl), 0.4 μl forward/reverse primer (10 μmol/l), 8.2 μl ddH_2O and 10 μl Luna Universal qPCR Master Mix. The cycles were according to the supplier's manual (40 cycles of 95 °C 15 s, 60 °C for 30 s). ACTIN2 was used as an internal control for gene expression normalization. The relative value of the gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

3. Results

3.1. Transcriptome assembly of RNA-seq datasets

To characterize the expression pattern of maca root, we sequenced 10 samples within a day (night-time and day-time) and among three months (Oct, Nov, and Dec). In total, 54,311,773,145 raw reads (135.8 Gb) were produced under the Illumina HiSeq 2500 platform. After removing adaptors, duplicated reads and low-quality reads, 47,585,030,640 clean reads (127.4 Gb) were generated (Table 1). Based on the deep sequencing data of maca root, we assembled the transcripts of maca root by Cufflinks pipeline (Trapnell et al., 2012) with a genome-guided method. In total, 108,745 transcripts corresponding to 106,458 loci were generated, with an average length of 1378 nt and N50 length 1902 nt (Table 2).

3.2. Identification of differential expressed genes of maca root

We used DESeq2 to perform DEGs analysis in maca root (see Supplemental File 2 for more details). Comparing to night-time, maca root up-regulated 990 genes and down-regulated 48 genes during day-time (Fig. 1A). The KEGG and GO terms enrichment analysis suggested that these up-regulated genes significantly enriched in plant immunity response. For example, significant KEGG terms (Table 3) including “plant hormone signal transduction” (ath04075) and “plant-pathogen interaction” (ath04626), and top GO terms (Fig. 2A) including “response to chemical stimulus” (GO:0042221), “response to stimulus” (GO:0050896), “response to chitin” (GO:0010200), “response to stress”

Table 2
Overview of maca transcripts identified by Cufflinks.

Item	Value
Total length (nt)	149,815,746
Total number	1378
N50 (nt)	1902
Mean length (nt)	1378
100–500 (nt)	22,649
500–1000 (nt)	27,133
1000–1500 (nt)	21,456
1500–2000 (nt)	14,695
> 2000 (nt)	22,821

(GO:0006950), "response to carbohydrate stimulus" (GO:0009743), and "response to organic substance" (GO:0010033). Although no significant KEGG terms enriched in these down-regulated genes, most top GO terms were found involved in ATP binding or similar proteins (Fig. 2B), such as "ATPase activity" (GO:0016887), "ATP binding" (GO:0005524), "adenyl ribonucleotide binding" (GO:0032559), "purine nucleoside binding" (GO:0001883), and "nucleoside binding" (GO:0001882).

DEGs of maca root among three months were summarized in Fig. 1B–D. Comparing to Oct, maca root up-regulated 746 genes in Nov and up-regulated 1263 genes in Dec. Comparing to Oct, nevertheless, maca root down-regulated 817 genes in Nov and down-regulated 1127 genes in Dec (Fig. 1B–C). Common up-regulated and common down-regulated gene numbers among three months were 403 and 395, respectively (Fig. 1D). To identify possible biological processes or pathways changed within three months, we performed KEGG and GO functional enrichment based on common up-regulated and common down-regulated genes. The results suggested that maca root up-regulated genes related to abiotic (cold/osmotic) stress tolerances from Oct to Dec. For example, the KEGG results (Table 3) suggested that common up-regulated genes enriched in metabolism of components related to cold/drought tolerance, such as "arginine and proline metabolism" (ath00330), "metabolic pathways" (ath01100) and "starch and sucrose metabolism" (ath01100). The GO functional enrichment results (Fig. 2C) suggested that common up-regulated genes were highly enriched in "response to cold" (GO:0009409), "response to abiotic stimulus" (GO:0009628), "response to osmotic stress" (GO:0006970), "cold acclimation" (GO:0009631), "response to water deprivation" (GO:0009414), and "response to water" (GO:0009415). Nevertheless,

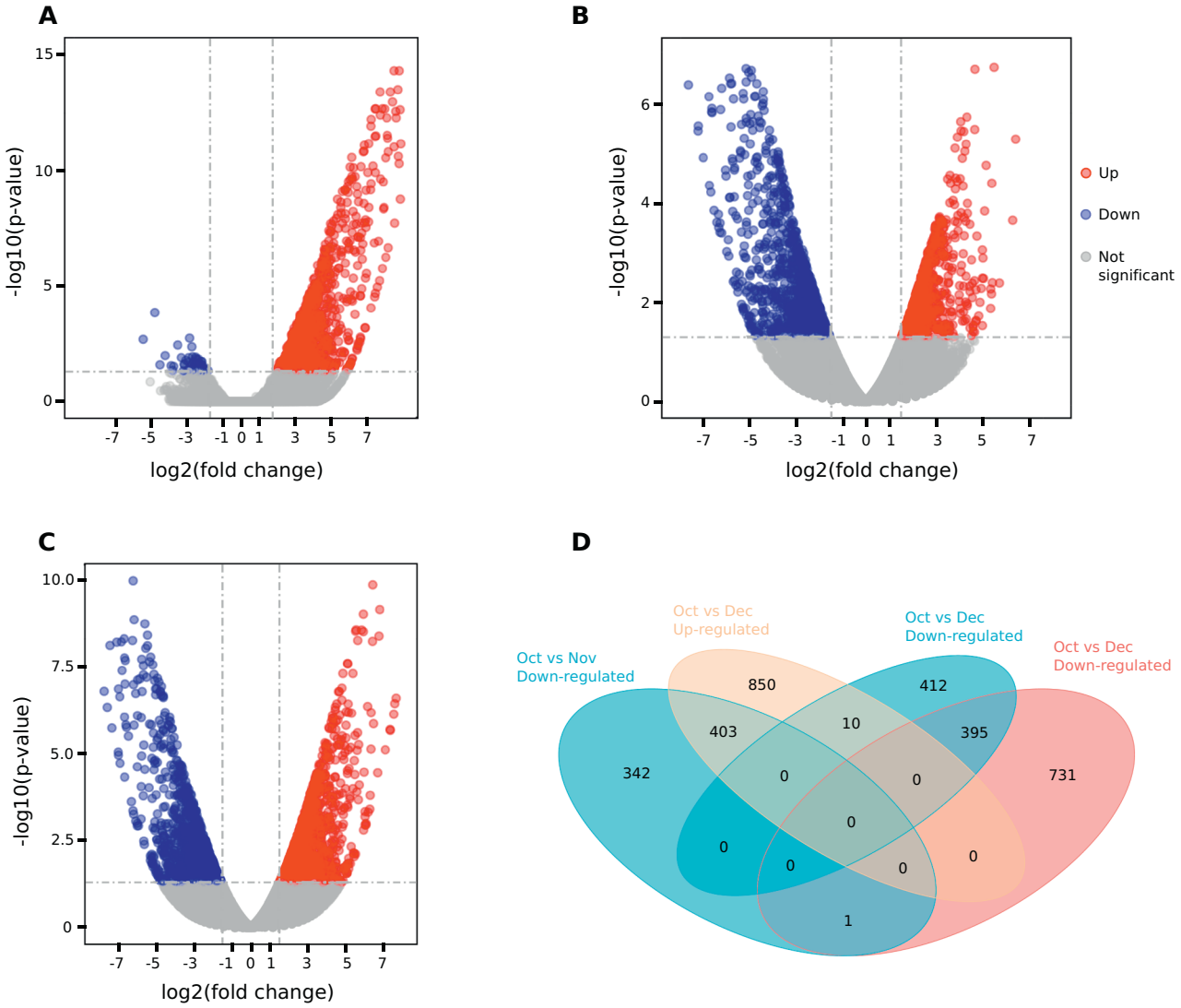

Fig. 1. Differential expressed genes (DEGs) of maca root within a day and among three months. Genes with log2(|fold change of FPKM|) ≥ 1.5 and false discovery rate (FDR) corrected *P*-value ≤ 0.05 from Fisher's exact test were considered as DGEs. (A) Volcano plot of DEGs: night-time vs day-time. (B) Volcano plot of DEGs: Oct vs Nov. (C) Volcano plot of DEGs: Oct vs Dec. (D) Venn diagram analysis of DEGs of maca root among three months.

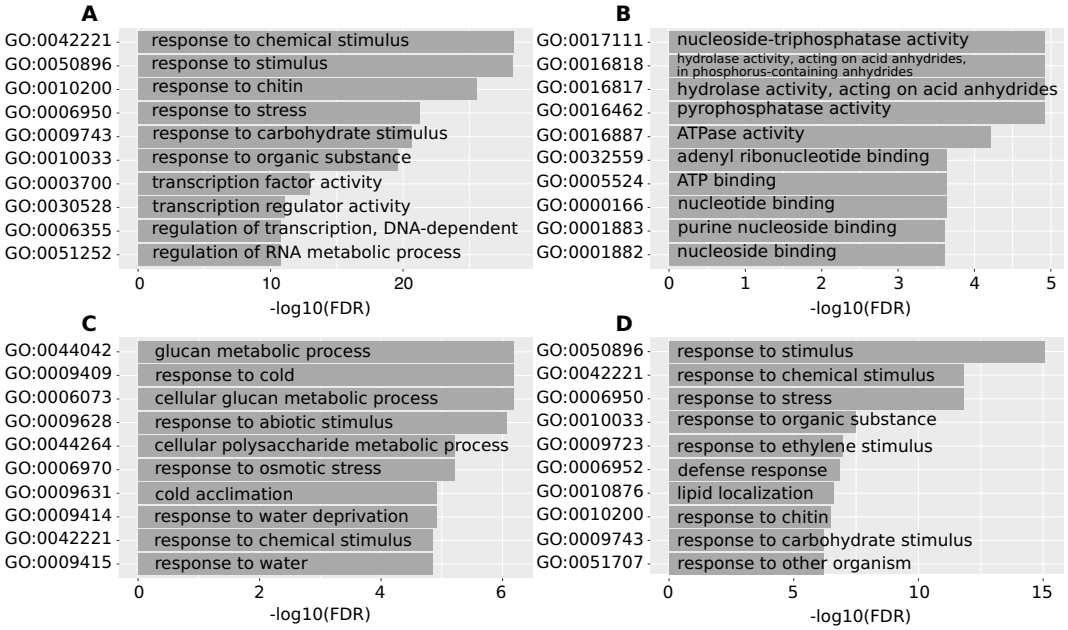

Fig. 2. Gene Ontology (GO) functional enrichment of DEGs within a day and among three months. In all analysis, only the top 10 false discovery rate (FDR) enrichment of GO terms were listed. (A) GO enrichment analysis for the significantly up-regulated genes within a day. (B) GO enrichment analysis for the significantly down-regulated genes within a day. (C) GO enrichment analysis for the common significantly up-regulated genes among three months. (D) GO enrichment analysis for the common significantly down-regulated genes among three months.

Table 3
Significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of differential expressed genes (DEGs) within a day and among three months.

Groups	KEGG pathway	ID	Count	Corrected *P*-value
Up-regulated genes during day-time	Plant hormone signal transduction	ath04075	15	1.19E−02
	Plant-pathogen interaction	ath04626	11	1.37E−02
	Carotenoid biosynthesis	ath00906	4	3.64E−02
Down-regulated genes during day-time[a]	NA	NA	NA	NA
Common up-regulated genes among three months	Arginine and proline metabolism	ath00330	5	7.99E−03
	Metabolic pathways	ath01100	32	3.34E−02
	Starch and sucrose metabolism	ath01100	7	4.96E−02
Common down-regulated genes among three months	Phenylpropanoid biosynthesis	ath00940	8	4.97E−03
	Biosynthesis of secondary metabolites	ath01110	24	5.89E−03
	Taurine and hypotaurine metabolism	ath00430	3	9.59E−03
	Plant hormone signal transduction	ath04075	9	2.62E−02
	Glutathione metabolism	ath00480	5	3.38E−02

[a] NA indicates that no significant KEGG pathways have been detected.

KEGG and GO enrichment of common down-regulated genes suggested that maca root down-regulated plant immunity related genes from Oct to Dec. For example, top KEGG terms of these genes including “plant hormone signal transduction” (ath04075), and top GO terms (Fig. 2D) of these genes including “response to stimulus” (GO:0050896), “response to chemical stimulus” (GO:0042221), “response to stress” (GO:0006950), “response to organic substance” (GO:0010033), “response to ethylene stimulus” (GO:0009723), “defense response” (GO:0006952), “response to chitin” (GO:0010200), “response to carbohydrate stimulus” (GO:0009743), and “response to other organism” (GO:0051707).

3.3. Filtration of positively selected genes

Using gKaKs pipeline (Zhang et al., 2013), we identified 12,228 orthologous unigenes between maca and *Arabidopsis*, of which 44 genes were identified as positively selected genes (Ka/Ks > 1, Supplement Table 1). After deleting the null ratio data and Ka/Ks < 1.5, 17 genes remained as positively selected genes. Based on annotation from DAVID web server (Dennis et al., 2003), we found that they are significantly enriched in genes related to mitochondrion or RNA editing (Table 4).

3.4. Alternative splicing events in maca

To investigate possible post-transcriptional regulatory mechanisms in maca, we extensively studied its AS profile and detected the changes under different conditions. In total, 74,599 AS events from 21,566 genes were identified, implying that nearly 20.26% of maca multi-exonic genes (21,566/106,458) underwent AS. The ratio of AS genes is relatively low compared to other plants (see Discussion below). Five types of AS events were extracted by ASTALAVISTA (Table 5), including A3SS (39.62%), A5SS (22.24%), MXE (4.74%), IR (23.17%), and ES (10.23%). MXE is the least frequent AS events, and A3SS were almost two-folds of A5SS events, which are consistent with most other plants (Lida et al., 2004; Ner-Gaon and Fluhr, 2006; Wang and Brendel, 2006; Barbazuk et al., 2008; Filichkin et al., 2010). Surprisingly, A3SS rather than IR predominate AS events in maca, which is in contrast to most other plant species, implying potential specific AS regulation in maca. The changes of AS between different groups were shown in Supplemental Fig. 1. Comparing to night-time, all 5 types of AS events decreased in day-time. From Oct to Dec, all 5 types of AS events also decreased, with a sharper decrease in Dec. Nevertheless, the A3SS events were always the most prevalent AS events.

Table 4
Gene set enrichment of positively selected genes by DAVID web server.

Category	Term	Genes	*P*-value	FDR
UP_KEYWORDS	Mitochondrion	acl, nadh, ccmB(ccb206), ccmC(ccb256), ccmFc(ccb452), atp4(orf25), mttB(orfX), rpl2, rps4	3.37E−07	3.10E−06
UP_KEYWORDS	RNA editing	ndhB, nad5, ccmB(ccb206), ccmC(ccb256), ccmFc(ccb452), atp4(orf25), mttB(orfX), rps4	4.74E−07	8.71E−06
GOTERM_CC_DIRECT	Mitochondrial membrane (GO:0031966)	nad1, ccmB(ccb206), ccmC(ccb256), atp4(orf25), mttB(orfX)	1.71E−04	2.56E−03

Table 5
Summary of five types of alternative splicing (AS) events between different species.

Species[a]	Latin name	A3SS[b]	A5SS[b]	MXE[b]	IR[b]	ES[b]
Arabidopsis	*Arabidopsis thaliana*	32.69%	17.59%	4.96%	36.05%	8.72%
Brachypodium	*Oryza sativa*	32.44%	15.51%	1.57%	42.89%	7.58%
Maize	*Zea mays*	22.80%	14.21%	2.26%	49.83%	10.91%
Populus	*Populus trichocarpa*	27.74%	17.60%	7.11%	39.14%	8.40%
Potato	*Solanum tuberosum*	18.25%	9.79%	1.06%	61.68%	9.22%
Rice	*Oryza sativa*	26.76%	14.35%	1.43%	48.42%	9.04%
Sorghum	*Sorghum bicolor*	34.39%	16.96%	1.66%	39.10%	7.89%
Maca	*Lepidium meyenii*	39.62%	22.24%	4.74%	23.17%	10.23%

[a] All data sets were re-analyzed using the same method with identical parameters.
[b] A3SS: Alternative 3′ Splice Site, A5SS: Alternative 5′ Splice Site, MXE: Mutually eXclusive Exons, IR: intron retention, ES: Exons Skip.

We performed KEGG and GO functional enrichment of genes under DAS which were identified by rMATS. The top significant enriched GO terms were illustrated in Supplemental Fig. 2. Their GO hierarchical structure (Supplemental Fig. 3) suggested that most genes under DAS clustered into ATPase related genes, including “ATP binding” (GO: 0005524), “ATPase activity” (GO: 0016887), “helicase activity” (GO: 0004386), and “catalytic activity, acting on RNA” (GO: 0140098). The KEGG enrichment results (Supplemental Fig. 4) suggested that most genes were involved in AS or eliminating aberrant mRNA transcripts, including “spliceosome” (ath03040), “mRNA surveillance pathway” (ath03015), “RNA degradation” (ath03018). The genes in “mRNA surveillance pathway” (ath03015) were shown in Fig. 3.

3.5. Identification of serine/arginine-rich proteins under alternative splicing

The SR proteins are one type of important RNA splicing regulators. They are involved in pre-mRNA splicing processes and play important roles in regulating AS by changing the splice site selection in a concentration- and phosphorylation-dependent manner (Graveley et al., 1999). In this study, 44 SR orthologous genes were identified in maca according to the annotation results. The heatmap (Supplemental Fig. 5) shows expression of the 44 SRs. Among them, 22 SRs were found under AS (Supplement Table 2), most of which belongs to SC35-like (SCL) subfamily, one of the three plant-specific SR subfamilies. Our result is consistent with *Arabidopsis* (Cruz et al., 2014), implying shared splicing regulation mechanisms under stress condition between maca and *Arabidopsis*.

3.6. Identification of long non-coding RNAs under differential alternative splicing

We identified 3396 lncRNAs in total using three software (CNCI, CPC, and Pfam-scan). Comparing to mRNA, lncRNAs generally expressed at a lower level, and were shorter in both total length and ORF length (Fig. 4). Transcripts with only one exon were found most prevalent both in mRNAs and lncRNAs. However, lncRNAs processed fewer exons, with 14 exons in max. All identified lncRNAs sequences are listed in Supplemental File 3. Moreover, lncRNAs under DAS were also filtered using rMATS. In total, 172 lncRNAs were identified to be differential alternatively spliced (five examples between Oct and Dec were illustrated in Fig. 5). The heatmap (Supplemental Fig. 6) shows expression of the 172 lncRNAs. Out of 172 lncRNAs, 117 lncRNAs have no orthologues in other plants (Supplement Table 3), implying potential maca-specific lncRNAs.

3.7. Validation by real-time quantitative PCR

Most of our RT-qPCR results were consistent with the RNA-seq data (Supplemental File 1). Of the 8 DEGs, 7 exhibited consistent expression patterns. Of the 5 lncRNAs and 5 SR proteins, all exhibited consistent expression patterns.

4. Discussion

To investigate how maca adapts to the high-altitude environment, we used RNA-seq to explore the transcriptional and post-transcriptional mechanisms of maca. We investigated DEGs in maca roots between different time spans (within a day and among three months), identified several maca genes under positive selection, explored AS profile of maca, and detected its changes under different conditions. Moreover, we identified components involved in AS and nonsense-mediated mRNA decay (NMD). In short, we investigated the mechanisms of high-altitude adaptation based on transcriptional and post-transcriptional evidence.

4.1. The DEGs of maca root within a day and among three months

We found that the up-regulated genes of maca root within a day mainly enriched in biotic stress responses (plant immunity genes), while up-regulated genes of maca root among three months mainly enriched in abiotic stress responses (cold/osmotic stress response genes). Comparing to night-time, up-regulated genes during day-time significantly enriched in plant immunity (Table 3, Fig. 2A). This could be the effect of dramatic variation of soil temperature between day and night (Supplemental Fig. 7A). The cold temperature during night-time may decrease the activity of plant pathogen. However, maca could activate more regulation factors related to plant immunity during day-time, such as WRKY, ERF, and NAC, most of which have been reported in *Arabidopsis* and/or rice (Pandey and Somssich, 2009; Gutterson and Reuber, 2004; Wang et al., 2009). In accordance with the present results, the previous study of two high-altitude plants in China, *Notopterygium incisum* and *N. franchetii* (Jia et al., 2017), have demonstrated that genes involved in plant-pathogen played important roles in high-altitude adaptation. The down-regulated genes results suggested that the reduction of ATPase activity, ATP binding and some other related proteins could be good for cold tolerance, which is similar to rice (Shen et al., 2014a).

Among three months, maca root up-regulated genes involved in abiotic stress (cold/osmotic stress), while down-regulated DEGs involved in plant pathogen defense (Table 3, Fig. 2C, D). Alpine environment is characterized by severe abiotic conditions that change dramatically. For example, the average soil temperature around maca decreased dramatically from Oct to Dec (Supplemental Fig. 7B). The

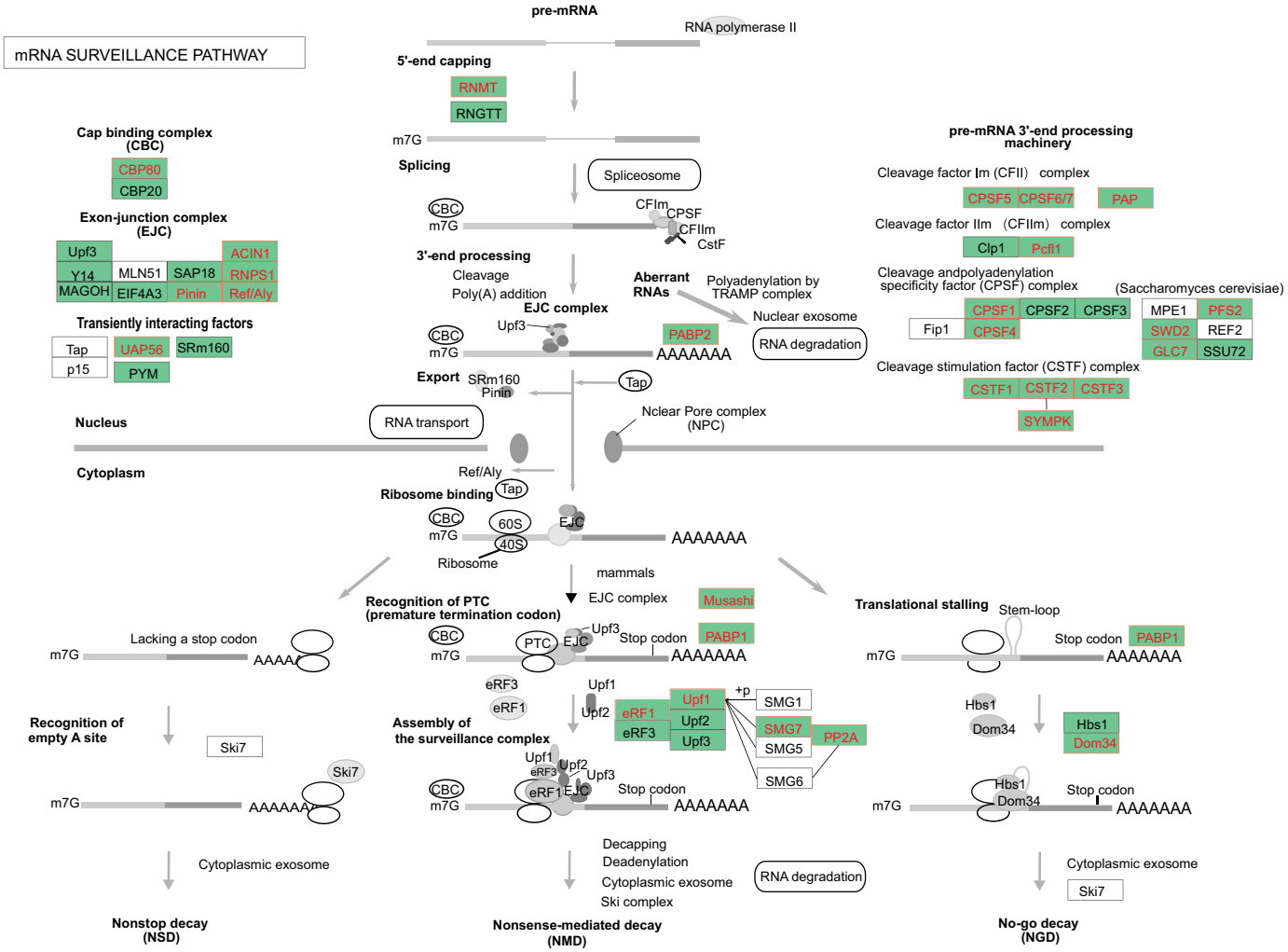

Fig. 3. The mRNA surveillance pathway adapted from the Kyoto Encyclopedia of Genes and Genomes (KEGG).

colder the soil temperature is, the fewer the pathogens exist around maca root. Thus, maca root is expected to reduce gene expression related to plant pathogen defense from Oct to Dec. According to our field observation, the soil around maca root was frozen in Dec during nighttime and thawing during day-time. Even short-term frozen may disable or injure plant tissue if not properly acclimated. Moreover, the damage may also occur during thawing (Levitt, 1980). Repeated cycles of freezing and thawing will amplify the injury (Alonso-Amelot, 2008). Thus, to adapt to these conditions, maca root up-regulated genes related to drought stress and cold tolerance, suggested that maca may have developed highly specialized physiological processes or particular strategies of cold adaptation, which is similar to other alpine plants (Alonso-Amelot, 2008).

4.2. Identification of positively selected genes

We identified 17 positively selected genes in maca, which could be involved in mitochondrion or RNA editing (Table 4). RNA editing is a mechanism to alter RNA sequence which is different from its DNA sequences (Shikanai, 2006). In plants, RNA editing happened on plastid genome (most events were found in mitochondria and a few events were found in chloroplasts), where mainly modified cytidines (C) to uridines (U) at specific sites (Covello and Gray, 1989). Besides to produce more proteins, RNA editing plays an essential role in correcting aberrant mutations in plastids and mitochondria (Smith et al., 1997; Gray, 2012). In some plants, RNA editing could regulate the mitochondrial gene expressions. For example, the intron splicing and/or RNA editing in wheat mitochondrial cox2 transcripts have been reported to be regulated by low temperature (Kurihara-Yonemoto and Handa, 2001). However, few robust evidence is documented about whether or not RNA editing and is associated with stress tolerance. Thus, it could be quite interesting to investigate the relationship between RNA editing and high-altitude adaptation in maca.

4.3. The alternative splicing of maca

AS is a pre-mRNA splicing process which produced more than one unique mRNA from one single gene (Baralle and Giudice, 2017). AS is a common mechanism of eukaryotic protein-coding genes. In this study, 74,599 AS events involving 21,566 genes were identified, which suggested 20.26% of maca multi-exon genes were alternatively spliced. The ratio of AS genes is lower than other plants, such as *Arabidopsis* (61%) (Marquez et al., 2012; Zhang et al., 2016b), rice (33%) (Zhang et al., 2010), *Glycine* max (63%) (Shen et al., 2014b), *Physcomitrella patens* (50%) (Chang et al., 2014) and *Zea mays* (40%) (Thatcher et al., 2014). Comparing to its relative species, the ratio of AS genes in maca reduced, partially could be attributed to the polyploidization of maca. This is consistent with the functional sharing model (Su et al., 2005) of duplicated/AS gene evolution, which suggests that polyploidization increases duplicated genes and decreases the AS events per gene.

The proportion of 5 types of AS events in maca is quite different from most other plant species. IR is considered as the most AS events in

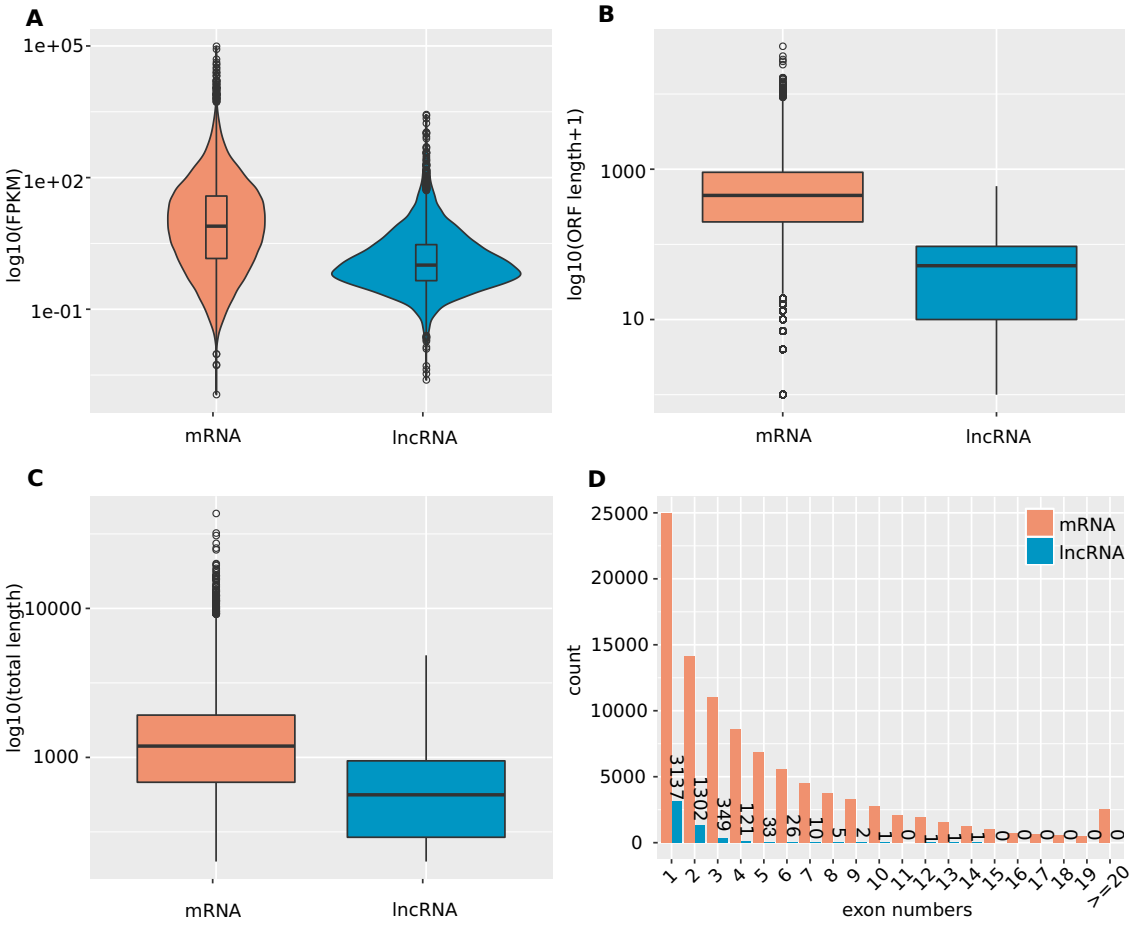

Fig. 4. Comparison of expression level and gene architecture between long non-coding RNAs (lncRNAs) and mRNAs. (A) Expression level indicated by log10(FPKM) of lncRNAs and mRNAs. (B) Distribution of open reading frames (ORF) length in lncRNAs and mRNAs. (C) Distribution of the total length of transcripts of lncRNAs and mRNAs. (D) Distribution of the exon number in lncRNAs and mRNAs. The counts of lncRNA with specific exon numbers are indicated above the bar.

both monocotyledons and dicotyledons, such as *Arabidopsis* (36.05%), *Brachypodium* (42.89%), Maize (49.83%), *Populus* (39.14%), Potato (61.68%), Rice (48.42%), and Sorghum (39.10%, Table 5, reviewed in (Mandadi and Scholthof, 2015)). However, our results suggest that it is not always the case. The most prevailed AS type in maca is A3SS (39.62%) rather than IR (23.17%), which is in contrast to most previous findings (Lida et al., 2004; Ner-Gaon and Fluhr, 2006; Wang and Brendel, 2006; Barbazuk et al., 2008; Filichkin et al., 2010) (but see teosinte (Huang et al., 2015)). This could be attributed to various reasons. AS has previously been thought to be regulated by splicing enhancers and silencers (Smith and Valcárcel, 2000; Chen and Manley, 2009). Recently, however, increasing evidence indicates that regulation of AS is a complex process and involves several factors and different mechanisms (reviewed in (Chen and Manley, 2009)). For example, AS events are highly associated with epigenetic features (Zhou et al., 2012). Comparing to un-methylated genes, the gene with methylation has more AS events, which have been discovered in invertebrate species (Flores et al., 2012). Thus, although further investigation is needed for a detailed mechanism, we cannot rule out the possibility that the epigenetic or DNA methylation features of maca may be unique and significantly different from other plant species.

The number of AS events in maca changed a lot under different conditions (Supplemental Fig. 1), supporting the idea that AS is frequently associated with stress environment (Mazzucotelli et al., 2008). Alpine environment changes dramatically in a short time. To survive the unfavorable conditions, plants employ pre-mRNA splicing as a mechanism to cope with stress via transcriptome plasticity (Dubrovina et al., 2013). For example, AS genes ratio in wheat increased from 12% to 40% under drought/heat stress (Liu et al., 2018). AS events were changed in *Arabidopsis* (Gan et al., 2011), *Vitis vinifera* (Vitulo et al., 2014) and *Gossypium davidsonii* (Zhu et al., 2018) when they were exposed to stress environments. Thus, the extreme environments in high altitudes have influenced the AS in maca significantly, which supports the argument that AS-based mechanisms are crucial in the plant adaptation to adverse environmental conditions (Dubrovina et al., 2013).

4.4. The AS coupled to NMD contributed to maca's high-altitude adaptation

We found that most genes under DAS were important components in NMD, supporting NMD as an essential mechanism for maca to cope with stress conditions. As a process conserved in eukaryotes, NMD can recognize and remove erroneous transcripts produced by AS, such as premature termination codons (PTC)-containing transcripts (Dubrovina et al., 2013; Arciga-Reyes et al., 2006). Aberrant AS transcripts increase under stress environments. Thus, plants employ NMD to degrade these aberrant mRNAs to maintain the stabilization of transcriptome. Both GO (Supplemental Figs. 2–3) and KEGG results (Fig. 3, Supplemental Fig. 4) supported that most DAS genes were factors involved in mRNA surveillance pathway, especially the NMD pathway (Fig. 3). For example, the UP-Frameshift protein 1 (UPF1) is an ATP-dependent factor which switches on mRNA degradation and is essential for normal NMD function (Kurihara et al., 2009; Kurosaki et al., 2014). UPF1 binds to UPF2 and UPF3 to format the surveillance complex which triggers its catalytic domain and helicase activity, and initiated mRNA degradation (Chakrabarti et al., 2011). These are consistent with GO results, which

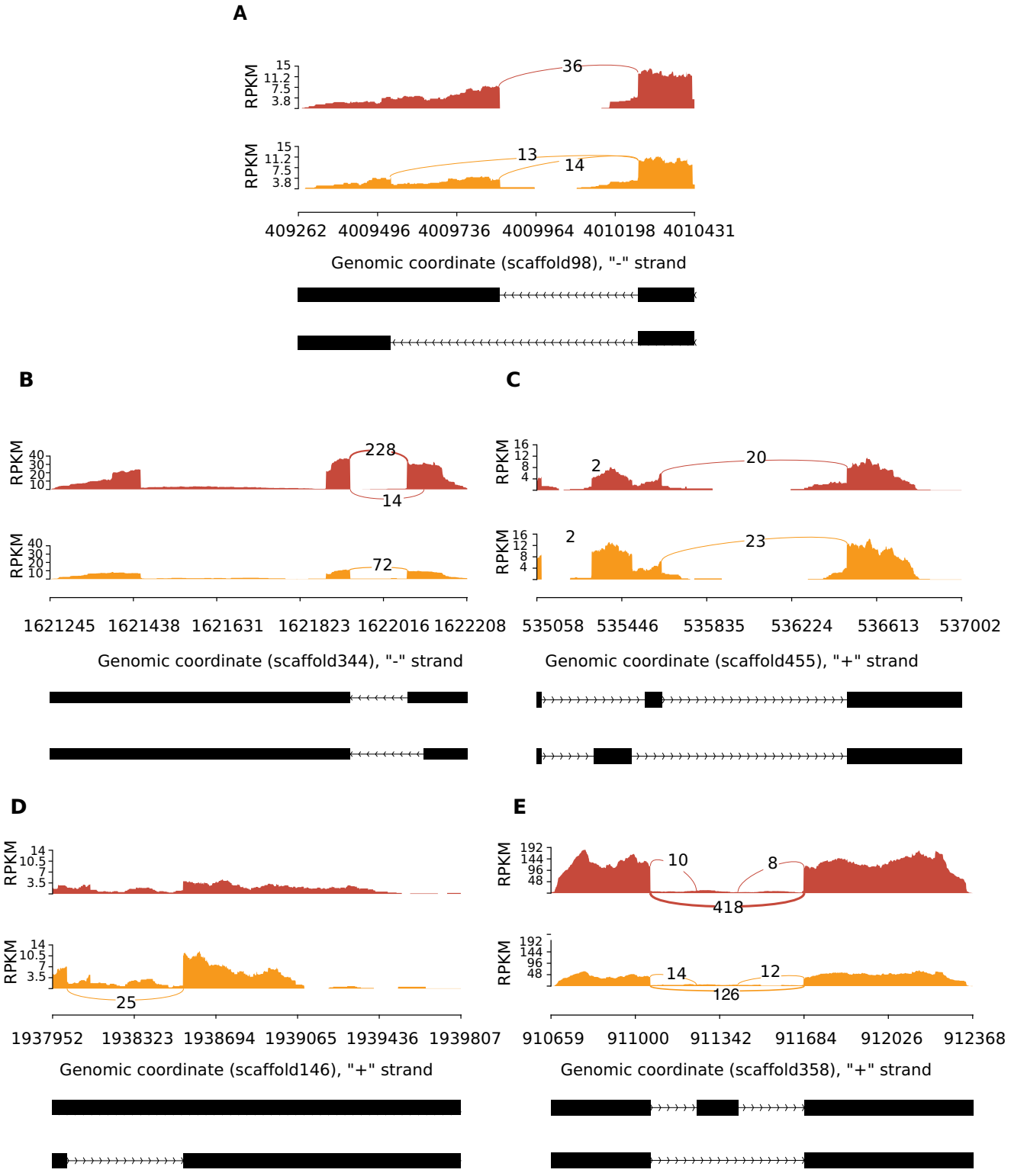

Fig. 5. Five examples of sashimi plots for differential alternative splicing (DAS) events of identified lncRNAs. Y-axis: per-base expression, x-axis: genomic coordinates, red: Oct, orange: Dec, numbers: junction spanning read counts averaged over two replicates, bottom: AS isoforms (exons in black, introns as lines). (A) A3SS events of lncRNA TCONS_00200978; (B) A5SS events of lncRNA TCONS_102549; (C) MXE events of lncRNA TCONS_00130413; (D) IR events of lncRNA TCONS_00026141; (E) ES events of lncRNA TCONS_00105822. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

suggest ATPase related genes significantly enriched. Other essential factors involved in NMD were also discovered in maca transcriptome, such as eRF1, SMG7 and PP2A. Therefore, NMD pathway may be crucial for maca to survive the high-altitude environments.

In maca transcriptome, we also discovered SR proteins as another important component in NMD. Most SR proteins function as bona fide

splicing factors in plants (Lopato et al., 1999; Ali et al., 2007), which bind specific splicing enhancer or suppressor sequences in the pre-mRNA (Erkelenz et al., 2013). By selecting different splice sites, SR proteins contribute to the AS process significantly (Manley and Tacke, 1996; Valcárcel and Green, 1996). Under stress conditions, SR proteins could regulate their expression by introducing PTC-containing transcripts, which could be recognized and degraded by NMD (Reddy, 2007; Ding et al., 2014a). In this study, we found 44 orthologous of *Arabidopsis* SR genes in maca, out of which 22 SRs were found under AS (most were classified into SCL subfamily, Supplement Table 2). Our results match those observed in earlier studies in *Arabidopsis* (Ding et al., 2014a) and *Gossypium davidsonii* (Zhu et al., 2018). Therefore, rather than regulated directly, these SR proteins in maca may regulate genes related to stress response mainly via splicing pathways, and may play critical roles in NMD.

Besides SR proteins, lncRNAs in maca may also play vital roles in AS regulation under stress conditions. LncRNAs refer to a class of RNAs that do not encode proteins with total length of > 200 nt. We identified 3396 lncRNAs in maca, which is comparable to other plants (Ou et al., 2017; Wang et al., 2015; Deng et al., 2018). The basic characteristics of maca lncRNAs are also similar to other plants (Zhu et al., 2017; Lv et al., 2016), such as lower expression, shorter ORF length, shorter total length, and enrichment of single exon transcripts (Fig. 4). Plants respond to external stress not only relied on protein-coding genes (Mittler, 2006; Hirayama and Shinozaki, 2010), but also relied on lncRNAs. LncRNA can hijack splicing regulators to modulate AS (Bardou et al., 2014). Recently, increased lncRNAs with multiple splice forms in plants have been characterized as stress-responsive pathways, such as COLDAIR, COOLAIR, LDMAR, At4/IPS1, npc48, and npc536 (Ding et al., 2012; Shin et al., 2006; Franco-Zorrilla et al., 2007; Ben Amor et al., 2009; Ding et al., 2014b). Here, we identified 172 lncRNAs in maca under DAS (five examples are illustrated in Fig. 5), 117 of them have no orthologous in other plants (Supplement Table 3), implying potential maca-specific lncRNAs. Thus, although more solid molecular evidence is needed, it is reasonable to speculate that these lncRNAs in maca may be essential for high-altitude stress responses in an AS manner.

In summary, AS coupled NMD could be essential for maca to cope with stress, which is crucial for high-altitude adaptation. Maca up-regulated plant immunity genes within a day, and up-regulated abiotic stress responses genes (cold/osmotic stress response genes) from Oct to Dec. Previous researches have established that plants may employ NMD-controlled gene expression to coordinate biotic and abiotic stress response, such as pathogen responses (Rayson et al., 2012), temperature, drought and salt response (Kalyna et al., 2011). Since important components, such as lncRNAs and SR proteins, involved in NMD were identified under DAS/AS, it is safe to conclude that DEGs could be regulated results of AS coupled to NMD mechanisms in maca's stress responses. Although further research is needed to clarify the involvement of AS coupled to NMD in maca's stress responses, our data provided ample evidence for a major function of AS-coupled NMD in shaping the maca transcriptome, having fundamental implications not only in eliminating aberrant mRNAs but also in plant stress response and high-altitude adaptation.

5. Conclusions

In conclusion, we investigated the transcriptional and post-transcriptional mechanism of maca to unveil its alpine plant adaptations. At transcriptional level, we found that maca root up-regulated plant immunity genes during day-time and up-regulated stress tolerance genes from Oct to Dec. We also found 17 positively selected genes which could be involved in mitochondrion. At post-transcriptional level, we found that maca had specifically characterized AS profile, which could be influenced by stress environments. Importantly, we identified a lot of components under DAS which are involved in the NMD pathway, suggested that the AS coupled to NMD would be an essential regulatory mechanism for maca's stress response and is crucial for high-altitude adaptation. Here, based on transcriptional and post-transcriptional evidence, our study offers a valuable insight to understand the adaptation of high-altitude plants, and provides invaluable resources to further explore mechanisms of harsh environment adaptation in plants.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2018.12.082.

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Author contributions

Chengjun Zhang and Runguang Xue conceived and designed the experiments; Zechun Su, Hong Yang and Guihua Jin performed the experiments and collected the data; Yong Shi, Hong Yang and Wenzhi Wang analyzed the data; Liangsheng Zhang, Guiqing He contributed materials; Yong Shi wrote the paper; Abu N. Siddique and Andan Zhu revised the manuscript; all authors revised and approved the final draft.

Conflicts of interest

The authors declare no conflict of interest.

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