Elevated CO₂ concentration enhance *Oryza sativa* seed shattering and affects seed-shattering gene expression

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Abstract

Weedy rice (*Oryza sativa* L.) is widely recognized as a major constraint in cultivated rice systems globally. Seed shattering is related to the invasiveness and persistence of weedy ecotypes in field and exacerbates its control in cultivated rice systems. Shattering traits are controlled genetically and by different environmental conditions. At present, a rapidly changing environment, including rising levels of carbon dioxide [CO₂], could alter shattering frequency, with subsequent effects on weed seed input and competition. The objective of the current study was to evaluate the interaction between weedy rice seed shattering and the transcriptional seed shattering-regulation genes as affected by weedy rice genotypes and atmospheric CO₂ concentrations. We examined seven biotypes and two atmospheric CO₂ concentrations: ambient {a[CO₂]} and enhanced {e[CO₂]} concentration, 400 and 700 µmol mol⁻¹ respectively. Our results indicate that e[CO₂] increases weedy rice seed shattering. The gene expression analysis demonstrates an effect of [CO₂] in the expression of all gene shattering-related genes (OsCPL1, qSH1, Sh4, SHAT1, OsXTH8, OSH15, and SH5), with high variability observed between genotypes. Here we showed that increased CO₂ concentration affects greatly seed shattering in weedy rice and in minor effect cultivated rice, by modulation of seed shattering-related genes and weedy genotypes showed the highest upregulation level of this genes. Thus, increased CO₂ concentration positively affect panicle number and grain yield mainly in cultivated rice.

Background

Among pernicious weeds in cultivated rice, weedy or red rice (*Oryza sativa* L.) is recognized as among the most competitive and significantly reduce rice yields even at small densities (Smith, 1988). Traits that can enhance weedy rice persistence and competitiveness include seed shattering and dormancy (Burgos et al., 2014; Durand-Morat et al., 2018; Engku et al., 2016; Fogliatto et al., 2011).

Seed shattering has been widely studied to understand rice domestication (Cheng et al., 2016; Dong and Wang, 2015; Li et al., 2006; Thurber et al., 2010; Zhang et al., 2017). In rice, seed shattering is dependent on the proper formation and subsequent degradation of an abscission layer in the joint between lemma and pedicel (Dong and Wang, 2015). This layer's degradation begins in the grain ripening process, caused by ethylene's production, which inhibits auxin synthesis. Synthesis of enzymes that degrade and proteins that remodel the cell wall occurs, including β-1,4-glucanase, polygalacturonase, xyloglucan-endotransglycosylase/hydrolase, and expansin (Taiz et al., 2017). As a consequence of hydrolytic enzymes' action, the middle lamella and the cell wall are degraded, causing the grain's fall (Roberts et al., 2002).

A number of genetic factors including several quantitative trait locus (QTLs) are associated with shattering in rice (Balanza et al., 2016; Zhou et al., 2012). The gene *qSH1* have been described in *Oryza japonica* subspecies (Konishi et al., 2006) and *SH4* in the *Oryza indica* subspecies (Li et al., 2006); these genes together are responsible for almost 70% of the shattering in rice. The *SH4* encodes a transcription factor with a Myb3 DNA binding domain and promotes abscission layer cells' hydrolyzing during the

abscission process (Li et al., 2006; Lin et al., 2007). The *SHAT1* gene encodes a transcription factor with an APETALA2 domain and acts on abscission layer cell differentiation (Zhou et al., 2012). There are also genes which can inhibit shattering. *OsCPL1*, which encodes a carboxy-terminal domain phosphatase-like protein, represses abscission layer development (Ji et al., 2010).

Although there are a number of descriptions regarding genetic regulation of abscission and seed shattering (Maity et al., 2021), there is little information about the effect of environmental factors in regulating these genes and the consequences for abscission and seed shattering (Patharkar and Walker, 2019). Among these factors it is worth investigating the role of rising CO₂ concentration [CO₂], which has increased by almost 30% since the 1960s and is expected to increase another 50% by century's end. It is widely recognized that CO₂ plays an essential role in plant morphology, fecundity, and development.

Recent and projected increases in [CO₂] can affect secondary metabolic processes, acting on different routes and interfering with metabolism at the cellular level (Kimball, 2016; Mhamdi and Noctor, 2016; Xu et al., 2015). During the abscission of tomato leaves, the transcriptional activation of multiple genes related to ethylene synthesis and heat shock proteins was induced by elevated CO₂ (800 µmol mol⁻¹) (Pan et al., 2019). Previous studies demonstrate that in addition to the vital role as a substrate in photosynthesis, CO₂ plays an essential role in cell homeostasis and hormonal signaling (Shi et al., 2015). Druart et al., (2006) reported that with the increase in the environment [CO₂], there is an effect in the repression of genes related to cell wall formation and cell growth. As far as we know, this is the first study to evaluate rice shattering in different [CO₂] and demonstrate the influence of [CO₂] on breaking tensile strength (BTS).

The current study was undertaken to evaluate the effect of projected, future CO₂ concentration on seed shattering and in the transcriptional regulation of seven genes known to be related to seed shattering in rice, cell wall synthesis, tissue degradation of the abscission layer, and genes that influence lignin biosynthesis. The study was conducted for both cultivated and weedy rice types to assess if differences to CO₂ were evident and if a CO₂ by genotype interaction was observed.

Results

Effect of [CO₂] in phenotypic evaluation

Weedy rice biotypes AVAR, AV53, and AV60 showed higher BTS in *a*[CO₂] than *d*[CO₂] (Fig. 1). Batatais, IRGA 417 and Nipponbare rice cultivars presented high BTS in *a*[CO₂]. In contrast, for IRGA 424 RI, no difference for [CO₂] was observed, with phenotypic evaluation showed BTS values ranging between 14 to 167 (gf). Weedy rice biotypes had lower BTS at *d*[CO₂] than *a*[CO₂]. In the rice cultivars, mainly Nipponbare, there was lower shattering, with higher BTS at *d*[CO₂].

Panicle number, grain yield, and above-ground dry mass

No difference was observed in panicle number of AVAR and AV60 weedy rice biotypes when submitted to different $[\text{CO}_2]$ (Fig. 2A). Otherwise, a higher panicle number was observed in the AV53 biotype under $a[\text{CO}_2]$. For rice cultivars, Batatais and IRGA 417 also showed a greater number of panicles in $a[\text{CO}_2]$, while no difference was detected for IRGA 424 and Nipponbare. Variability in panicle number and grain yield was observed for most of the weedy rice biotypes tested. Weedy rice biotypes are widely described to present high variability in several traits. For rice cultivars, the results were different, most responded with a greater panicles number and grain yield in $a[\text{CO}_2]$, except for the IRGA 424 RI genotype, in which there was no difference.

The total grain yield per pot for the weedy rice biotype AV53 was not affected by $[\text{CO}_2]$ (Fig. 2B). However, it is essential to note that the AV60 weedy rice genotype presented a higher grain number at $a[\text{CO}_2]$. Considering the rice cultivars, Batatais, IRGA 417, and Nipponbare showed the highest grain yield in $a[\text{CO}_2]$, while the $[\text{CO}_2]$ did not affect IRGA 424 RI grain yield. Concerning the above-ground dry mass an increase for AV60, Batatais and Nippobare in $a[\text{CO}_2]$ was observed (Fig. 3).

Gene expression

Taking into account the effect of the different $[\text{CO}_2]$ on BTS value, we also tested the effect of different $[\text{CO}_2]$ in the transcriptional regulation of seed shattering-related genes (Table 2). The target genes are associated with the different abscission layer formation phases, from cell differentiation to lignin deposition regulation and abscission layer cell separation.

The genes *qSH1*, *OSH15*, *SH4* and *SHAT1* are involved with abscission layer formation therefore, displaying a role in seed shattering (Konishi et al., 2006). At $a[\text{CO}_2]$ condition (Fig. 4), *qSH1*, *OSH15* and *SHAT1* were upregulated in two of the three weedy rice biotypes (AVAR and AV53) which also showed high seed shattering compared to cultivated rice genotypes. Despite AV60 weedy rice also shown high seed shattering at $a[\text{CO}_2]$ condition, *qSH1*, *OSH15*, *SH4* and *SHAT1* were downregulated. Batatais also showed a similar expression pattern observed in AVAR and AV53 respect to the upregulation of *qSH1*, *OSH15* and *SHAT1*. However, Batatais showed the lowest seed shattering level among cultivated rice at $a[\text{CO}_2]$ condition and a downregulation of *SH4*. In IRGA 417 and IRGA 424 RI, *qSH1*, *OSH15* and *SHAT1* were downregulated at $a[\text{CO}_2]$ condition while *SH4* was upregulated in both genotypes. With exception of Batatais that showed a transcriptional regulation of seed shattering genes similar to weedy rice genotypes, Irga 417 and Irga 424 RI showed *SH4* upregulation in both CO_2 conditions while most of weedy rice presented *SH4* upregulation only at $a[\text{CO}_2]$. At $a[\text{CO}_2]$ condition *qSH1*, *OSH15*, *SH4* and *SHAT1* were upregulated in all analyzed genotypes, except *qSH1* in IRGA 417 and *SH4* in AVAR. The expression pattern observed for *qSH1*, *OSH15*, *SH4* and *SHAT1* agree with the increasing in

seed shattering observed at $a[CO_2]$ condition. Among these genes, *OSH15* showed the higher transcript accumulation (Fig. 4).

OsCPL1 was upregulated in all analyzed genotypes, except in AV53, at $a[CO_2]$ condition. At $d[CO_2]$ condition, *OsCPL1* showed higher transcript amount respect to $a[CO_2]$ condition mainly in AV53 and AV60 that showed increases of 8 and 2.5 times, respectively. For *SH5* at $a[CO_2]$ condition was downregulated in all genotypes except in AVAR weedy rice genotype showing upregulation. As observed for the genes involved with abscission layer formation, *SH5* was strongly upregulated at $d[CO_2]$ condition in all analyzed genotypes. *OsXTH8* was upregulated at $a[CO_2]$ condition in AVAR and AV53 weedy rice biotypes and also in Batatais. Interestingly, *OsXTH8* transcript accumulation was conserved at $d[CO_2]$ condition in AVAR and AV53 and increased in Batatais. AV60, IRGA 417 and IRGA424 RI showed *OsXTH8* upregulation at $d[CO_2]$ condition.

Considering the molecular effects, the cultivated rice genotype Batatais displays a similar profile to those identified in AVAR and AV53 weedy rice genotypes under $a[CO_2]$ condition. Also, the weedy rice AV60 has a similar profile as observed for cultivated rice genotypes at $a[CO_2]$ condition.

Discussion

CO₂ concentration affects seed shattering in cultivated and weedy rice

Here we showed that weedy rice biotypes demonstrated lower BTS at $d[CO_2]$ than $a[CO_2]$, and rice cultivars, presented lower shattering with higher BTS at $d[CO_2]$. Nunes et al., (2015) reported low shattering capacity for rice cultivars, while for weedy rice biotypes, a high level of shattering was reported. The low shattering observed for rice cultivars is related to the crop's domestication, which is linked to reducing the natural shattering of seeds (Li et al., 2011).

The mechanistic basis for the increase in shattering at the higher CO₂ concentration is not clear. The increase in [CO₂] has been reported to influence several biochemical and physiological plant processes, affecting secondary metabolic processes, acting in different pathways, and interfering with cellular metabolism (Kimball, 2016; Noctor and Mhamdi, 2017; Xu et al., 2015). The shattering process involves the degradation of a specific zone between the pedicel and the grain, coordinated by methods involving the action on hydrolytic enzymes, where the middle lamella and the cell wall are degraded (Roberts et al., 2002). Also, a hormonal balance between ethylene and auxin influences the beginning of the shattering process, and environmental factors can affect this balance. Another critical point is the wide genetic and phenotypic diversity present in weedy rice biotypes (Singh, 2013). All these factors indicate that many processes can influence shattering characteristics and that environmental by [CO₂] interactions will require additional study.

The positive effect of high CO₂ in increasing cultivated rice yield while decreased in weedy rice

Different profiles at $e[CO_2]$ was observed between cultivated rice yield and weedy rice. Variability in panicle number and grain yield was observed for most of the weedy rice biotypes tested. Weedy rice biotypes are widely described to present high variability in several traits. For rice cultivars, the results were different, most responded with a greater panicles number and grain yield in $e[CO_2]$, except for the IRGA 424 RI genotype, in which there was no difference. Similar results were observed by Xu et al., (2018), indicating no difference in the production of panicles number and grain yield. Observing the rice cultivars, except for IRGA 424 RI, they were responsive to $e[CO_2]$, which corroborates with studies developed with rice culture (Ainsworth et al., 2006; Cai et al., 2016; Hasegawa et al., 2013).

The increase in grain yield under $e[CO_2]$ is related to increased photosynthetic rate, production of organic compounds by plant, and source-sink ratio (Cai et al., 2016). With enhance of $[CO_2]$, there is an increase in carboxylation, and suppression of the oxygenation activity of ribulose-1,5-bisphosphate carboxylase/oxygenase decreases photorespiration (Busch et al., 2018); thus, there is an increase in photosynthetic rate. Li et al., (2017) obtained an increase in the panicle number and grain yield in rice plants grown in $e[CO_2]$. In the present study, cultivars were more responsive to $[CO_2]$ than weedy rice biotypes. Weedy rice results were also found in evaluating rice genotypes where the seed mass similar and the above-ground mass was increased in the CO₂ (Wang et al., 2020). In the present study, weedy rice biotypes tended to produce more biomass but were inefficient in the source-sink for grain production.

Increased CO₂ concentration affects seed shattering by altering gene expression pattern

The molecular control of seed shattering has been unveiled and genes involved in abscission layer cell differentiation, lignin deposition and abscission layer cell separation were detected in rice. *qSH1* is the major QTL for seed shattering in japonica rice and encodes for a BELL1-type homeobox protein and is involved with abscission layer cell differentiation (Konishi et al., 2006). *qSH1* and *OSH15*, a KNOX protein, form a dimer that promote abscission layer cell differentiation (Yoon et al., 2017). The dimer *qSH1* and *OSH15* transcriptionally regulates *SHAT1* (Zhou et al., 2012). *SHAT1* encodes a transcription factor with an APETALA2 domain and regulate downstream genes involved in cell abscission layer differentiation. *SHAT1* can also be activated by *SH4* to activates *qSH1* to maintain *SH4* expression (Zhou et al., 2012). This fine-tuned network promotes abscission layer cell differentiation, the first phase of seed shattering. Here, we show that the transcriptional regulation of these genes is affected by increasing in CO₂ concentration. We also show that in $e[CO_2]$ condition all analyzed genotypes, weedy and cultivated rice, increase shattering level.

Specifically related to *qSH1* transcriptional regulation, no expression pattern was detected between weedy and cultivated rice (Fig. 4A). A previous study also evaluated the Batatais cultivar, AV60, and other weedy rice genotypes and found that the *qSH1* gene was not differentially expressed among the genotypes (Nunes et al., 2014) and that the SNP associated with seed shattering was not present in the weedy rice (Nunes et al., 2015). Here, the *qSH1* was expressed mainly in weedy rice biotypes in $a[CO_2]$ condition and all evaluated genotypes in $e[CO_2]$ condition, including those with a low shattering level. Thus, it seems that *qSH1* is not a suitable marker gene for seed shattering in $e[CO_2]$ condition. The high *qSH1* expression in genotypes showing low shattering does not agree with the phenotype and may be an effect of other genes acting repressing the shattering (Htun et al., 2014).

As expected, *OSH15* show a similar profile as observed for *qSH1* in $a[CO_2]$ and $e[CO_2]$ conditions (Fig. 4B). However, it is interesting to note the higher transcript amount of *OSH15* related to *qSH1* in $e[CO_2]$ condition where all genotypes also showed increased shattering level. It can be explained by the fact that *OSH15* displays two different roles in regulating seed shattering, in forming a dimer with *qSH1* to promote abscission layer cell differentiation and also in forming a dimer with *SH5*, a BELL homeobox protein acting to inhibit lignin biosynthesis in abscission layer (Yoon et al., 2017). The interaction between *OSH15* and *SH5* may explain the upregulation of *OSH15* in those genotypes showing low shattering degree (Batatais) (Fig. 1). *SHAT1* is affected by $e[CO_2]$ rice and weedy rice genotypes and its expression pattern at $a[CO_2]$ condition in most of weedy rice genotypes highlight its role in seed shattering (Fig. 4D). The upregulation of *SHAT1* in cultivated rice genotypes, mainly in Batatais, may be due the fact that *SHAT1* can display other roles independent of *SH4* as previously reported by Zhou et al., (2012). *SH4* has a different transcriptional profile from the other players involved in AZ cell differentiation. The alternated profile identified in weedy rice and Batatais where *SH4* was downregulated in $a[CO_2]$ and upregulated in $e[CO_2]$ corroborates with the increased seed shattering. The low seed shattering profile of cultivated rice at $a[CO_2]$, despite *SH4* upregulation, may be due the high expression of *OsCPL1*.

If in one hand, *qSH1*, *OSH15*, *SH4* and *SHAT1* work together to promote abscission layer formation; the *OsCPL1* works oppositely. *OsCPL1* is a recessive shattering gene that codifies for a carboxy-terminal domain (CTD) phosphatase-like 1 protein that represses the differentiation of the abscission layer reducing seed shattering (Ji et al., 2010). *OsCPL1* show increase transcript accumulation in almost all genotypes and $[CO_2]$ conditions (Fig. 4E). Higher *OsCPL1* transcript amount in $e[CO_2]$ and the high shattering level in all genotypes in $e[CO_2]$, maybe a case of post-transcriptional or post-translational modifications affecting *OsCPL1* product in play its role in repressing abscission layer cell differentiation (Nunes et al., 2014).

Homologous to *qSH1*, the *SH5* gene encodes a BEL1-type homeobox protein that inhibit lignin biosynthesis at abscission layer and therefore promotes seed shattering (Yoon et al., 2014). Interestingly, *SH5* is downregulated in almost all genotypes analyzed here at $a[CO_2]$ condition, while *SH5* is upregulated in all analyzed genotypes under $e[CO_2]$ condition (Fig. 4F). Therefore, the transcriptional regulation of *SH5*

is sensitive to $[\text{CO}_2]$ changes, with a vital effect on seed shattering in a future environment with increased $[\text{CO}_2]$. That effect was highlighted in Batatais genotype that have a high shattering level in $d[\text{CO}_2]$ (Fig. 1). In addition, the *SH5* sensitivity to increased $[\text{CO}_2]$ is also harmful since *SH5* can also induce the expression of *SHAT1* and *Sh4*, two important players for the proper abscission layer formation (Yoon et al., 2014). Also, considering that the abscission layer is a specialized cell layer located in the rachilla and is composed of small cells with thin nonlignified walls surrounded by larger lignified cells (Yu et al., 2020), the expression of *SH5* and *OSH15* can be acting directly in inhibiting the deposition of lignin, increasing shattering. Moreover, these genes effects can stand out from the others, resulting in suffer repression due to the expression of others genes, since shattering has a complex and polygenic character (Yoon et al., 2017).

Finally, abscission layer cell separation is the last step for seed abscission. Cell wall degrading enzymes can be involved in this process. *OsXTH8* is a cell wall remodeling enzyme that catalyzes cleavage of xyloglucan polymers (Jan et al., 2004). *OsXTH8* was reported to be highly expressed in abscission layer, and it is proposed to facilitates the separation of the grain (Nunes et al., 2014). As observed for *SH5*, *OsXTH8* expression was also highly sensitive to $[\text{CO}_2]$ changes (Fig. 4G). The high expression of *OsXTH8* in Batatais cultivar, which has low shattering, suggests that *OsXTH8* maybe not the main factor acting in seed shattering and/or some post-transcriptional or post-translational mechanism may be acting avoiding *OsXTH8* effect.

Seed shattering in a future atmosphere CO_2 concentration

The exact and detailed mechanism of how abscission layer formation and degradation occur is not fully understood. The genes identified in these processes may not act alone since other processes, such as synthesizing new cells and enzymes, can have a fundamental role. Also, a hormonal gradient between ethylene and auxin interconnects the process, acting precisely in the synthesis and secretion of enzymes that act on cell wall degradation and proteins that remodel the cell wall (Taiz et al., 2017). However, studies that underlie phytohormones' involvement in the specification of the abscission layer suggest that a precise balance between their biosynthesis and responses is of fundamental importance (Dong and Wang, 2015). Still, there is no complete elucidation of the molecular mechanisms and interactions of plant hormones underlying the abscission layer differentiation.

The plant's machinery works powered by the photosynthetic process, from where it obtains energy toward the formation of fundamental organic compounds toward the plant. When the plant is exposed to $d[\text{CO}_2]$, gene expression is altered (Leakey et al., 2009; Tallis et al., 2010) indicating that changes in gene regulation can be a mechanism linked to adaptation to increased $[\text{CO}_2]$ (Watson-Lazowski et al., 2016). Some transcriptomic studies have identified processes that respond to increased $[\text{CO}_2]$ with changes in gene expression such as photosynthesis (De Souza et al., 2008), respiration (Leakey et al., 2009) and leaves development (Ainsworth et al., 2006). In the latter, transcripts

for ribosomal proteins, cell cycle, and cell wall loosening, necessary in cytoplasmic growth and cell proliferation, were highly expressed in growing soybean leaves grown in $e[CO_2]$. In rice plants, gene expression obtained from plants grown in $e[CO_2]$ revealed to many gene expression, including senescence-associated protein 5, a gene associated with leaf senescence that triggers cell growth and structure (Fukayama et al., 2009). Thus, the expression of genes from the senescence-associated family can influence the shattering process in rice. Still, in a study developed with tomato leaves (Pan et al., 2019) the transcription of multiple genes related to ethylene synthesis and heat shock proteins was induced by elevated CO_2 ($800 \mu\text{mol mol}^{-1}$), acting in abscission in leaves. Therefore, there could be an effect of CO_2 on the hormonal balance in the abscission layer, affecting rice shattering.

Here, we identified that seed shattering-related genes are sensitive to changes in $[CO_2]$. We detected substantial increases in transcript accumulation of *qSH1*, *OSH15*, *SHAT1*, *OsCPL1*, *SH5* and *OsXTH8* which are related to abscission layer formation, lignin biosynthesis inhibition, and abscission layer cell separation in $e[CO_2]$ condition. Besides, the abscission layer inhibitor showed a smaller increase in its expression in the $e[CO_2]$ condition. Moreover, the weedy rice biotypes showed high seed shattering-related gene expression in $e[CO_2]$. This expression profile corroborates with the BTS values detected in the $e[CO_2]$ condition, whereas all analyzed genotypes showed increased shattering. Altogether, we demonstrate the harmful shattering profile, mainly related to weedy rice, in a future increased $[CO_2]$ environment (Fig. 5).

Atmospheric carbon dioxide directly determines the rate of photosynthesis in plants, affecting plant productivity and fitness, and can act as a selective pressure, driving evolution. Changes in gene expression, linked to critical adaptive characteristics, represent phenotypic plasticity and offer clues to the selection targets during long-term (multigenerational) adaptation. Also, it is possible that, in addition to gene regulation, the mutation acts to give rise to new locally adapted alleles (Watson-Lazowski et al., 2016). The availability of new molecular tools, particularly high-throughput and inexpensive RNA and DNA sequencing, suggests that further studies can be developed, using previously impossible approaches that combine phenotyping, functional genomics, and population genetic analysis in non-model systems.

Conclusion

The present study demonstrated that $e[CO_2]$ increases seed shattering in rice genotypes (estimated by the BTS measure), presenting a greater phenotypic effect for weedy rice biotypes. This was the first study that evaluated the effect of $[CO_2]$ on the shattering process. The mechanism associated with elevated CO_2 and shattering is unknown, as such, more approaches are needed to investigate other factors to elucidate this effect.

The gene expression analysis demonstrates an effect of $[CO_2]$ in the expression of all shattering-related genes (*OsCPL1*, *qSH1*, *SH4*, *SHAT1*, *OsXTH8*, *OSH15*, and *SH5*), presenting high variability between evaluated genotypes.

Methods

Plant material and growth conditions

Three weedy rice and four cultivated rice genotypes were analyzed (Table 1), previously phenotyped in other studies (Nunes et al., 2015, 2014). The experimental unit consisted of 8-L pots filled with soil (Albaqualf), fertilized at sowing, and it was seeded ten, and, after emergence, plants were trimmed to four plants per pot. The experiment was performed in a growth chamber maintained at 28/25°C day/night temperature. The cultural practices were based on rice production's technical recommendations for the south of Brazil (SOSBAI, 2018). The experimental design was completely randomized with three replications. The treatments included two [CO₂] levels: ambient (*a*[CO₂]) at 400 ± 20 µmol mol⁻¹ and elevated (*e*[CO₂]) at 700 ± 20 µmol mol⁻¹.

Table 1

Weedy rice and rice cultivars identification, species, and sub-species with origin in this study.

Genotype	Type	Species/Subspecies	Seed Shattering	Origin
AVAR	Weedy rice	<i>Oryza sativa</i>	Hight	Pelotas/Brazil
AV53	Weedy rice	<i>Oryza sativa</i>	Hight*	Porto Alegre/Brazil
AV60	Weedy rice	<i>Oryza sativa</i>	Hight*	Porto Alegre/Brazil
Batatais	Cultivated rice	<i>Oryza sativa</i> sub. <i>indica</i>	Low*	Porto Alegre/Brazil
IRGA 417	Cultivated rice	<i>Oryza sativa</i> sub. <i>indica</i>	Medium*	Pelotas/Brazil
IRGA 424 RI	Cultivated rice	<i>Oryza sativa</i> sub. <i>indica</i>	Medium	Pelotas/Brazil
Nipponbare	Cultivated rice	<i>Oryza sativa</i> sub. <i>japonica</i>	Low*	Porto Alegre/Brazil

*Described by Nunes et al., (2014)

Seed shattering phenotyping

Seed shattering was evaluated according to (Nunes et al., 2015). Briefly, the quantitative evaluation of the BTS was obtained at seed maturity using force gauge equipment connected by a hook to the seed until the seed's release from the pedicel. The force direction was exerted longitudinally to the petiole and the

grain. Four panicles per genotype and 10 grains from the panicle median part were evaluated. At the end of the experiment, the number of panicles, seed yield, and above-ground dry weight (ADW) per pot was determined. Data were analyzed by the two-tailed Student's t-test was used for statistical analyses (** indicates p -value<0.01 and * indicates p -value<0.05).

RNA extraction and cDNA synthesis

RNA was extracted from rice and weedy rice genotypes described in Table 1. Four panicles were selected per pot and marked with plastic tags at anthesis. Ten days after anthesis, 30 flower-pedicle junctions were chosen in the middle of the panicle, consisting of approximately 30 mg of plant material and one replicate. This material was immediately placed in liquid nitrogen and kept at -80°C until the RNA extraction occurred. Each collected flower-pedicle structure consisted of a 1 mm region of the pedicle and 1.5 mm of the flower, corresponding to the abscission layer zone (Ji et al., 2006; Li et al., 2006).

The total RNA was extracted using PureLink™ Plant RNA Reagent (Invitrogen) according to the manufacturer's recommendations. RNA quantity and purity were verified by spectrophotometry in NanoVue (GE Healthcare) and integrity by agarose gel electrophoresis. The cDNA was synthesized with the reverse transcriptase SuperScript™ First-Strand Synthesis System III (Invitrogen) using oligo(dT). The quality of the cDNA was assessed using an RT-qPCR reaction in the LightCycler® 480 Instrument II thermocycler (Roche) using SYBR Green I (Invitrogen) and oligonucleotides for the reference gene *Actin 1* (Table 2). Test reactions were performed before conversion into cDNA (to confirm digestion) and after conversion to cDNA. In both tests, genomic DNA was used as a positive control since the oligonucleotide for *Actin 1* was designed in exon junctions and had a greater amplicon in the presence of an intron serving as good control of the absence of genomic DNA.

Quantitative reverse transcription PCR (RT-qPCR)

The quantification of gene expression in RT-qPCR was performed according to the MIQE Guidelines (Bustin et al., 2009) using oligonucleotides for target and reference genes (Table 2). Each oligonucleotide's amplification efficiency and specificity were determined through validation experiments for each oligonucleotide using four cDNA dilutions. Oligonucleotides that presented efficiency between 90 and 110% and a single peak in the dissociation curve were used to quantify gene expression. The gene expression experiments were performed on the LightCycler® 480 Instrument II (Roche) thermocycler with three biological replicates and three technical replicates.

The reactions were performed containing 1 µL of cDNA at 1:25 dilution (determined in the validation experiment), 11.0 µL of UltraPure™ DNase / RNase-Free Distilled Water (Invitrogen), 0.25 µL of ROX Reference Dye (Invitrogen), 2.0 µL 10X PCR Buffer, 1.5 µL 50 mM magnesium chloride, 0.05 µL Platinum™ Taq DNA Polymerase, 0.2 µL dNTPs, 3.0 µL SYBR Green I (Invitrogen) and 1.5 µL of each oligonucleotide (forward and reverse) in a 20 µL of final volume. PCR reactions were performed under the

following conditions: initial denaturation at 95° C for 5 minutes, 45 cycles of 95° C for 20 seconds, 60° C for 15 seconds, and 72° C for 20 seconds on LightCycler® 480 Multiwell plates Plates 96 (Roche).

The quantification of gene expression was calculated using the comparative $\Delta\Delta CT$ method (Livak and Schmittgen, 2001), using a baseline the expression of the low seed shattering genotype Nipponbare in each treatment and normalized for the reference genes *OsACT1*, *OsEF1 α* , and *OsUBQ5*.

Table 2
Oligonucleotides used in RT-qPCR assay.

Gene	Oligonucleotide forward (5'-3')	Oligonucleotide reverse (5'-3')	Reference
<i>Housekeeping genes</i>			
<i>OsACT1</i>	CCTTCAACACCCCTGCTATG	CAATGCCAGGGAACATAGTG	(Zhou et al., 2012)
<i>OsEF1α</i>	TTTCACTCTTGGTGTGAAGCAGAT	GACTTCCTTCACGATTTTCATCGTAA	(Zhou et al., 2012)
<i>OsUBQ5</i>	ACCACTTCGACCGCCACTACT	ACGCCTAAGCCTGCTGGTT	(Zhou et al., 2012)
<i>Seed shattering target genes</i>			
<i>OsCPL1</i>	TATCTTGCCATGCAGCAGTC	TTGCTTGGGGAGTAGGACAG	(Nunes et al., 2014)
<i>OsH15</i>	TACCCCTCAGAGACGGAGAA	TGTGGGTGAAAACCTTCCAT	(Yoon et al., 2017)
<i>OsqSH1</i>	GCAAGGGTTAGGTTGTGGAA	CTGATGATGCACGCTATGCT	(Konishi et al., 2006)
<i>OsSH4</i>	CACAACGCCTCCGTTTCTTG	CATTACACCACACACCTGC	
<i>OsSH5</i>	TTCGGACTGGAGGACGTTAG	CCAACAAAGTCATGGAGCAA	(Yoon et al., 2014)
<i>OsSHAT1</i>	CAACCGCTACAGCAGCTGCA	GACGATGAATGCAGCGATCTTG	(Zhou et al., 2012)
<i>OsXTH8</i>	CCATCCATTCGTTCTCCATT	GTGCTCACTTGCTCAGCATC	(Nunes et al., 2014)

Abbreviations

BTS: breaking tensile strength; PCR: Polymerase chain reaction; RT-qPCR: Quantitative reverse transcription PCR;

Declarations

Ethics approval and consent to participate:

Not applicable.

Consent for publication:

Not applicable.

Availability of data and material:

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests:

The authors declare that there is no conflict of interest.

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Authors' contributions:

LAA, AMJr, CM and LHZ designed the research. AB, VEV, ARF, MVF and TH performed the experiments. LAA, AB and VEV analyzed the data. LAA, AB and VEV wrote and revised the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Effect of CO₂ concentrations ($a[\text{CO}_2] = 400 \mu\text{mol mol}^{-1}$ and $e[\text{CO}_2] = 700 \mu\text{mol mol}^{-1}$) in breaking tensile strength (BTS) of seeds from the pedicel of the weedy rice biotypes AVAR, AV53 and AV60 and rice cultivars Batatais, IRGA 417, IRGA 424 RI and Nipponbare at physiological maturity. Error bars correspond to standard error. The two-tailed Student's t-test was used for statistical analyses (** indicates p-value<0.01 and * indicates p-value<0.05).

Figure 2

Effect of CO_2 concentrations ($a[\text{CO}_2] = 400 \mu\text{mol mol}^{-1}$ and $e[\text{CO}_2] = 700 \mu\text{mol mol}^{-1}$) in the number of panicles (A) and grain yield (B) of the weedy rice biotypes AVAR, AV53 and AV60 and rice cultivars Batatais, IRGA 417, IRGA 424 RI and Nipponbare. Error bars correspond to standard error. The two-tailed Student's t-test was used for statistical analyses (** indicates $p\text{-value} < 0.01$ and * indicates $p\text{-value} < 0.05$).

Figure 3

Effect of CO_2 concentrations ($a[\text{CO}_2] = 400 \mu\text{mol mol}^{-1}$ and $e[\text{CO}_2] = 700 \mu\text{mol mol}^{-1}$) in above-ground dry weight (g pot⁻¹) (ADW) of the weedy rice biotypes AVAR, AV53 and AV60 and rice cultivars Batatais, IRGA 417, IRGA 424 RI and Nipponbare. Error bars correspond to standard error. The two-tailed Student's t-test was used for statistical analyses (** indicates $p\text{-value} < 0.01$ and * indicates $p\text{-value} < 0.05$).

Figure 4

Effect of CO₂ concentrations (*a*[CO₂] = 400 μmol mol⁻¹ and *e*[CO₂] = 700 μmol mol⁻¹) in weedy rice genotypes and rice cultivars 10 days after polinization. Relative expression of *qSH1*(A), *OSH15* (B), *SH4* (C), *SHAT1*(D), *OsCPL1* (E), *SH5* (F) and *XTH8* (G) genes and the relationship between gene expression and seed shattering as function of CO₂ concentrations (H).

Figure 5

The schematic model, the effects of CO₂ increased concentrations on seed shattering based on the gene expression profile of seed shattering-related genes in increased CO₂ concentrations (700 μmol mol⁻¹) in rice and weedy rice. The expression values were obtained from Batatais (rice cultivar) and AV60 (weedy rice biotype) since they were the most sensitive genotypes related to relative gene expression in comparison between CO₂ concentration of 400 μmol mol⁻¹ (normal condition) and CO₂ concentration of 700 μmol mol⁻¹ (a future condition). The genetic interactions were obtained from Zhou et al., (2012), Yoon et al., (2014), and Yoon et al., (2017).