APICAL SPIKELET ABORTION (ASA) Controls Apical Panicle Development in Rice by Regulating Salicylic Acid Biosynthesis

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Panicle degradation causes severe yield reduction in rice. There are two main types of panicle degradation: apical spikelet abortion and basal degeneration. In this study, we isolated and characterized the apical panicle abortion mutant *apical spikelet abortion* (asa), which exhibits degeneration and defects in the apical spikelets. This mutant had a pleiotropic phenotype, characterized by reduced plant height, increased tiller number, and decreased pollen fertility. Map-based cloning revealed that *OsASA* encodes a boric acid channel protein that showed the highest expression in the inflorescence, peduncle, and anther. RNA-seq analysis of the asa mutant vs wild-type (WT) plants revealed that biological processes related to reactive oxygen species (ROS) homeostasis and salicylic acid (SA) metabolism were significantly affected. Furthermore, the asa mutants had an increased SA level and H₂O₂ accumulation in the young panicles compared to the WT plants. Moreover, the SA level and the expression of *OsPAL3*, *OsPAL4*, and *OsPAL6* genes (related to SA biosynthesis) were significantly increased under boron-deficient conditions in the asa mutant and in *OsASA*-knockout plants. Collectively, these results suggest that the boron distribution maintained by *OsASA* is required for normal panicle development in a process that involves modulating ROS homeostasis and SA biosynthesis.

Keywords: apical panicle abortion, anther, *Oryza sativa*, reactive oxygen species, salicylic acid

INTRODUCTION

Rice (*Oryza sativa*), one of the most important food crops in the world, is the staple food of half the world’s population. The rice yield is mainly a result of the panicle number per unit area, grains per panicle, and grain weight, which are influenced by plant type, panicle type, and grain development (Xing and Zhang, 2010). Regarding one of the factors that affect the rice yield, panicle formation involves complex physiological and biochemical processes, including axillary meristem development, inflorescence establishment, and grain development. Many important genes related to panicle development have been reported, involving the regulation of axillary meristem initiation,
spatiotemporal transformation related to meristem development, branch elongation, and the inflorescence structure (Teo et al., 2014). The aberrant panicle organization 1 (apo1) mutant exhibits altered inflorescence architecture and changes in the control of floral organ identity, with a severely reduced number of spikelets and grains; in contrast, APO1 overexpression increases the inflorescence branches and spikelets (Ikeda et al., 2005, 2007; Ikeda-Kawakatsu et al., 2009). The IPA1 quantitative trait locus (QTL) encodes SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE 14 (OsSPL14) and is regulated by the microRNA OsmiR156 in vivo; higher expression of OsSPL14 in rice in the reproductive stage promotes panicle branching, increases the grain yield, accelerates flowering, and decreases the number (Jiao et al., 2010; Miura et al., 2010; Luo et al., 2012). The larger panicle (lp) mutant has significantly increased panicle size, improved plant architecture, more inflorescence branches (especially primary branches), and more grains per panicle (Li et al., 2011). The lax1 mutant exhibits severely reduced initiation and maintenance of rachis-branches, lateral spikelets, and terminal spikelets (Komatsu et al., 2001). Additionally, LAX2 regulates the branching of the aboveground parts of rice except for the primary panicle branch; together, LAX2 and LAX1 regulate the process of axillary meristem formation (Tabuchi et al., 2011). In the rice frizzy panicle (fzp) mutant, floret formation is replaced by sequential rounds of branching; FZP (the ortholog of the maize BD1 gene) is required to prevent axillary meristem formation within the spikelet meristem and permit the subsequent establishment of floral meristem identity (Komatsu et al., 2001, 2003). Lastly, a gain-of-function DENSE AND ERECT PANICLE 1 (DEP1) mutation has been reported to enhance meristematic activity, reduce the inflorescence internode length, and increase the number of both primary and secondary panicle branches (Huang et al., 2009).

Panicle degradation reduces the yield per plant, seriously affecting rice production. There are two main types: apical spikelet abortion and basal degeneration. Temperature, humidity, and other environmental factors during panicle differentiation affect the degeneration degree, causing difficulties in genetic and biomolecular research on panicle degradation. Several QTLs related to panicle spikelet abortion have been reported. On chromosomes 1, 10, and 11, there are three main-effect QTLs underlying floret abortion of the rice panicle before flowering (Yamagishi et al., 2004). SHORT PANICLE 1 (SPI1), which encodes a peptide transporter (PTR) family member, regulates panicle branch elongation and is related to basal degeneration, with the sp1 mutant exhibiting degenerated basal branches and spikelets (Li et al., 2009). Apical spikelet abortion is more prevalent than basal degeneration in rice production. Several QTLs related to spikelet degeneration have been identified (Tan et al., 2011). The panicle apical abortion 8 (pap8) mutant exhibits degenerated spikelets that are associated with excess H$_2$O$_2$ accumulation (Cheng et al., 2011). The rice panicle degenerative mutant tutou1 (tut1) exhibits severe defects; with apical panicle degeneration, TUT1 can activate the protein complex actin-related protein 2/3 to promote actin nucleation and polymerization in vitro (Bai et al., 2015). Mutation of OsALMT7, which encodes a putative aluminum-activated malate transporter, leads to programmed cell death in apical spikelets and spikelet degeneration in the apical portion of panicles (Heng et al., 2018). SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE 6 (SPL6) acts as a negative regulator of the inositol-requiring enzyme 1 (IRE1)-mediated endoplasmic reticulum (ER) stress signaling outputs, so that the spl6-1 mutant with IRE1 hyperactivation exhibits apical panicle abortion (Wang Q. L. et al., 2018). Knockout of CALCINEURIN B-LIKE PROTEIN-INTERACTING PROTEIN KINASE 31 (OsCIPK31) causes a pleiotropic phenotype that includes apical panicle abortion (Peng et al., 2018). Lastly, DEGENERATED PANICLE AND PARTIAL STERILITY 1 (DPS1) plays a vital role in regulating reactive oxygen species (ROS) homeostasis and anther cuticle formation, with the dps1 mutant displaying apical panicle degeneration and reduced fertility (Zafar et al., 2019).

Salicylic acid (SA) is a key plant defense hormone with critical roles in various aspects of plant immunity (Zhang and Li, 2019). It also plays an important role in the regulation of plant growth, development, and responses to abiotic stresses (Hara et al., 2012). In higher plants, SA biosynthesis involves two distinct and compartmentalized pathways with multiple steps: the isochorismate synthase and phenylalanine ammonia-lyase pathways (Dempsey et al., 2011). ROS are crucial regulators of metabolism and plant responses to biotic and abiotic environmental stimuli, and ROS are also important for plant development (Waszczak et al., 2018). SA maintains root meristem activity in rice by promoting ROS accumulation (Xu et al., 2017). Additionally, WRKY75, SA, and ROS form a tripartite amplification loop to accelerate leaf senescence (Guo et al., 2017).

Boron (B) is an essential micronutrient for plant growth and development. Its principal known function in vascular plants is to maintain cell wall structure by cross-linking the pectic polysaccharide rhamnogalacturonan II (RG-II) (Kobayashi et al., 2017). Boron is required for plant growth, development, and responses to abiotic stresses (Shorrocks, 1997; Lordkaew et al., 2011; Durbak and Drummond, 2014; Routray et al., 2018).

In this study, we isolated and characterized an apical panicle abortion mutant, designated the apical spikelet abortion (asa) mutant, that exhibited degeneration of the top spikelets in the early stage of panicle development. Map-based cloning indicated that the gene responsible for apical spikelet abortion in the asa mutant, OsASA (LOC_Os10g36924), encodes a boric acid channel. It plays an important role in panicle development by regulating ROS homeostasis and SA biosynthesis.

**MATERIALS AND METHODS**

**Plant Materials and Growing Conditions**

The rice asa mutant was a spontaneous mutant in line Q179, derived from the F$_4$ progenies of a cross between BobaiB and RW11. It was crossed with Nipponbare [the wild-type (WT)]
plant], and the resultant F$_1$ plants were selfed to produce F$_2$
seeds for the mapping population. To allow fine mapping,
first, a recessive individual from F$_2$ exhibiting apical spikele
abortion was selected to successively backcross with the recurrent
parent Nipponbare to construct the BC$_2$F$_1$ population; the BC$_2$F$_2$
population was derived from a BC$_2$F$_1$ individual in which the
region around OsASA was heterozygous, and almost all other
regions were homozygous. Meanwhile, to evaluate the agronomic
traits, an asa recessive individual from the BC$_2$F$_2$ population
whose agronomic traits were similar to the recurrent parent was
crossed with Nipponbare to produce the BC$_3$F$_2$ population.
The F$_2$, BC$_2$F$_1$, BC$_2$F$_2$, and BC$_3$F$_2$ populations were planted
in the experimental field of the Rice Research Institute, Fujian
Academy of Agricultural Sciences in Fuzhou, Fujian Province,
China. Germinated seeds were sown in seed beds in mid-
May, and 25-day-old seedlings were transplanted to the field.
There was 20 cm between each plant in each row, and
the rows were 20 cm apart. Field management, including
irrigation, fertilizer application, and pest control, essentially
followed normal agricultural practices. Additionally, transgenic
plants were grown in pots in a greenhouse under standard
growth conditions.

Measurement of Agronomic Traits
To explore the morphological characteristics of the asa mutant
with apical spikelet degeneration, the BC$_3$F$_2$ population was
used to measure agronomic traits. This population was divided
into three subpopulations: asa mutant (asa/asa), heterozygous
(asa/Asa), and WT (Asa/Asa) populations by screening using the
insertion–deletion (InDel) markers ID17. The agronomic traits
(including plant height, panicles per plant, and panicle length)
of the three plant types were assessed at the mature stage. Plant
height was measured from the soil surface to the panicle tip of
the main tiller (excluding the awn); the number of panicles per
plant was evaluated by counting the panicle number of each plant;
panicle length was scored from the neck node up to the tip of the
last spikelet; each variable was averaged over 12 plants. Source-
size traits, including flag leaf width and flag leaf length, were also
measured at 5 days after heading.

Microscopic Observation
For scanning electron microscopy (SEM) examination of young
spikelets and pollen, samples were fixed in 2.5% (w/v) glutaraldehyde
[in 0.1 M phosphate-buffered saline (PBS)] for 2 h and then washed three times with 0.1 M PBS. Following
derhydration using an ethanol series at 4°C, the samples were
subjected to critical-point drying, coated with palladium-gold
using a sputter-coater, and observed under a scanning electron
microscope (JSM-6390LV; JEOL).

Additionally, spikelets at various developmental stages were
fixed in 2.5% (w/v) glutaraldehyde solution, post-fixed in 1%
Os$_4$O$_4$ solution, dehydrated using an acetone series, embedded
in Spurr resin, sectioned, and stained with 0.1% toluidine blue.
The spikelet sections were observed with a light microscope
(DM4B; Leica) and a transmission electron microscope (HT-
7800; Hitachi).

Map-Based Cloning
Using the bulked segregant analysis (BSA) method, polymorphic
markers were employed to analyze the mutant and WT DNA
pools. Three simple sequence repeat (SSR) markers (RM25756,
RM171, and RM147) co-segregated with the OsASA locus. A total
of 169 F$_2$ recessive individuals were used for the linkage analysis.
The OsASA locus was located on chromosome 10 between
RM25756 and RM171. Further markers with polymorphisms
between the two DNA pools were then used to assess the 169 F$_2$
recessive individuals and thereby narrow down the OsASA locus
to the region between the InDel markers ID14 and ID16. For
fine mapping and phenotyping, approximately 4500 individuals
in the BC$_2$F$_2$ population were assessed. Among them, 978 OsASA
recessive individuals were selected to screen for recombinants
among InDel markers within the ID14–ID16 interval based on
rough mapping of the F$_2$ population. The recombinants were
used for fine mapping, with newly developed markers between
ID14 and ID16 being used to narrow down the OsASA locus to a
10.05-kb region between markers ID36 and ID16. Primers for the
InDel markers are listed in Supplementary Table S1.

RNA Isolation and Real-Time (RT)-PCR
Analysis
Total RNA was isolated from the WT and asa mutant plants using
an Eastep Universal RNA Extraction Kit (Promega). First-
strand cDNA was synthesized by reverse transcription using an
M-MLV First Strand Kit (Invitrogen). Quantitative RT-PCR
was performed using SYBR Premix Ex Taq II (TaKaRa) on an
ABI Step One Real-Time PCR system (Applied Biosystems). The
rice UBIQUITIN gene was used as an endogenous control. All
primers for RT-PCR are listed in Supplementary Table S2. The
comparative threshold cycle (CT) method was used to analyze the
relative expression levels.

Plasmid Construction and Transformation of WT Plants
To analyze the expression pattern of the OsASA gene, a 2077-bp
promoter fragment was amplified and then cloned into the binary
vector pCXGUS-P, to generate the pOsASA-GUS construct. To
create an OsASA overexpression vector, the full-length coding
sequence of OsASA was amplified from the Nipponbare genome
and then cloned into the binary vector pCXUN-FLAG by TA
cloning. This created the pUBi:OsASA-FLAG plasmid, which was
introduced into the asa mutant. To create the clustered regularly
interspaced short palindromic repeats (CRISPR)-OsASA (cr-
OsASA) transgenic plants that had the OsASA gene knocked
out, a single guide RNA (sgRNA) sequence was designed
using the CRISPR-PLANT website1. Primers containing the BsaI
digestion site were ligated into pU3-sgRNA, and the sgRNA
fragment was then cloned into the binary vector pH-Ubi-cas9-7
using the LR recombination reaction. All transgenic rice plants
were generated using Agrobacterium-mediated transformation
of rice calli. All primers for plasmid construction are listed in
Supplementary Table S2.

1http://www.genome.arizona.edu/crispr/CRISPRsearch.html
**β-Glucuronidase (GUS) Staining Assay**

For the GUS staining assay, tissues were collected at various developmental stages, and the staining was performed as previously described (Jefferson, 1987). Images were captured using a stereoscope or a camera.

**Boron Treatment of Rice Seedlings**

The seeds of the WT, *asa* mutant, and *cr-OsASA* (*OsASA-knockout*) transgenic plants were soaked and sown in 96-well plates. They were grown in an incubator at 30°C in the light and at 25°C in the dark, with 10/14-h light/dark cycles. After 1 week, the seedlings were moved to 24-well plates, and the hydroponic culture solution was changed every 2 days. After 3 weeks of hydroponic culture, the seedlings were used for assessment of plant height, root length, and shoot and root fresh weight, along with assessing the SA level and the expression of *ISOCHORISMA TE SYNTHASE 1* (*OsICS1*) and nine *PHENYLALANINE AMMONIA-LYASE* (*OsPAL*) genes. The hydroponic culture solution was prepared as described previously (Fukuda et al., 2004), with three boron concentrations (0, 15, and 150 μM) being used for each of the three types of plants (WT, *asa* mutant, and *cr-OsASA*).

**DAB Staining for H₂O₂**

Spikelets collected at various developmental stages were incubated at 25°C with 1 mg/ml 3,3′-diaminobenzidine (DAB) plus 0.05% Tween-20, and they were subjected to vacuum infiltration for 10–15 min. After 12–24 h, destaining buffer (ethanol:acetic acid:glycerol = 3:1:1) was added. Images were captured using a stereoscope.

**Quantitative H₂O₂ Assays**

The concentration of H₂O₂ in panicles was quantitatively determined using an H₂O₂ assay kit (Beyotime) according to the manufacturer’s instructions. Absorbance was measured with a spectrometer at a wavelength of 560 nm.

**Hormone Profiling**

Hormones (including SA) were extracted as previously described. First, 100 mg of panicles was mixed with 1 ml of 80% methanol with internal standards (250 pg [5H₆] ABA for ABA, SA, SAG, and 1A; 250 pg [5H₃] IAA for IAA and JA-ILE). Extraction was performed twice using a laboratory rotator for 2 h at 4°C. After 10 min of centrifugation at 18,000 g and 4°C, the supernatant was collected and dried using nitrogen gas. The pellet was then dissolved in 300 μl of 30% methanol. The hormones (including SA) were separated using a C18 column and analyzed using a triple quadrupole mass spectrometer as previously described (Šimura et al., 2018).

**RNA Sequencing**

Young panicles of about 3 cm in length were collected from the WT and *asa* mutant plants to compare global gene expression changes between the WT and *asa* mutant. RNA extraction and RNA-seq analysis were performed by BioMarker (Qingdao, China), with three biological replicates.

**RESULTS**

**asa Mutant Exhibits an Apical Panicle Degeneration Phenotype**

The rice *asa* mutant involved a spontaneous mutation found in line Q179, derived from the F₄ offspring of a cross between *Bobai B* (*O. sativa* L. ssp. *indica* cv) and *RW11* (*O. sativa* L. ssp. *indica* cv) under natural conditions. Compared to WT plants, the *asa* mutants showed severe apical panicle degeneration at the heading stage; the apical spikelets exhibited various degrees of degeneration, while the middle and bottom of each panicle developed normally (**Figures 1A–D** and **Supplementary Figure S1**). The abortion rates for the primary and secondary branches in the *asa* mutants were about 55% and 40%, respectively, while scarcely any degenerated spikelets were observed in the WT plants (**Figure 1E**). In addition, the plant height was lower for the *asa* mutants than the heterozygous and WT plants (**Figure 1F**). Moreover, the panicle length and panicle number decreased significantly in the *asa* mutants, while these agronomic traits displayed no difference between the heterozygous and WT plants (**Figures 1G,H**). The flag leaf length and width were also obviously reduced in the *asa* mutants (**Figures 11,J**). Thus, the *asa* mutant exhibits a pleiotropic phenotype, with severe apical panicle degeneration, and this significantly reduced the grain yield.

**asa Mutant Displays Defect in Spikelets**

A mature WT rice spikelet consists of a pair of glumes, a pair of sterile lemmas, two lodicules, six stamens, and one pistil with a bifurcated stigma (**Figures 2A,F**). Compared to the WT plants, the *asa* apical spikelets were defective, including hooked or enlarged lemmas and palea (**Figures 2B–E**), twisted or whitish anthers with indefinite numbers, glume-like lodicules, and three-forked stigmas (**Figures 2G–L**).

To characterize the differences in spikelet meristem development, we used SEM to examine the primordia of the WT and *asa* mutant plants at various developmental stages. At the spikelet specification developmental stage, in the WT and *asa* mutant plants, there were several protrusions similar to palea or lemma primordia, and the sterile lemmas were drastically differentiated and larger than in the WT plants (**Figure 2M**). In contrast, the *asa* mutants exhibited non-uniform stamen primordia, with varied numbers, irregularly surrounding the pistil primordium (**Figure 2N**). During the stamen and pistil primordium differentiation stage, the stamen and pistil primordia were enclosed by palea and lemmas and two sterile lemmas were located at the base of the spikelet in the WT plants (**Figure 2O**). However, in the *asa* mutants, there were several protrusions similar to palea or lemma primordia, and the sterile lemmas were drastically differentiated and larger than in the WT plants (**Figure 2P**). In the late stage of spikelet development, the WT palea and lemmas were wrapped around the floral organs, and the palea and lemma outer surfaces were composed of epidermal cells with neat papillary bulges; glume hairs and spiny tenuous hairs were located in the depressions between these papillary bulges (**Figure 2Q**). In contrast, the *asa* mutants had extra lemma-like organs and abnormal sterile lemmas,
OsASA Controls Apical Panicle Development

FIGURE 1 | Phenotypic characterization of the asa mutant. (A) Phenotypic comparison between mature wild-type (WT) and asa mutant. Immature panicles phenotypic comparison between WT (B) and asa mutant (C). (D) Mature panicles phenotypic comparison between WT and asa mutant. (E) Statistics of seed setting rate of WT and asa mutant. PB, primary branch; SB, secondary branch. Comparison of plant height (F), panicle length (G), panicle number (H), length of flag leaf (I), and width of flag leaf (J) between WT, hybrid type (asa/ASA), and asa mutant. Values are mean ± SD (n = 12); asterisks indicate significant differences (*P < 0.05, **P < 0.01) according to the Student’s t-test compared with the WT.

and the glume hairs were unevenly distributed on the surfaces (Figure 2R). Iodine-potassium iodide (I₂-KI) staining showed that the pollen grains from asa apical spikelets were partly sterile compared to the WT plants (Figures 2S,T). SEM was used to further observe the pollen morphology. Compared to the normal pollen grains in the WT plants, the asa mutants had shrunken
and irregularly shaped pollen grains (Figures 2U,V). These observations suggested that spikelet development was abnormal in the asa mutant.

**asa Mutant Exhibits Decreased Pollen Fertility**

To further confirm the defects in the asa pollen grains, we prepared semithin sections of WT and asa anthers at various developmental stages. At the middle stages of microspore development in the WT plants, the spherical microspores exhibit multiple small vacuoles, with the tapetal cells becoming more vacuolated (Figure 3A). In the asa mutants, the pollen cavity accumulated unidentified substances, the microspores were irregularly shaped, and the tapetum was abnormal, without vacuolization (Figure 3B). Consequently, the WT plants had mature pollen grains, while some of the asa mutant microspores were degenerated, with a sickle shape and without starch accumulation (Figures 3C,D). To characterize the defects in the anther development, we used SEM examination of WT and
**asa** anthers. Consistent with the findings of light microscopy of the transverse spikelet sections, the spherical microspores from WT plants formed multiple small vacuoles (Figures 3E,F). The **asa** pollen cavity accumulated unidentified substances, irregularly shaped microspores adhered to the inner side of the tapetum, and the tapetum was abnormally enlarged and thicker, without vacuolization (Figures 3H,I). The WT tapetum was condensed, with proliferated ER in the tapetal cells (Figure 3G). In contrast, almost no ER but abundant vacuoles and liposomes were observed in the **asa** tapetal cells (Figure 3J). These results indicated that **OsASA** may affect tapetal cell degradation and thereby control microspore formation.

**Map-Based Cloning and Characterization of **OsASA**

In the BC_{2}F_{2} population, there were 383 WT and 87 mutant plants, with the frequencies fitting the expected Mendelian ratio (3:1) for single-locus segregation ($\chi^2 = 1.07$). We inferred that the **asa** trait was controlled by a single recessive gene.

To map the **OsASA** locus, 331 SSR markers on the 12 chromosomes (with a mean interval between markers < 5.0 cM) were selected to screen for polymorphic markers between the two parents, and for suspected markers linked to the **OsASA** locus, using the BSA method. We found that three SSR markers (RM25756, RM171, and RM147) co-segregated with the **OsASA** locus. Subsequently, 169 F_{2} recessive individuals were used for linkage analysis using the above three markers. The results showed that **OsASA** was located on chromosome 10 between SSR markers RM25756 and RM171, at distances of 1.2 and 4.7 cM, respectively. To narrow down the region, 29 InDel markers and five SSR markers were used. Of the 34 markers, 11 markers with polymorphisms between the mutant and WT DNA pools were used to map the 169 F_{2} recessive individuals, and linkage analysis showed that the **OsASA** locus was between InDel markers ID14 and ID16 at distances of 1.8 and 0.3 cM, respectively, while ID17 co-segregated with **OsASA**.

For fine mapping of the **OsASA** QTL, an **OsASA** recessive individual was selected to successively backcross with the recurrent parent **Nipponbare** to construct the BC_{2}F_{1} population. Approximately 4500 individuals in the BC_{2}F_{2} population (which was derived from a BC_{2}F_{1} individual with a **Nipponbare** genetic background and a heterozygous region around **OsASA**) were
screened using the markers RM25756 and RM171. Among the 4500 individuals, 978 Os ASA recessive individuals were selected to undergo screening for recombinants using the InDel markers ID14 and ID16. As a result, 19 recombinants were detected between ID14 and ID16, and then eight InDel markers within the two markers with polymorphisms were used to detect the 19 recombinants. Thus, the locus was delimited to a 10.05-kb interval between the InDel markers ID36 and ID16 (Figure 4A). The target region contains only one predicted gene, LOC_Os10g36924, based on the Rice Genome Annotation Project website. Comparing the coding sequence of the Os ASA genes revealed a two-nucleotide substitution of GC to CT in the first exon, and a two-nucleotide deletion in the second exon in the asa mutant (Figure 4B), which results in an amino acid substitution (Ala to Leu) and a frameshift. The Os ASA gene comprises three exons and two introns, encoding a putative aquaporin NIP3;1 protein. Structural prediction showed that the Os ASA protein has six transmembrane domains and two conserved Asn-Pro-Ala (NPA) motifs, while Osasa only contains two transmembrane domains and one NPA motifs (Supplementary Figure S2).

To confirm that the mutation in LOC_Os10g36924 was responsible for the asa mutant phenotype, its full-length coding sequence driven by the rice UBQUITIN promoter was introduced into an asa mutant background. All positive transformants (T<sub>1</sub>) displayed fertile spikelets, mimicking the WT phenotype (Figures 4D,E). This observation confirmed that mutation of LOC_Os10g36924 (referred to as Os ASA) underlies apical panicle abortion in the asa mutants. In addition, we transformed a CRISPR-Cas9 construct that targets the first exon of Os ASA into the WT plant (Nipponbare) to knockout Os ASA (Figure 4C), creating transgenic cr-Os ASA plants. These plants exhibited the apical panicle abortion phenotype (Figure 4F).
Collectively, these results demonstrated that the OsASA mutation was responsible for the asa mutant phenotype.

**OsASA Is Preferentially Expressed in Inflorescence**

To elucidate the function of OsASA, we analyzed its expression pattern by subjecting the WT and asa mutant plants to qRT-PCR. In WT plants, OsASA expression was detected in all tissues analyzed, with relatively high expression in young panicles (0–4, 4–8, and 8–12 cm stages), especially the 4–8 cm stage, and lower expression in the young leaf, stem, and mature leaf (Figure 5A). However, the asa mutant had relatively low expression in 4–8 cm stage panicles and increased expression in the young leaf and 0–4 cm stage panicles (Figure 5A). The expression pattern of OsASA was further evaluated in transgenic plants harboring a pOsASA::GUS reporter vector, with the reporter gene driven by the 2077-bp promoter sequence of OsASA. GUS signals were observed in panicles at various developmental stages, with strong staining in young panicles and peduncles (1.5 mm, 5 mm, 7 mm, 9.5 mm, 2.5 cm, 3.5 cm, 5 cm, 7.5 cm, and 13 cm) (Figures 5B–G and Supplementary Figure S3). During spikelet development, strong GUS signals were observed in the anthers until the spikelets matured, and then the signals were only detected in the glumes (Figure 5H). Consistent with the asa phenotype, the OsASA expression was highest in the panicles. These results demonstrated that OsASA has a key role in panicle development.

**Transcriptome Analysis Highlights the Role of OsASA-Regulated ROS Homeostasis and SA Metabolism**

We observed increased ROS levels in the degenerated asa apical spikelets by using DAB staining for H$_2$O$_2$ (which causes reddish brown precipitation) compared to that in the WT apical spikelets (Figure 6A). The DAB staining appeared in the asa apical spikelets, being mainly distributed in several apical spikelets of the primary and secondary branches in the 3-cm panicles (Supplementary Figure S4). This suggested increased ROS accumulation at the early stage of asa panicle development. Quantitative measurements of H$_2$O$_2$ levels also showed that ROS levels were significantly higher in asa panicles than WT panicles (Figure 6B). These findings suggest a possible correlation between the ROS level and panicle degeneration.

To further explore the molecular processes and functional pathways modulated by OsASA during panicle development, RNA-seq analysis of 3-cm panicles was used to compare global gene expression changes between the WT and asa mutant plants. There were 1505 differentially expressed genes (DEGs), with 655 down-regulated genes and 850 up-regulated genes in the asa mutants. Based on the annotated gene functions, 117 DEGs were involved in anther and pollen development (41 down-regulated and 76 up-regulated), 54 were involved in ROS homeostasis (22 down-regulated and 32 up-regulated), and 125 were related to SA metabolism (32 down-regulated and 93 up-regulated) (Supplementary Figure S5). The RNA-seq data were confirmed by performing qRT-PCR for five genes (Figures 7C–G). These results indicated that OsASA is involved in panicle development by regulating the ROS level and SA metabolism.

To further identify the pathways associated with the DEGs, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was performed. This revealed that 135 (8.97%) DEGs were enriched in 73 KEGG pathways, including plant hormone signal transduction, plant–pathogen interaction, and phenylpropanoid biosynthesis (Figure 7A). To explore the possible phytohormone regulation of apical panicle abortion in the asa mutant, the endogenous levels of free SA, SA glucoside (SAG), indole-3-acetic acid (IAA), abscisic acid (ABA), and jasmonic acid (JA) in 3-cm stage panicles were measured. The free SA and SAG levels were significantly up-regulated in the asa mutants compared to the WT plants, while the IAA, ABA, JA, and JA-isoleucine (JA-ILE) levels did not differ (Figure 7B). These results suggested that SA is likely to play an important role in panicle development.

**OsASA Regulates SA Biosynthesis Under Boron-Deficient Conditions**

To verify whether the asa mutants were defective in boron absorption, asa and WT seedlings were cultured for 3 weeks in hydroponic culture solution with various boron concentrations (0, 15, and 150 µM). In the 15 and 150 µM groups, the growth of asa mutants was indistinguishable from that of WT plants. However, in the boron-deficient group, the growth of asa mutants was seriously inhibited compared to that of WT plants (Figures 8A–C). In asa mutants under boron-deficient conditions, the plant height and shoot fresh weight clearly decreased compared to the values in the WT plants, while there were no significant differences in the length and fresh weight of roots (Supplementary Figures S6A–D). These findings indicated that asa mutants are sensitive to boron deficiency. Thus, OsASA may play an important role in boron transportation.

Salicylic acid level was significantly up-regulated in the young panicles of the asa mutant. To assess whether boron deficiency can affect SA metabolism, we measured the free SA and SAG levels and found that both of them were significantly up-regulated in asa and cr-OsASA plants under boron-deficient conditions (Figures 8D,E). We further examined the expression level of OsICS1 and nine OsPAL genes, which are associated with SA biosynthesis, under boron-deficient conditions, and we found that OsPAL3, OsPAL4, and OsPAL6 were up-regulated (Figures 8F–H), whereas OsICS1, OsPAL1, OsPAL2, OsPAL5, OsPAL7, OsPAL8, and OsPAL9 showed no differences (Supplementary Figures S6E–K). Taken together, these results suggest that boron deficiency induces SA biosynthesis via the phenylalanine ammonia–lyase pathway.

**DISCUSSION**

**OsASA Is Involved in Panicle Development**

Panicle degradation causes low seed setting and reduced grain yield. Several genes that are related to apical panicle degeneration,
**FIGURE 5** | Expression pattern of OsASA. (A) Relative expression of OsASA in various tissues between wild-type and asa mutant. Rice UBQUITIN gene was used as an internal control. 0–4, 4–8, and 8–12 cm present developing panicles at the 0–4, 4–8, and 8–12 cm stages. Values are mean ± SD. (B) Promoter activity of OsASA by GUS staining at early developmental stages of panicle. Different stages are shown as indicated by panicle length. Bars = 1 mm. (C–G) Promoter activity of OsASA by GUS staining at late developmental stages of panicles. Different stages are shown as indicated by panicle length, 2.5 cm (C), 3.5 cm (D), 5 cm (E), 7.5 cm (F), and 13 cm (G). Bars = 1 cm. (H) Promoter activity of OsASA as shown by GUS staining in spikelets. Bars = 1 mm.
namely, *TUT1*, *OsALMT7*, *OsCIPK31*, *SPL6*, and *DPS1*, have been characterized (Bai et al., 2015; Heng et al., 2018; Peng et al., 2018; Wang Q. L. et al., 2018; Zafar et al., 2019). In this study, we isolated an apical panicle abortion mutant, the *asa* mutant, which exhibits apical panicle degeneration, abnormal spikelet development, and sterile pollen grains at the apical portion of panicles. *ASA* encodes a boric acid channel protein, identical to *NIP3;1* and *DTE1*, which plays an important role in regulating boron distribution in rice (Hanaoka et al., 2014; Liu et al., 2015; Shao et al., 2018). However, the mechanism of this gene in regulation rice reproductive development remains largely unclear. In this study, we discovered that the *asa* mutant harbors a mutation in *OsASA*, which encodes a boric acid channel (though the mechanism of this protein regarding the regulation of reproductive development in rice remains largely unclear). *OsASA* was preferentially expressed in the inflorescence. Additionally, in the *asa* mutant, genes involved in SA biosynthesis (*OsPAL3*, *OsPAL4*, and *OsPAL6*) were up-regulated, leading to increased SA levels and ROS accumulation, and thus affecting spikelet development and pollen fertility.

Panicle outgrowth in rice involves a rapid elongation process that requires higher rates of nutrient and energy supply (Heng et al., 2018). In particular, nitrogen nutrition and malate are required for normal panicle development (Fu et al., 2011; Heng et al., 2018). Boron is an essential micronutrient for the growth and development of vascular plants. Loss of function of boron influx channels or efflux transporters, such as in *bor1*, *nip5;1*, *nip6;1*, *ret1*, and *tsb1* mutants, leads to plants with abnormal phenotypes under limited boron conditions, with reduced leaf and root growth, inflorescence defects, and sterility (Noguchi et al., 1997; Takano et al., 2006; Tanaka et al., 2008; Chatterjee et al., 2014; Durbak et al., 2014). Cereals such as rice and maize are especially sensitive to boron deficiency during reproductive development (Hu et al., 1996; Shorrocks, 1997; Lordkaew et al., 2011). The symptoms of boron deficiency first become visible in the growing tips of the plant, as boron is mainly required in developing tissues (Jiao et al., 2005; Krug et al., 2009; Lordkaew et al., 2011). We found that the *asa* mutant developed degenerated spikelets at the apical portion of the panicles (Figure 1). Meanwhile, it also exhibits similar phenotypes like retarded growth and increased number of tillers as *dte1* (Liu et al., 2015). Furthermore, spikelet meristem formation was defective in the *asa* mutant (Figure 2). *OsASA* was preferentially expressed in the inflorescence, peduncle, and young spikelet (Figure 5). Together, these results suggested that *OsASA* might support the preferential distribution of boron in inflorescence that is required to maintain panicle development.

**OsASA Plays a Role in Regulating ROS Homeostasis During Spikelet and Pollen Development**

Reactive oxygen species are important signaling molecules that participate in a diverse range of biological processes (Mittler, 2017). ROS accumulation beyond a certain level often leads to apical panicle degeneration, defective anther development, and decreased pollen fertility (Hu et al., 2011; Bai et al., 2015; Zafar et al., 2019). ROS is an important trigger of tapetal programmed cell death, which is involved in tapetal degeneration during normal anther development (Li et al., 2006; Hu et al., 2011; Zhang and Yang, 2014). We identified many DEGs between the WT and *asa* mutant involved in the ROS network and anther development (Supplementary Figure S5), suggesting that *OsASA* may play a role in ROS homeostasis. Consistent with the *OsASA* expression profile analysis, higher H$_2$O$_2$ levels were detectable in the young panicles of the *asa* mutant, especially in the apical spikelets (Figure 6B). Increased DAB staining was observed in the apical panicles, mainly in multiple apical spikelets of the primary and secondary branches (Figure 6A and Supplementary Figure S4). These findings indicate that higher ROS accumulation is associated with the defective phenotype. In the *asa* mutant, the pollen cavity accumulated unidentified substances, the tapetum was abnormally enlarged and thicker, without vacuolization, and irregularly shape microspores adhered to the inner side of the tapetum, which may partly be the result of higher ROS levels during pollen development (Figure 3). Furthermore, the *asa* tapetal cells displayed features associated with oxidative stress, including intense cytoplasmic condensation and abundant vacuoles and liposomes (Figure 3). These findings indicate that the phenotype of *asa* mutant is accompanied by higher ROS accumulation in the apical spikelets, which causes the defective spikelets and sterile pollen development.

**OsASA Regulates SA Biosynthesis and Thereby Regulates Panicle Development**

Salicylic acid is widely distributed in plants and plays an important role in the regulation of plant growth and development (Klessig and Malamy, 1994). It has been proposed that SA may also be involved in the regulation of spikelet degeneration under abiotic stress (Wang Z. Q. et al., 2018). Exogenous application of SA to heat-stressed plants significantly reduced spikelet degeneration and increased pollen viability (Zhang et al., 2017; Zhao et al., 2018). Other studies have also reported that SA plays important roles in regulating pollen viability,
floretn fertility, and the development of normal female flowers (Ostergaard et al., 2002; Liu et al., 2019). It has been reported that phenylpropanoid can provide the key precursor for SA biosynthesis (Arrom and Munné-Bosch, 2012). In our study, 125 DEGs between the WT and asa mutant plants were involved in SA metabolism (Supplementary Figure S5). Additionally,
the KEGG analysis revealed that biological processes related to phenylpropanoid biosynthesis were significantly enriched in the *asa* mutant (Figure 7A). Furthermore, SA accumulated in the young panicles of the *asa* mutant (Figure 7B). Under boron-deficient conditions, SA was significantly enriched in *asa* and *cr-OsASA* plants, and three genes (*OsPAL3*, *OsPAL4*, and *OsPAL6*) involved in SA biosynthesis were up-regulated (Figure 8). These observations suggest that SA accumulation suppresses apical spikelet development and is thus involved in spikelet degeneration.

In summary, our results showed that *OsASA*, which encodes a boric acid channel, regulates ROS homeostasis and SA biosynthesis, thus affecting spikelet development and pollen fertility.
DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA680625.

AUTHOR CONTRIBUTIONS

LC and MZ supervised the project and revised the article. LC, MZ, and DZ designed the experiments. DZ performed most of the experiments, analyzed the data, and wrote the article. The other authors assisted in experiments and analyzed the data. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by grants from the National Key Research and Development Program of China (2016YFD0100903), and the Joint Fund of the National Natural Science Foundation of China and the Karst Science Research Center of Guizhou Province (U1812401).

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ACKNOWLEDGMENTS

We are thankful to Xiaoling Guo (College of the Environment and Ecology, Xiamen University) for generously providing technical assistance and Dr. Luming Yao and Dr. Caiming Wu (School of Life Sciences, Xiamen University) for generously providing technical assistance with electron microscopy. We thank Dr. Kewei Zhang (College of Chemistry and Life Sciences, Zhejiang Normal University) for help with mass spectrometer measurements.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.636877/full#supplementary-material
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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