Integrative hormone and transcriptome analyses underline the role of abscisic acid in seed shattering of weedy rice

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Abstract

Abstract Backgrounds: Weedy rice is one of the most severe weeds in paddy fields, strongly characterized by its high seed shattering level. Abscisic acid (ABA) serves as an abscission-accelerating signal and plays a critical role during abscission. However, mechanisms that link ABA and seed shattering remain elusive. In this study, we compared WR04-6 a shattering, and SN9816 non-shattering rice variety for genetic expression and ABA levels in the abscission zone (AZ) and the spikelet. Results : ABA content in WR04-6, particularly in AZ, was significantly higher than that in SN9816, and it increased remarkably prior to abscission. Transcriptomic analysis and qRT-PCR showed that the expression of NCED , the key gene in ABA biosynthesis, coincided with increased ABA content in AZ and increased significantly during the seed shattering process. Additionally, the expression of genes related biosynthesis and metabolism of IAA, GA, and ETH showed the greatest fold change. Phytohormone levels associated with ABA co-expression-prediction revealed a potential signal transduction network among plant hormones involved in regulating seed abscission. Conclusions: Altogether, our data strongly indicated that ABA contributes to seed shattering and appears to transiently cooperate with other hormones, triggering a hormone imbalance that leads to the downstream activation of AZ

Background

Taxonomically classified as the same species as cultivated rice (*Oryza sativa* L.) [1, 2], weedy rice (*Oryza. sativa* f*. spontanea*), is one of the most dominant and aggressive weeds found in paddy fields worldwide. It competes for nutrients, water, sunlight and other resources with cultivated rice and consequently affects crop production [3, 4]. Thus, for example, in China, weedy rice can reduce rice crop yield by 10 % - 50 %, and consequently cause serious economic loss [5]. Furthermore, it has many morphological and physiological traits related to weediness, and is strongly characterized by its high seed shattering rate [6, 7]. Weedy rice has evolved a system to control and adapt the time when the seed population reaches final maturity in relation to their nutritional status, thus allowing seeds to separate from the parent plant to facilitate seed dispersal and long persistence in the field. Excessive seed shedding in cereal crops is a major cause for yield loss, and consequently, for the loss of interest from the point view of farmers. Moreover, controlling the degree of grain shattering is an important challenge during cereal crop breeding. Therefore, studying the mechanism of seed shattering in weedy rice is important for an efficient regulation of crop productivity and effective management of weedy rice.

Seed shattering primarily occurs in a systemically regulated way at predetermined an anatomically distinct cell layers collectively called the abscission zone (AZ), further, seed shattering occurs in response to developmental, hormonal, and environmental cues [8]. Previous studies on wild rice (*Oryza rufipogon*) and *O.indica* identified several genes involved in seed shattering, which are necessary for AZ formation in the pedicel. Thus, *Shattering4* (*Sh4*) encodes a transcription factor with homology to Myb3 and is necessary for the development of a functional abscission layer in the pedicel [9], while the underlying gene, *qSH1*, encoding a BEL1-type homeobox transcription factor, often considered responsible for seed shattering in the *indica* subspecies [10]. Mutations in *qSH1* or *Sh4* show moderate shattering or even a

non-shattering phenotype due to the impairment of AZ development. Conversely, *SH5*, another BEL1-type homeobox gene, is highly expressed in AZ, and silencing it suppresses AZ development and inhibits seed shattering [11]. The *OsSHAT1* gene, which encodes an APETALA2 transcription factor, is also required for seed shattering by specifying AZ development in rice [12], and *OsCPL1* was the first recessive shattering gene to be identified, which encodes a carboxy- terminal domain phosphatase-like protein that acts as a repressor of AZ differentiation, thereby reducing seed shattering [13]. Additionally, other quantitative trait loci determine whether rice seeds shatter from or persist on the spikelets, such as *qSH3*, *SSH1* [14-16].

Phytohormones, such as abscisic acid (ABA), are considered to play important roles in regulating organ abscission [17]. The role of ABA as a possible activator of the abscission process was postulated as endogenous levels of ABA increase temporarily or constitutively during abscission, while the application of ABA-biosynthesis inhibitors, such as nordihydroguaiaretic acid (NDGA), reduced flower abortion in *Lupinus luteus* [18]. Transcriptomic evidence indicated that abscission-related ABA is biologically active, and its increased biosynthesis is associated with the induction of a specific ABA-responsive 9-cis-epoxycarotenoid dioxygenase gene (*NCED*) [19, 20]. These findings have led to the hypothesis that abscission is started by ABA activation. There is considerable controversy concerning the roles of ABA in the promotion of abscission. Several researchers view ethylene (ETH) as the primary regulator of abscission, citing correlations of abscission with ETH production, and the inability of exogenous ABA to accelerate abscission in many cases [21]. Moreover, the effect of ABA on abscission seems to depend on its interaction with auxin (IAA) or ETH, rather than being directly involved all by itself. Thus, ABA could have an intermediary role [22, 23]. Furthermore, ABA is believed to be the main regulator of ripening, playing a key role in the control seed maturation, in desiccation tolerance and dormancy. Thus, the determination of how ABA accumulation in seeds influences dormancy or seed abscission is crucial. However, knowledge about the role of ABA in the abscission process of crop species, such as rice, is extremely rudimentary, and questions remain unanswered about how ABA cross-talk with other hormones induce abscission in plant systems and how this should be interpreted.

To provide a comprehensive understanding of ABA-associated seed abscission at the molecular level in weedy rice, we measured endogenous ABA levels in spikelets of unpollinated flowers to the time of abscission, and in AZ at 15 days post anthesis (DPA) in shattering weedy rice, WR04-6, and in *temperate japonica* non-shattering SN9816 rice accessions. We further performed transcriptome analysis to examine the expression profiles of ABA biosynthesis genes during seed shattering and we verified the expression patterns deciphered by quantitative real-time (qRT)-PCR. Based on hormone levels associated with ABA co-expression network, we characterized and discussed the function of ABA during seed shattering. Our findings lay a solid foundation for in-depth understanding of the regulation of seed abscission.

Results

Identification of seed shattering phenotype

The two rice varieties under study differed significantly in their seed shattering and seed persistence abilities. Five days after pollination, the BTS value of WR04-6 had increased slightly increased and peaked at 85.75 g (Fig. 1A). As seeds developed, BTS dropped sharply to 22.70 g at 12 DPA. At this point, there was no natural abscission phenotype, but seeds would shatter with a slight external force. By 17 DPA, WR04-6 showed complete loss of BTS and grain dispersal, and stayed at this level until 30 DPA. Concomitantly, a small number of seeds completed the transition from milk to wax stage and the hull color changed to dark brown (Fig. 1B).

On the other hand, at anthesis, the BTS value of SN9816 was close to that of WR04-6, at 73.96 g, and similarly it decreased slowly from 5 to 17 DPA, i.e., values were nearly one-third of those shown at the earlier stage, although it were not low enough as to allow grain shattering.

AZ anatomy

Different degrees of seed shattering may be accompanied by different anatomical structures, therefore. Here, we used SEM to analyze such differences. AZ generally forms in rice pedicel tissue 16–20 days before heading [24, 25], therefore, AZs of -5 (booting stage), 0, and 7 DPA were used for SEM. We found no obvious phenotype difference in WR04-6 between booting and anthesis (Fig. 1C). However, while developing into seed shattering phenotype, part of the seeds of WR04-6 showed a narrow gap under the palea and the lemma, with only a small portion of the tissue being connected (Fig. 1C). In contrast, the rachilla of SN9816 remained unchanged from -5 to 7 DPA (Fig. 1C). In summary, AZs of WR04-6 and SN9816 were morphologically different.

The biological function of ABA during seed shattering

ABA concentrations in AZ tissue of WR04-6 and SN9816 at 15 DPA were 30.97 and 9.15 ng/g, respectively, i.e., a three-fold difference (Fig. 2A).

ABA level dynamics during spikelet development of the two varieties were detected (Fig. 2B). ABA concentration significantly increased from 5 to 12 DPA (7.70 and 19.17 ng/g, respectively), consistently with BTS values measured at 12 DPA. Subsequently, ABA concentration decreased continuously during mid- to late-phase embryogenesis, and the lowest concentration was detected at 35 DPA (3.88 ng/g; Fig. 2B). Further, ABA concentration trend from -5 to 12 DPA was similar in SN9816 and WR04-6, initially decreasing in both genotypes and then increasing, although the ABA concentration was lower in SN9816 than in WR04-6 during the same period. Then, ABA concentration in SN9816 reached 17.72 ng/g at 17 DPA, which was 1.18 times that of WR04-6 (14.96 ng/g), and when the seeds reached physiological maturity, ABA level in SN9816 was higher than in WR04-6.

OsVP1, a global regulator in the integrated seed maturation and germination developmental program, mediated the ABA response during mid- to late-phase embryogenesis [26-28]. Thus we performed immunoblot analysis and found that there was no significant difference in VP1 protein content at different stages of seed development between WR04-6 and SN9816 (Fig. 2C-D, Fig. S1).

Comparative transcriptome analysis in AZ tissues

To elucidate the molecular basis of the observed differences in seed abscission, we performed RNA-Seq to investigate the genome-wide gene expression profile in WR04-6 and SN9816 at 15 DPA. After filtering out genes with low expression, we identified a total 7082 differentially expressed genes (DEGs), including 2926 up-regulated and 4156-down-regulated DEGs (fold change >2, false discovery rate <0.05; Fig. 3A, Table S1), which were classified into 128 biochemical pathways, mainly including biosynthesis of secondary metabolites, metabolic pathways, phenylpropanoid biosynthesis, alpha-Linolenic acid metabolism, starch and sucrose metabolism, and fatty acid metabolism (Fig. 3B).

As hormones act as internal cues to initiate the abscission process, hormone biosynthesis- and signaling-related DEGs were analyzed in detail. Overall, 39 DEGs involved in ABA biosynthesis/signaling were detected (Table 1, Table S2). *OsNCED3* and *OsNCED5* genes were detected as up-regulated in RNA-Seq analysis (Fig. 3C). Furthermore, among the six DEGs encoding a putative aldehyde oxidase that catalyzes the conversion of abscisic aldehyde to ABA, five genes were down-regulated and only one was up-regulated (Table S2). In addition, two DEGs, *OsPYL3* and *OsPYL7*, encoding ABA receptor family (PYR/PYL), were down-regulated simultaneously. Moreover, the expression of genes encoding PP2C family (OsABIL1, OsABIL3, OsSIPP2C2) and SnRK2 family (OsSAPK1, OsSAPK6), which are involved in ABA signal transduction, were significantly higher in AZ tissue. Furthermore, two types of ABA-responsive protein genes (*HVA22E* and *GEM*) responded differently to the seed shattering process.

Genes related to plant hormone biosynthesis and signaling pathways

The greatest differential expression of genes among the most abundant AZ-enriched transcripts was found in those involved in phytohormone synthesis and metabolism. We found that 17, 21, and 9 genes were related to IAA, ETH and GA, respectively (Table 1, Table S3). Among the 17 IAA-related DEGs, there were 16 down-regulated genes encoding IAA biosynthesis intermediates (*OsFIB*, *OsYUCCA3*, *OsYUCCA5*, *OsYUCCA7*, *OsYUC9*), GH3 protein (*OsGH3-2*, *OsGH3.1*, *OsGH3-11*) (Table S3) and AUX/IAA protein among others, and only one up-regulated gene. Among ethylene-related genes, i.e., four genes (*ACO1*, *ACO2*, *ACO5* and *ACO7*) and four putative *ACO* genes (*LOC_Os09g39720*, *LOC_Os08g30100*, *LOC_Os08g30080*, and *LOC_Os03g63900*) encoding for 1-aminocyclopropane-1- carboxylate oxidase (ACO), which is the key enzyme in ethylene biosynthesis, all were strikingly down-regulated, except for *ACO2* and *LOC_Os08g30100*. In this study, nine GA-related genes were found to be involved in seed shattering. GA 2-oxidase, encoded by *OsGA2ox* genes, can deactivate bioactive GAs or their precursors irreversibly to reduce bioactive GA levels. Our analysis showed that *OsGA2ox* were up-regulated. Furthermore, expression of *OsKS*, *OsKOS* and *OsGA20ox* genes, which are responsible for the GA biosynthesis pathway, were down-regulated. In addition, we found five and six genes related to jasmonic acid (JA) and cytokinin (CK), respectively. All JA -related DEGs were up-regulated whereas the four DEGs involving in CK signaling were down-regulated (Table S3).

To provide a general view on the functions and processes that change in AZ tissue at the last stage of abscission, protein–protein interaction (PPI) network for the phytohormone related proteins was

predicted. The gene expression data (log2 fold change, i.e., log2FC) for DEGs were mapped to the PPI network. The network included genes related to ABA/IAA/ETH/GA biosynthesis and transduction pathways (Fig. 3D). *NCED3* had a strong connectivity with *GA2ox5* and *GA2ox9* genes of the GA pathway, while *NCED1* and *NCED4* genes were correlated with IAA-related genes *OsIAA1* and *OsSAUR8*.

Spatio-temporal expression pattern of ABA biosynthesis related genes

To monitor changes in ABA biosynthesis pathway, we quantified the gene expression levels of the ABA biosynthesis pathway in AZ and the spikelet from -5 to 15 DPA. Significant differences were observed in the expression patterns and magnitude of induction for some of the key genes. Quantitative real-time (qRT)-PCR analysis showed that the expression of *OsZEP* and *OsNCEDs* remained at a basal level in the spikelet and were specifically up-regulated in the AZ of WR04-6 and SN9816. In the AZ of WR04-6, the expression level of *OsNCED2* and *OsNCED4* were barely detectable, while *OsNCED1*, *OsNCED3* and *OsNCED5* expression exhibited a similar trend, increasing throughout the abscission stage (Fig. 4). The expression level of *OsNCED3* decreased after 15 DPA, while the expression of *OsNCED1* and *OsNCED5* still increased. In SN9816, *OsNCEDs* expression was lower than that in WR04-6 during the studied period.

Measurement of IAA, GA_3, and ETH levels in spikelet

To further verify RNA-Seq analysis and investigate the role of other hormones in the abscission process, we quantified the levels of IAA, GA_3, and ETH in the spikelet at different development stages in WR04-6 and SN9816 (Fig. 5). Accumulation of IAA, GA_3, and ETH in WR04-6 exhibited similar patterns to those in SN9816, while they were lower in WR04-6 than in SN9816.

Discussion

High seed shattering is an adaptive trait of weedy rice for seed dispersal and is the main trait related to the co-evolution of weedy rice and cultivated rice. Therefore, weedy rice represents a unique model system for studying the genetic basis of seed shattering. Consequently, extensive research has been conducted on the abscission characteristics of weedy rice [29, 30]. Genetic studies on AZ have been most extensive in rice, identifying several genes that, when mutated, lead to loss of abscission. Previous studies mostly focused on one or a few genes in the genetic pathway controlling AZ development. However, the physiological and biochemical aspects underlying the abscission process remain poorly known. Thus, understanding the mechanisms that regulate seed abscission is of great significance to weedy rice management, which is subject to high seed abscission rates during maturation stage.

In our study, BTS was similar for weedy rice accession (WR04-6) and cultivar (SN9816) at anthesis. However, at 12 DPA, WR04-6 showed a remarkable decrease in BTS accompanied by an increased seed shattering level. The seed shattering time trend observed in WR04-6 was consistent with several weedy rice accessions, and BTS values were in a similar range to those previously reports in others studies, where seed shattering ranged between 120 and 20 [10], or 220.1 and 4.5 g [31], suggesting that most weedy rice accessions may have a similar growth and development process. Rice AZ is located in the

rachilla, whereby we hypothesized that AZs should be anatomically different between WR04-6 and SN9816. SEM showed that, AZ displayed significant change one week post anthesis in WR04-6, with part of the seed having a narrow gap between lemma and rachilla, whereas there was no change in the control cultivar, SN9816 (Fig. 1C). Although previous studies showed that AZ formation is a universal prerequisite for abscission, the difference in AZ anatomy may be another phenotypic trait of weedy rice adapted to seed shattering [32].

The physiological model of abscission has basically four steps: differentiation and development of the AZ tissue, acquisition of competence to respond to abscission signals, activation and execution of abscission, formation of a protective layer and post-abscission trans-differentiation [33]. The responses of AZ cells to internal and external abscission-triggering signals are mediated by phytohormones [17]. Generally, ABA accumulation in seeds is low during early embryogenesis but increases during the transition when the developing embryos to the maturation phase, usually peaking around mid-maturation. ABA levels usually decline abruptly during late seed development, particularly during the maturation drying phase [34]. To verify our initial hypothesis that ABA is involved in the regulation of seed shattering in weedy rice, in this study, ABA levels of both spikelet and AZ were determined. In spikelets, ABA accumulation of the both varieties showed a similar trend (Fig. 2B). However, both in AZ and spikelet (Fig. 2A-B), ABA levels in WR04-6 were higher than those in SN9816, indicting ABA content variation, particularly in the AZ, may be associated with abscission. The situation seems to be similar to that in apple, in which case, a statistically significant correlation was calculated between fruitlet abscission and ABA content [19]. Additionally, ABA plays a fundamental role in acquiring embryonic dormancy during seed maturation [35]. As OsVP1 serves as a seed specific transcription factor functioning primarily on late embryo functions such as desiccation tolerance and dormancy [36], its level was further investigated by western blot in this study. Comparison of gray value, VP1 protein level in WR04-6 was slightly lower than that in SN9816 at different stages of spikelet development, implying that increasing ABA in the spikelet might be responsible for seed abscission rather than seed dormancy (Fig. 2C-D). Together, differences in hormone level and protein expression pattern between accessions suggest that ABA was involved in the process of signal perception and transduction in the last stage of organ abscission.

ZEP and the NCED catalyze the first committed steps of ABA biosynthesis, producing xanthoxin, which is thought to be the main rate-limiting reaction [37]. It is worth noting that transcript abundances of genes encoding the enzymes responsible for ABA biosynthesis (*OsNCED3*, *OsNCED5*) were significantly increased during seed abscission. We confirmed gene expression with qRT-PCR analysis and found the same results as in the RNA-Seq experiment. Unlike the ABA biosynthesis pathway, the ABA signaling pathway was stimulated by ABA in AZ, which was evident by the increased observed in expression of *PP2C* (*OsABIL1*, *OsABIL2*, *OsSIPP2C2*) and *SnRK2* (*OsSAPK1*, *OsSAPK6*) genes acting immediately downstream of the receptor genes and the decrease in *PYL* (*OsPYL3*, *OsPYL7*), the ABA receptor gene. These results agree with previous studies on tomato [38], citrus [39] , *Lupinus luteus* [8], and sugarcane [40] organ abscission, which showed differences in ABA biosynthesis (*NCED*) and signal transduction (*PYR/PYL*, *PP2C*, *SnRK2*). Thus, gene expression and transcriptomic profiling studies have reinforced the

view that seed shattering appears to rely on up regulation of ABA level and the results further confirmed that ABA participated in important regulation during abscission in different species or organs [18-20].

Having established a clear correlation between ABA levels and abscission, we asked how ABA cross-talk with other hormones inducing abscission in plant systems may occur, as this may be more important than merely ABA absolute concentration. For years, the idea that ABA itself activated organ separation has been controversial and cooperation with other hormones in controlling generative organ abscission cannot be excluded. Wide-ranging studies are helpful for understanding the complicated mechanisms regulating the process of organ detachment, and interactions among ABA, IAA, ETH, and GA at all developmental stages of different plants have been well documented. Thus, for example, excess ethylene accumulation were observed in tomato and maize mutants characterized by an ABA deficit [41, 42]. Contrastingly, ETH and IAA are well-known pivotal effectors of plant organ abscission, and have been shown to act antagonistically to control organ abscission [43]. In this study, profiling of global gene expressions in AZ revealed DEGs involved in IAA, ETH, and GA pathways (Table 1, Table S3). Furthermore, PPI predictions (Fig. 3D) associated with hormone levels of ABA, IAA, ETH, and GA (Fig. 5) revealed a potential signal transduction network of plant hormones involved in regulating seed abscission. Based on these results, we hypothesized that increasing ABA levels could inhibit the expression of genes related to the IAA/GA pathway, thereby affecting plant hormone homeostasis in response to seed abscission.

Conclusion

In summary (Fig. 6), transcriptomic analysis revealed a gene expression profile in AZ that shows the typical characteristics of the phytohormone response to seed shattering, manifested by up-regulation of ABA, as well as inactivation of IAA/GA-related genes. These results were validated by measurement of phytohormone level and qRT-PCR detection. The results reported herein support a model for the function of ABA in seed shattering regulation of weedy rice. Further work is necessary to ascertain the biological significance of ABA-related genes and to elucidate the molecular mechanisms underlying the interaction among phytohormones to control abscission in weedy rice.

Methods

Plant Materials and Growth Condition

WR04-6 (*Oryza. sativa* f. *spontanea*), with seed shattering, red pericarp, and black hull phenotype, is a typical weedy rice in Liaoning Province. The seeds of WR04-6 were collected and preserved by our institute. *Temperate japonica* (*Oryza sativa*) cultivar Shennong9816 (SN9816) is a non-seed shattering variety, which was bred by our institute and used as a control plant in this study. The experimental materials were grown in the germplasm resources field at Rice Research Institute of Shenyang Agricultural University, Liaoning Province, China. Seeds were sown on April 15, with seedlings transplanted to their final locations on May 26. Plants were spaced 30.0 × 13.4 cm apart, and fertilizer and water management followed the local standard management.

Owing to variation in heading dates in the test populations, booting initiation and heading dates were recorded to ensure the correct timing of phenotypic evaluation and sampling. The emergence of flag leaf was marked as the beginning of booting stage. Booting stage was sampled at a stage when anthers were fully developed, and was referred to as -5 DPA. To detect the dynamic changes of ABA content during the growth and development of WR04-6 and SN9816 and verify the biological function of ABA during seed shattering, at -5, 5, 10, and 15 DPA, nine individuals (three biological replicates with three plants per replicate) were randomly sampled from each variety and AZ tissues were collected by manually cutting at approximately 2 mm of the abscission fracture for qRT-PCR detection. To reduce the influence of some residual seeds in the sampling period on ABA concentration and distinguish whether ABA accumulation in spikelet of weedy rice acts on organ abscission or seed dormancy, and spikelets from -5, 5, 12, 17, and 35 DPA were sampled (three biological replicates with three plants per replicate) for hormone level and western blot analysis. All the samples were quick frozen using liquid nitrogen and stored at -80 °C.

Seed shattering measurment

BTS was used as a quantitative standard to evaluate seed shattering at 0, 5, 12, 17 DPA. BTS is inversely proportional to seed shattering and measures the maximum amount of weight (g) a single flower or grain can hold before releasing [31, 44]. To value seed shattering, five spikelets or grains were selected from the main panicles in five plants for shattering tests using a digital force gauge (aiPLi, China). An individual seed was detached from the panicle by holding the seed with a clip, and the peak measurement on grain removal was recorded.

Morphological analysis of AZ

To observe the morphological differences at abscission zones (the rachilla below the floret) between WR04-6 and SN9816, spikelet samples from both varieties were collected at -5 (booting stage), 0, and 7 DPA for SEM analysis and observed using a Hitachi TM3030 (Japan). At least three inflorescences per variety were collected and dissected.

ABA , IAA, GA_3, and ACC extraction and analysis

The levels of ABA, IAA, GA_3, and ACC were determined by Zoonbio Biotechnology Co., Ltd. Approximately 0.5 g of fresh sample was finely ground in liquid nitrogen and extracted with 5 mL extraction buffer composed of isopropanol/hydrochloric acid and 8μL internal standards (1 μg/mL) were added to each sample tube. The mixture was shaken at 4 °C for 30 min. Then, 10 mL dichloromethane (CH_2cl_2) was added, and the sample was again shaken at 4 °C for 30 min. The sample was then centrifuged at 13,000 rpm for 5 min at the same temperature, and the lower, organic phase was extracted. The organic phase was dried under N_2, dissolved in 400 μL methanol (0.1% methane acid) and filtered through a 0.22 μm filter membrane. ACC determination was achieved by adding an external standard method.

The purified product was then subjected to high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis and the methods were modified from those described by You et al.

[45]. Three independent replicates were performed for each experiment containing three biological repeats.

Western blotting

A primary antibody targeting OsVP1 was generated by the GenScript (GenScript, Nanjing Co, Ltd.) using a synthetic peptide (SKQPKPSPEKPKPKC) derived from OsVP1. The anti- Bip-2 and secondary antibodies were obtained from TaKaRa (Dakin, China). Total protein of 5, 15, 25, and 35 DPA seeds were extracted using the Minute^{TM} Total Protein Extraction Kit for plants (Invent Biotechnologies, Inc., America). Protein concentration was determined with the BCA Protein Assay Kit (Nan Jing Key Gen Biotech Co□Ltd, China). The obtained proteins were separated on a 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoresis separation, the proteins were transferred to a polyvinylidene fluoride membrane for protein profile analysis or immunoblot analysis. Quantification analysis of the protein band intensities from immunoblot was performed by software Gel-Pro Analyzer 4 (Switzerland).

RNA- sequencing

AZs (≤ 2 mm in length) were collected at 15 DPA from WR04-6 and SN9816. Total RNA samples were isolated for library construction. The cDNA libraries were sequenced using Illumina HiSeq^{TM} 2500 system by Gene Denovo Co. (Guangzhou, China). RNA-Seq data were processed, assembled and annotated and RNA-Seq reads were examined to remove low-quality (Q-value < 20) reads. The reference genome for RNA-Seq analysis is Nipponbare genome (*Oryza sativa japonica*; http://plants.ensembl.org/Oryza_sativa/Info/Index). Cleaned short reads were aligned to all exon sequence by Bowtie2, and expression abundance was calculated by RNA-Seq by Expectation-Maximization with default parameters. Genes with an expression Log2 ratio and a false discovery rate < 0.05 were significant DEGs. Then, DEGs were subjected to enrichment analysis of GO function and KEGG pathways, and string database was used to construct the PPI network based on hormone related DEGs.

RNA Isolation and Real-Time qRT-PCR

Total RNA was isolated using TaKaRa MiniBEST Plant RNA Extration Kit (TaKaRa, Dakin, China), while PrimeScript ^{TM} RT Master Mix (TaKaRa) and TB Green ^{TM} Premix Ex Taq ^{TM} II (Tli RnaseH Plus) (TaKaRa) were used for synthesizing the first strand cDNA and quantitative real-time PCR (qRT-PCR), respectively. The transcript levels of genes were normalized to the reference gene *OsActin* following the $2^{-\triangle Ct}$ method. Three biological duplicates were quantified for qRT-PCR analysis. The gene specific primers used in the qRT-PCR are listed in Supplementary Table S4.

Statistical Analysis

Data were statistically analyzed by analysis of variance and for significance ($P < 0.05$) of treatment differences were tested using Duncan's test on SPSS software version 19.0. Results are presented as means ± standard deviation (SD) of three replicate.

Abbreviations

ABA: Abscisis acid; IAA: Auxin; ETH: Ethylene; NCED: 9-cis-epoxycarotenoid dioxygenase gene; ZEP: Zeaxanthin epoxidase; NDGA: nordihydroguaiaretic acid; RNA-Seq: RNA sequencing; qRT-PCR :Quantitative real-time; BTS: Breaking tensile strength; SEM: Scanning electron microscopy; PPI: protein-protein interaction

Declarations

Acknowledgments

Not applicable

Author's Contributions

D.M. and H.L. conceived the project. H.L. and Y.H. conducted gene expression phenotypic measurement; J.S., and F.L. analysed and interpreted the data; H.L. drafted the manuscript; D.M., J.S., and F.L. critically revised the manuscript. All authors read and approved the paper.

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Availability of data and materials

Data is being upload to NCBI database.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing Interests

The authors have no competing interests to declare.

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Supplementary Files Legend

Additional file 1: Figure S1. Determination of the accumulation of OsVP1 with specific antibody by immunoblot analysis.

Additional file 2: Table S1. Total differentally expression genes (DEGs) statistical data result of WR_AZ vs SN_AZ.

Additional file 3: Table S2. Differentially expressed genes involved in ABA metabolism and signaling.

Additional file 4: Table S3. Differentially expressed genes involved in IAA /ETH /GA /JA /CK metabolism and signaling.

Additional file 5: Table S4. Gene-specific primers for qRT-PCR analysis.

Table

Table 1 Differential expression patterns of plant hormone metabolism and signaling related genes in comparison to the AZ between WR04-6 and SN9816

Hormone	Total No. of DEGs	Up regulated	Down regulated	Function
ABA	39	5	9	Biosynthesis
		1	-	Catabolism
		14	10	Signal transduction
IAA	17	-	5	Biosynthesis
		-	3	Catabolism
		-	8	Signal transduction
ETH	21	3	6	Biosynthesis
		7	5	Signal transduction
GA	9	-	6	Biosynthesis
		2	1	Catabolism
JA	5	1	-	Biosynthesis
		4	-	Signal transduction
CK	6	1	5	Signal transduction

Figures

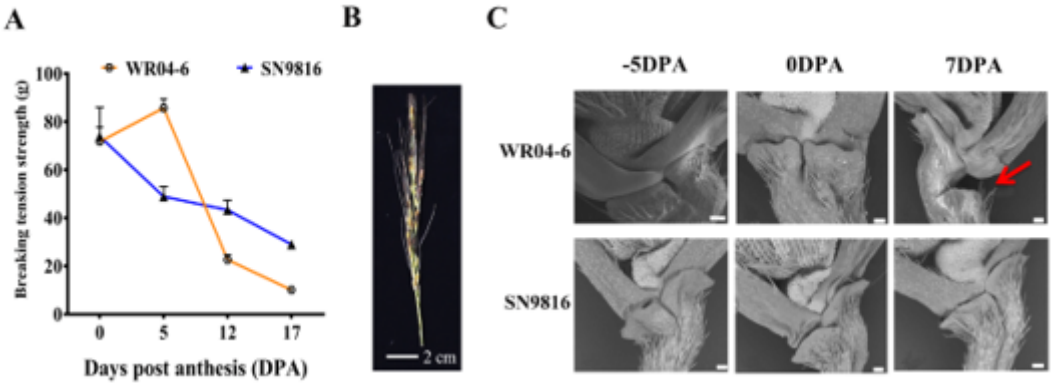

Figure 1

Phenotypic identification of seed shattering in weedy rice. (A) Breaking tensile strength (g) of seeds from the pedicel of WR04-6 and SN9816 at different days post anthesis (DPA). (B) Spikelet of WR04-6 at 17 DPA. (C) Anatomy of the abscission zone (AZ) in the grain pedicel. The red arrow represents the narrow gap under the palea and lemma observed in WR04-6. Photographs in each panel were taken at a 120× magnification. Bar= 50μm.

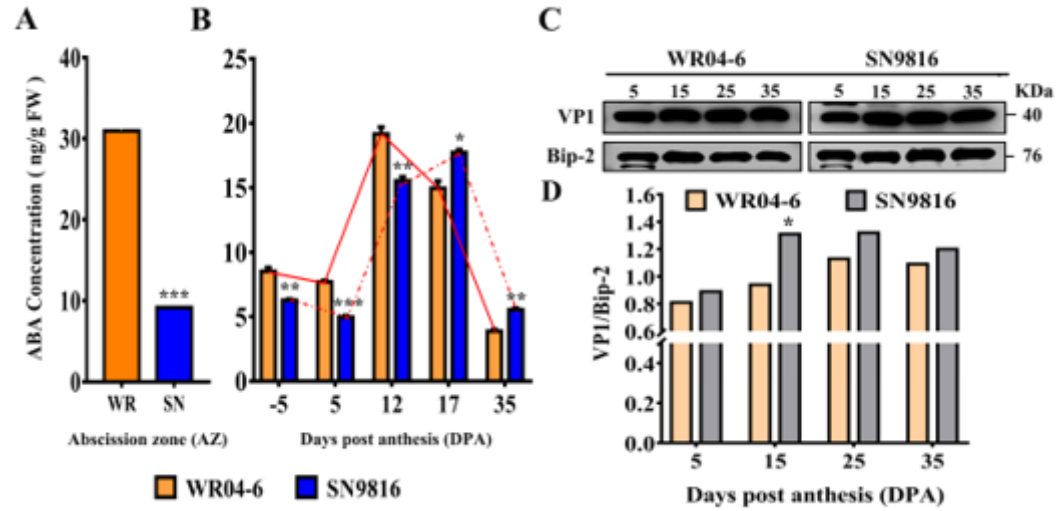

Figure 2

ABA level in the abscission zone (AZ) and spikelet and western blot analysis. ABA concentrations in AZ at 15 DPA (A) and spikelet (B) during the course of development. Standard error of three replicates. (C). Determination of the accumulation of OsVP1 with specific antibody by immunoblot analysis. The figures were cropped from the Fig. S1C. Bip-2 was used as the internal control. (D). Gray scale analysis of the western blot. The figures in (B), (C) and (D) represent samples that were collected at pre-established days post anthesis (DPA). Asterisks indicate a significant difference (Student's t-test: ***P ≤ 0.0005; ** P ≤ 0.005; * P ≤ 0.05).

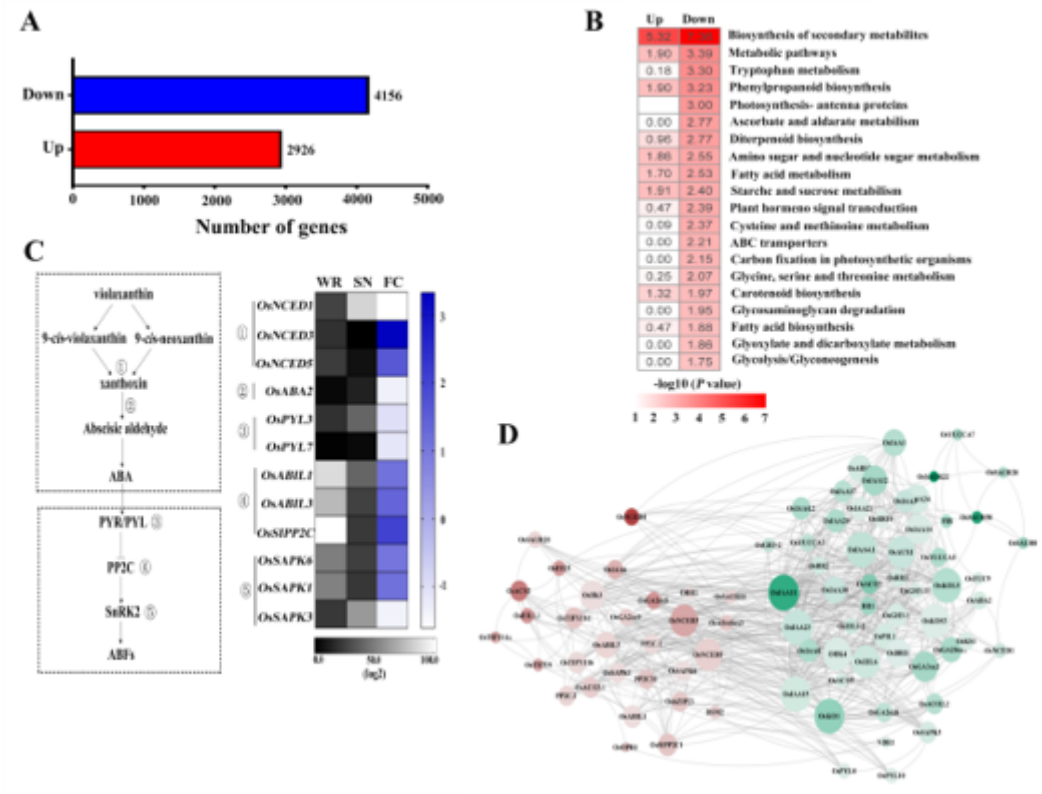

Figure 3

Transcriptional analysis of abscission zone (AZ) tissue during seed abscission. (A). The number of differentially expressed genes (DEGs) resulting from pairwise comparisons of AZ transcriptomes between WR04-6 (shattering) and SN9816 (non-shattering). (B). Diagrams showing significant terms by KEGG pathway enrichment analyses. (C). Comparison of gene expression levels of ABA biosynthesis and transduction-related genes. Average mRNA levels in the WR04-6 and SN9816 plants are indicated as single color gradient. Blue and white colors indicate higher and lower expression in WR04-6, respectively, based on their ratios (FC: fold change) between WR04-6 and SN9816. A simplified view of ABA biosynthesis and transduction pathways and genes involved in the indicated step are described in the left side of the expression data. (D). Gene expression level and protein-protein interaction (PPI) network for gene products related to ABA, IAA, ET, and GA pathways. Regulated protein/gene expression are illustrated by red or green color, indicating up- or down-regulation, respectively; dot size is proportional to connectivity.

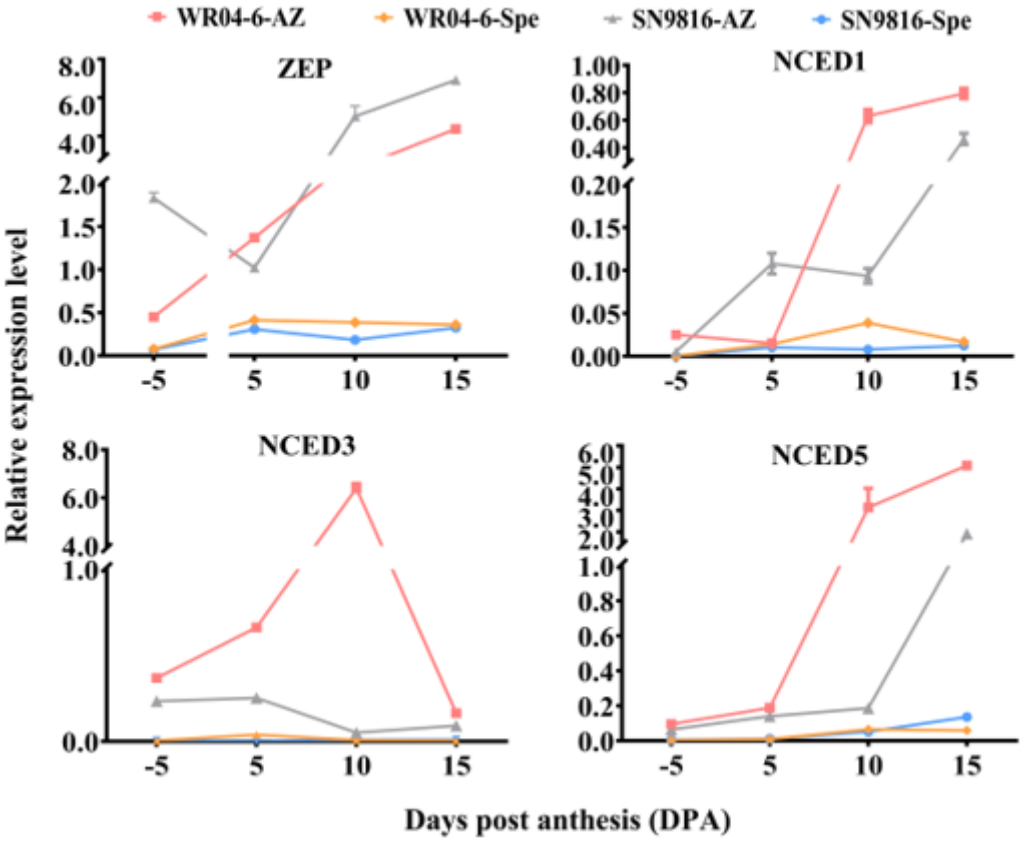

Figure 4

Expression patterns of ABA biosynthesis-related genes in the abscission (AZ) and spikelet during abscission by real-time quantitative PCR. Expression of OsActin was used as an internal control. Significance was assessed using p value < 0.05 (n = 3). AZ: abscission zone; Spe: spikelet

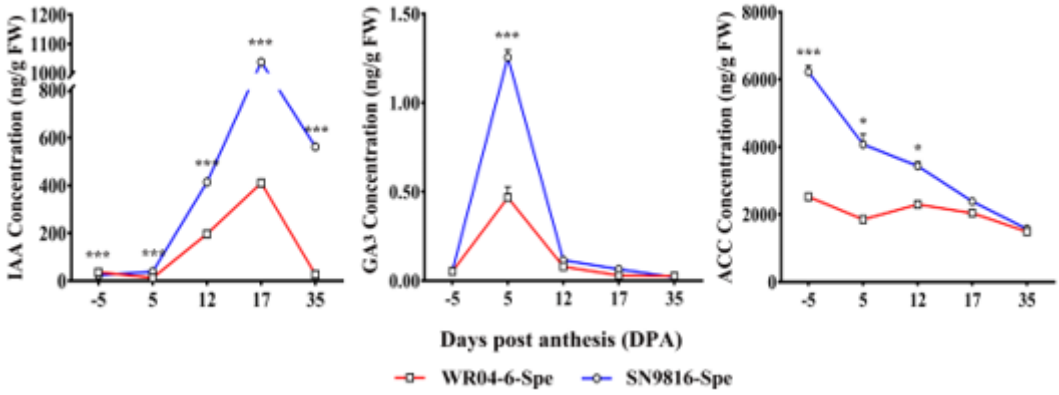

Figure 5

Changes in IAA, GA3 and ACC levels in spikelets during the course of development. Asterisks indicate a significant difference (Student's t-test: ***P ≤ 0.0005; ** P ≤ 0.005; * P ≤ 0.05).

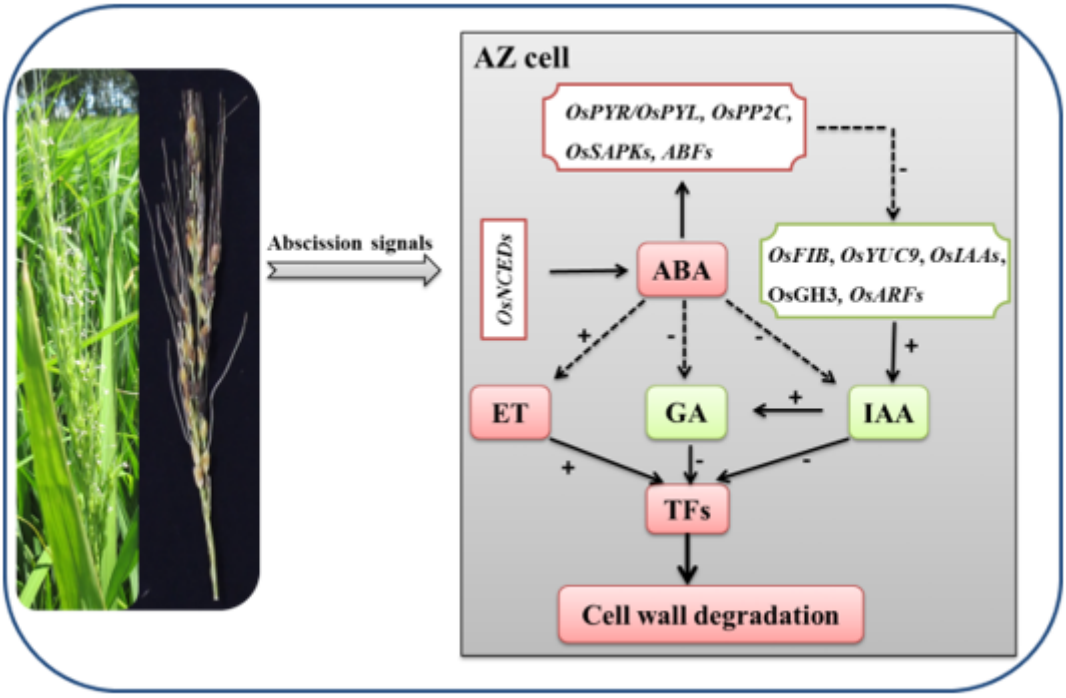

Figure 6

Hypothetical schematic model explaining the involvement of ABA within the regulatory network that leads to abscission induction based on gene expression data obtained from RNA-Seq analysis. “+”, promotion; “-”, suppression; solid arrow, direct regulation; dotted arrow, unknown direct or indirect regulation; red and green represent up-regulation and down-regulation, respectively.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.xls
- TableS3.docx
- FigureS1.docx
- TableS4.docx
- TableS2.docx