

Experimental methods for phenotypic and molecular analyses of seed shattering in cultivated and weedy rice

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Abstract: The seed shattering trait has been repeatedly reshaped during rice evolution. Reduced in cultivated rice and increased in weedy rice, shattering is of great agronomic importance because of its association with yield losses. Since its first descriptions, the phenotypic patterns and the genetic regulation of cultivated and weedy rice seed shattering have been extensively studied, with a variety of methods and techniques. The aim of this review is to discuss and recommend the most suitable experimental methods for phenotypic and molecular evaluation of seed shattering in cultivated and weedy rice. Rice seed shattering must be

quantified, preferably, by breaking tensile strength (BTS) assays, because other methods are more prone to human errors. The evaluation time is particularly important, and the developmental stages of the panicles measured need to be recorded. QTL analyses and GWAS studies are suitable for discovery of genes influencing shattering, but the resulting genes may only be relevant in the parental lines or the populations used. The variety of cultivated rice and evolutionary origin of weedy rice accessions has a great influence on results of rice seed shattering phenotypic and genotypic analyses and needs to always be taken into account.

Keywords: *Oryza sativa*; Breaking tensile strength; Abscission layer; Seed shattering; Weedy rice evolution

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1. Introduction

Shattering is the ability of the plant to naturally release its seeds, and is a trait with polygenic genetic inheritance of great evolutionary importance for spermatophyte plants (Dong, Wang, 2015). Shattering allows seed dispersal and maintenance in the soil seed bank, ensuring species perpetuation. In many cultivated grains, such as rice, shattering has been lost or reduced during the domestication process to facilitate harvesting and threshing. Conversely, weedy plants tend to shatter their seeds. Weedy rice plants compete with cultivated rice for water, nutrients and light, and produce roots, culms, leaves and grains similar to the cultivated rice. However, the grains of weedy rice constantly shatter before harvest and prevent the possibility of utilization by humans and removal during harvest. The cultivated rice grain yield losses caused by weedy rice may vary from 20 to 80% (Xu et al., 2018; Karn et al., 2020) depending on the level of infestation and rice variety (Dai et al., 2014).

Cultivated Asian rice (*Oryza sativa* L.) is a self-pollinating diploid species with 12 chromosomes (2n = 24), existing as two distinct subspecies – *O. sativa* subsp. *indica* (*indica* rice) and *O. sativa* subsp. *japonica* (*japonica* rice). *Indica* rice is further divided into *indica* and *aus* subgroups. *Indica* varieties thrive in tropical regions, primarily in Latin America and South Asia. *Aus* group varieties are stress-tolerant and generally have more easily shattering seeds (Lee et al., 2018). *Aus* rice cultivation is limited to specific regions in East India and Bangladesh (Garris et al., 2005; Zhou et al., 2022). *Japonica* rice is the predominant type of rice grown in North China, Europe, and the United States. It is subdivided into *tropical japonica*, *temperate japonica*, and *aromatic* subgroups. Weedy rice invades cultivated rice fields globally and has been shown to have multiple independent origins. Most weedy rice has arisen through feralization or de-domestication from cultivated rice and thus can be classified as *O. sativa*, but origins from wild ancestors or through hybridization have also been documented (Goulart et al., 2014; Ziska et al., 2015; Huang et al., 2018; Qiu et al., 2020; Hoyos et al., 2020; Li et al., 2022).

Recognized by its red pericarp, leading to the term “red rice,” weedy rice often exhibits distinct traits such as black, brown or straw-colored hulls, awned seeds, and taller plants; however, these features are not universal across all weedy rice populations (Huang et al., 2021; Roma-Burgos et al., 2021). Historically, weedy rice plants have often been taller than rice cultivars (Schwanke et al., 2008; Shivrain et al., 2010), but the introgression of genes from cultivars and selection pressure through hand weeding select for plants mimicking rice cultivars (Burgos et al., 2014; Piveta et al.,



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2021). Likewise, in South America, the old short grain characteristic of weedy rice changed into for long grain similar to the actual modern cultivated rice varieties (Goulart et al., 2014; Piveta et al., 2021). Nevertheless, the ability to shatter their seeds is a ubiquitous trait among weedy rice populations and, along with seed dormancy, is the most important trait related to weediness (Ziska et al., 2015). This trait is the basis of the Italian name for weedy rice, “riso crodo”, which means “rice that shatters” (Ferrero, Vidotto, 2004), and which better characterize this plant along with the morphological characteristic most associated with the damage in agricultural fields.

High seed shattering is a trait usually associated with wild and weedy plants. In cultivated plants, a moderate or low amount of shattering is desirable, to facilitate threshing; ideally seeds will detach from the plant easily at the harvest time, but not at other times to avoid yield losses. The reduction of seed shattering in crops is associated with domestication. Initially, this process occurred through unintentional selection, as grains collected during harvest were those retained until maturity (Purugganan, 2019). Subsequently, this process was improved through artificial selection, where desirable traits were identified and maintained in the population through diverse methods of plant breeding (Ishikawa et al., 2022). Two seminal studies published in 2006 cloned the first genes involved in regulation of seed shattering in rice: *qSH1* (Konishi et al., 2006) and *SH4* (Li et al., 2006a). These publications opened an avenue for further studies related to weedy rice evolution and potential development of control strategies. However, later studies have demonstrated that other major genes are also important in different populations of *japonica* and *indica* rice, as well in *japonica*-like and *indica*-like weedy rice accessions (Htun et al., 2014; Ji et al., 2010; Jiang et al., 2019; Nunes et al., 2015; Zhu et al., 2012), emphasizing the multi-regulation of seed shattering in rice and weedy rice, and the difficulties related to studying this trait.

The genetic basis of and the influence of environmental factors on seed shattering have been recently reviewed (Maity et al., 2021; Wu et al., 2023). However, there is no concise synthesis of the methods used to evaluate seed shattering in cultivated and weedy rice. The lack of common criteria for evaluating the magnitude of shattering can hinder comparisons between studies and potentially result in incorrect inferences about the timing of shattering, its anatomical basis, and gene regulation. The aim of this review is to discuss and recommend the most adequate experimental methods related to phenotypic and molecular evaluation of seed shattering in cultivated and weedy rice. The review briefly presented the basis of seed shattering in *Oryza* and points out the importance of precisely determining the seed shattering profile. We further discuss methodologies to characterize rice seed shattering phenotypically and genotypically and present a practical stepwise direction for cultivated and weedy rice seed shattering analysis.

2. The fundamentals of seed shattering in rice

Seed shattering in monocotyledonous plants occurs by a direct detachment of the seed from the pedicel. The abscission layer (AL; also, often referred to as the abscission zone or AZ) plays a fundamental role in shattering. In rice, the AL is usually a variable single layer of cells (Jin, 1986) and is located below the two sterile lemmas and above the rudimentary glumes (Figure 1). The cells that compose the rice AL are small, with dense cytoplasm connected by plasmodesmata, and characterized by the lack of lignin. The level of shattering is impacted by the formation and degradation of the AL (Thurber et al., 2011). Studies in *Arabidopsis thaliana* suggest that the process of general organ abscission consists of four main steps (Figure 2) (Patterson, 2001). However, these steps have not been verified in such detail in rice. The process of abscission can vary among populations and is influenced by environmental conditions and phenological stages of development, with the participation of plant hormones (mainly ethylene and auxins) and several molecular factors (Wu et al., 2023).

3. Phenotypic analyses of seed shattering

The seed shattering level of a certain cultivated and weedy rice accession can be qualitatively categorized as easy-shattering, moderate-shattering, and hard/non-shattering phenotypes. Seed shattering is also quantitatively assessed by the percentage of shattered seeds or the force necessary for a seed to detach. These phenotypic analyses, supplemented and correlated with genotypic analyses, can provide valuable insights into the mechanisms and factors underlying seed shattering.

3.1 How to quantify

Seed shattering can be evaluated using various methods, and the choice of method may depend on resources available and the specific objectives of the study. Regardless of the technique, enough replications are required for a valid evaluation, given the high variance of the trait: a minimum of two, preferably three, panicles per plant are ideal. To reduce variability and improve comparability across studies, it is crucial to evaluate seed shattering at a consistent developmental stage (see section 3.2). In rice, grain maturation occurs progressively from the tip of panicle toward the base. For the methods focusing on individual seeds, evaluation is recommended for the seeds located in the middle third of the panicles that are at comparable developmental stages. For the methods that evaluate the whole panicle, it is also critical to choose the panicles at similar maturation stages. Even with these precautions, shattering level can be influenced by the environmental conditions; thus, within-study comparisons tend to be more reliable than those between studies.

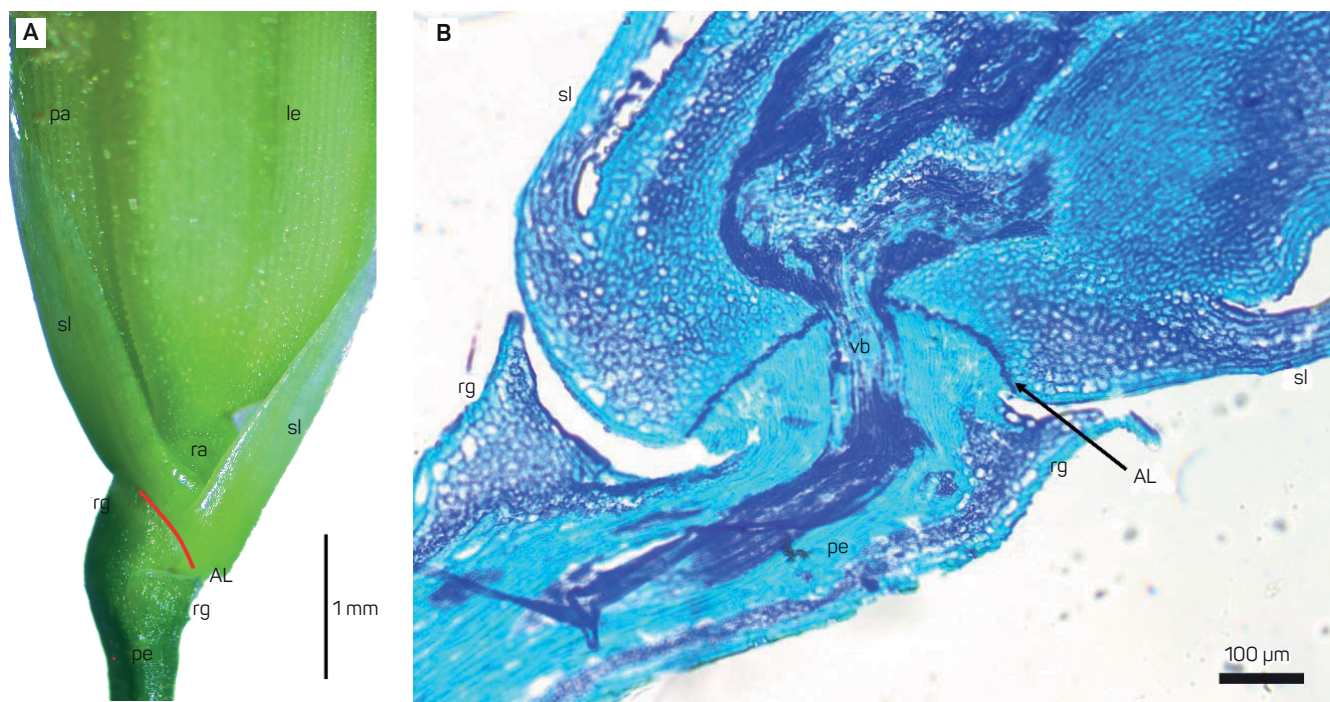


Figure 1 - Detailing the base of a rice spikelet. Rice spikelet under a light dissection microscope (A). Toluidine blue stained longitudinal section of a spikelet from weedy rice (B). The AL (abscission layer) is represented by a red line (A) or stained dark blue and indicated by the black arrow (B). pa: palea; le: lemma; ra: rachilla; sl: sterile lemma; pe: pedicel; vb: vascular bundle; rg: rudimentary glume

3.1.1 Hand method

This method entails gently rubbing or gripping the panicle manually and observing the extent of seed detachments. Typically, the seed shattering level obtained through this method is qualitative, categorized as “shatter” or “do not shatter” (Zhu et al., 2012), or as scales from 1 to 7, where 1 means difficult to shatter and 7 means easy to shatter (Qin et al., 2010). In some cases, the number of shattered seeds resulting from hand-based methods can be counted and compared to the total number of seeds from the same panicle, providing a percentage of shattered seeds. Variations of this method include hitting the panicle on a surface or clasping the panicle with one hand. Despite its convenience and feasibility, these hand techniques are highly subject to human error, varying according the skill and consistency of the performer, making it challenging to achieve consistent results when repeating the experiments or when comparing across studies.

3.1.2 Falling method

This method involves dropping the panicle from a predetermined height onto a hard surface, typically a concrete or metal plate. Similar to the hand method, the results obtained can be interpreted qualitatively, by categorizing shattering levels, or quantitatively, by measuring the percentage of shattered seeds. One advantage of the falling method is its less dependency on the consistency of the operator performing the

experiment. It is particularly useful for studies aiming to assess and compare the broad levels of seed shattering between the rice accessions with contrasting shattering abilities. However, this method is not appropriate for precise estimation of small variations in seed shattering because the velocity and impact of the fall is modified by slight variations in panicle weight.

3.1.3 Bag method

This method consists of placing the panicles at heading stage inside a plastic or paper bag, and then evaluating the number of shattered seeds in comparison with the total number of seeds at a specific timepoint. A minimum number of 4 panicles and 2 individuals per accession are recommended for this method. The timepoint must be the same among repetitions and between accessions. This method has been used in a recent study due to its simplicity and independence from human factor (Jiang et al., 2019). However, it is important to ensure that the panicles are not subjected to abrupt movements to avoid seed shattering overestimation. Additionally, there is a risk that motions associated with the bag removal can exacerbate seed shattering and distort estimates and may introduce variability associated with the human operator.

3.1.4 Breaking tensile strength (BTS)

The breaking tensile strength (BTS), in the units of gram-force (gf) or newtons (N), can be used to quantify

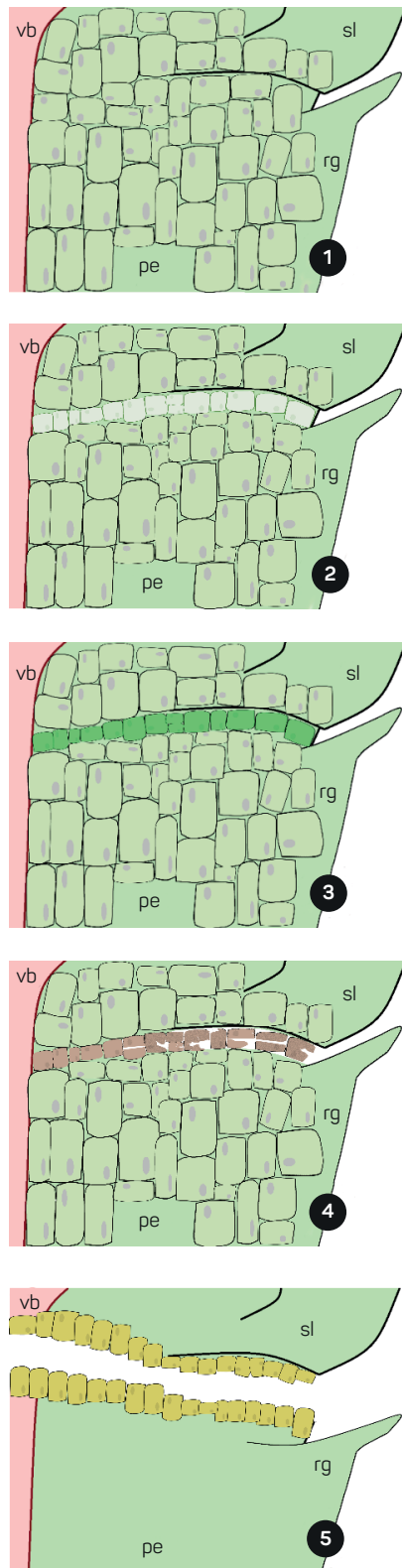


Figure 2 - Schematic representation of how rice grain shattering hypothetically proceeds based on studies in *A. thaliana*. 1) Cells prior to abscission start; 2) Differentiation of the cells of the abscission layer; 3) Acquisition of cellular competence for the perception of abscission signals; 4) Activation of the abscission; 5) Separation of the organs. sl: sterile lemma; pe: pedicel; vb: vascular bundle; rg: rudimentary glume

the force required to detach seeds from the panicle, and, consequently, determine the shattering levels in rice (Ji et al., 2006). A larger force needed to detach seeds indicates harder shattering – that is, lower levels of seed shattering. BTS can be measured using a digital force gauge (dynamometer) (Figure 3A). Researchers can either choose to affix the force gauge to the work surface or affix a panicle to the work surface. A small hook or a clamp is attached to the seed (Figure 3B) and then gentle pulling occurs until the seed is detached (Ji et al., 2006; Thurber et al., 2010). The maximum tensile strength at the moment of seed detachment is recorded by the instrument. Note that it is important to keep the force gauge and the direction of the applied force aligned with each other to avoid errors of measurement (Figure 3A). Any pulling at an angle changes the force being recorded. Besides this pulling strength (PS), some studies have also measured bending strength (BS), which consists of applying the force perpendicular to the pedicel, attaching the seed horizontally (Li et al., 2019; Qin et al., 2010). In general, the BS is much lower than PS, but these two measurements are correlated. For this reason, we consider that there is no reason to conduct both measurements simultaneously, and PS is more prevalent in the literature. As an alternative to a digital force gauge, BTS can be measured with small weights. In this case, the panicle should be fixed upside down and the hook placed at the bottom of the seed. The hook is connected to a plate where the weights are manually positioned (Figure 3C). The load that causes the seed detach is recorded and the weight of the plate should also be considered. In both cases, a minimum of 10 seeds per panicle should be evaluated to obtain a measure of variability. The BTS technique, especially using an automatic instrument, is the most precise and sensitive one among the existing methods.

3.1.5 Observations of seed bases

An indirect evaluation of seed shattering ability in archaeological and modern rice samples involves examination of the rice seed base under a light or scanning electron microscope (Fuller et al., 2009; Ishikawa et al., 2022; Qiu et al., 2017). Rice accessions characterized by high shattering levels, such as wild or weedy rice, often exhibit a smooth surface with a small sealed vascular bundle pore after detaching from the plant. In contrast, cultivated rice varieties with low shattering levels often display a rough and uneven surface, along with larger and more irregular pores resulting from the tearing of vascular strands (Fuller et al., 2009).

The rice seed base observation method can be effective in some degree in identifying highly shattering phenotypes. However, in our experience, it is not a reliable method for attempting to qualitatively categorize samples into various degrees of shattering/non-shattering. There is substantial variation in seed base morphology, even within single plants, which can be misleading about the shattering status

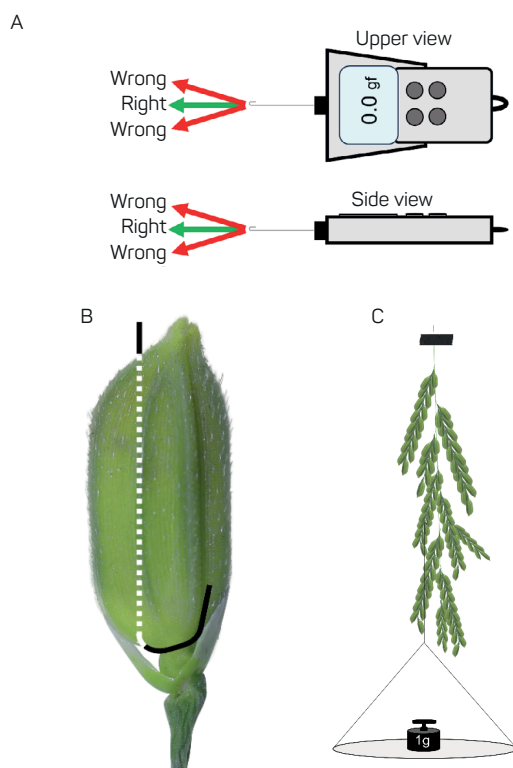


Figure 3 - Shattering level estimation based on breaking tensile strength (BTS). Demonstration of the digital force gauge used to evaluate the strength to detach the seeds and the correct operation of the instrument (A). Small hook connected vertically to the seed (B). Alternative way to determine BTS with weights (C)

of a given genotype. It is also challenging to precisely determine the degree of smoothness or roughness of the seed base, or to establish a correlation between these characteristics and shattering abilities. Some studies have also explored the surface of the pedicel after detachment as an additional means to estimate shattering abilities (Ishikawa et al., 2022; Ning et al., 2023), but the correlation between pedicel surface morphology and shattering levels have not been extensively evaluated.

3.2 When to quantify

The timing of measurement is very important in seed shattering studies since BTS values tends to decrease during development in all types of rice, although they stabilize after reaching a certain stage of maturity. Because of changes in BTS throughout development, it is imperative that all samples measured are at the same developmental stage. The ideal moment to evaluate seed shattering in rice depends on the choice of method and the specific research objectives. However, most studies seek to characterize the shattering level at seed maturity, as this is when seed dispersal is likely to occur naturally. Evaluations that occur too early risk underestimating the final shattering level of the plant. When present, the AL, which is crucial

for shattering, forms substantially before heading (Jin, 1986; Thurber et al., 2011). However, AL presence and morphology varies extensively between different cultivated rice varieties and weedy rice accessions (Konishi et al., 2006; Li et al., 2006a; Lin et al., 2007; Thurber et al., 2011; Zhou et al., 2012) so in and of itself, AL development does not provide guidance on the most suitable time to measure seed shattering.

Although, ideally, shattering measurements should be performed across several time-points to obtain a curve of how BTS changes over the course of development for each sample studied, the labor involved is too high. However, such time courses have been carried out for some samples of cultivated and weedy rice, and across most accessions examined, BTS levels off and stabilizes 20 days after heading (DAH) (Ji et al., 2006; Thurber et al., 2011). The timepoint of 30 DAH has been widely used to estimate the shattering levels (Cai, Morishima, 2000; Thurber et al., 2010; Zhou et al., 2012) as it constitutes a “safe” time point at which all rice types have mature grains and BTS has stabilized. To carry out shattering assessments correctly, then, it is crucial that the heading date of each individual panicle be noted to determine when 30 DAH is reached. A colored thread can be used to mark each day.

4. Genotypic analyses of seed shattering

Knowledge of genetic regulation of the seed shattering trait in cultivated and weedy rice has progressed since identification of the first two loci (Konishi et al., 2006; Li et al., 2006a), though many gaps remain. Existing studies have primarily sought to elucidate the genetic factors involved in the loss of seed shattering during the domestication of cultivated rice, or the reacquisition of this trait in weedy rice populations (Li et al., 2006b; Lin et al., 2007; Thurber et al., 2013; Subudhi et al., 2014; Yao et al., 2015; Li et al., 2022). QTL mapping has been widely used to identify the corresponding genomic regions for shattering. To date, at least 61 shattering QTLs and 12 genes with some degree of importance for shattering have been identified in cultivated rice, as summarized in Wu et al. (2023). In weedy rice, 40 chromosomal regions have been reported to have some degree of influence on the shattering trait (Table 1) (Bres-Patry et al., 2001; Gu et al., 2005; Subudhi et al., 2014; Thurber et al., 2013; Yao et al., 2015; Qi et al., 2015; Mispan et al., 2013; Oh et al., 2004; Sun et al., 2019).

It is paramount to clarify that the genetic regulation responsible for variation in seed shattering in *Oryza* is background-dependent. This means that differences in seed shattering may be attributed to different mechanisms in different cultivated varieties and in weedy rice with different evolutionary origins. For this reason, it is important to have knowledge of the genetic background of the material of interest. In cultivated and weedy rice, physical characteristics are not generally sufficient to infer genetic background. For cultivated rice, knowledge

of group membership (i.e. *indica*, *aus*, *temperate japonica*, *tropical japonica*, *aromatic*) can often be obtained from germplasm repositories or from breeders. For weedy rice, determination of group membership, or, more specifically, what ancestral group the weedy rice evolved from, usually requires genotyping and comparison with other known cultivated and weedy groups using phylogenetic or population structure analyses, unless the material has been characterized previously. In the following section, the latest knowledge on genetic regulation of seed shattering in cultivated and weedy rice is summarized and the specifics regarding genetic background are discussed.

4.1 Genetic regulation of *Oryza* seed shattering and possible targets for molecular studies

4.1.1 Known shattering loci involved in rice domestication

The *SH4* gene was the first rice shattering locus identified, initially through QTL mapping in an F_2 population from a cross between a shattering wild rice (*O. nivara*) and a reduced shattering *indica* cultivar (Li et al., 2006a). This locus was mapped to chromosome 4 and explained 69% of the shattering phenotypic variation observed in this cross. The *SH4* gene encodes a Myb transcription factor and is probably involved in AL development (Li et al., 2006a). The substitution of the amino acid lysine for asparagine at position 79 (Lys79Asn) in the *sh4* allele results in a reduced shattering phenotype (Li et al., 2006a). This mutation is considered of fundamental importance in rice domestication, as it has been found to be fixed in all cultivated rice varieties, consistent with strong selection for reduced shattering and ease of harvest. Simultaneously, an allele of *SH4*, denominated *SHA1* was identified in a cross between *O. rufipogon* wild rice and an *indica* cultivar. Distinguishing itself from *SH4* by a single residue change and a deletion of six residues, *SHA1* was proposed to encode a trihelix transcription factor and promote hydrolysis of the abscission layer. However, the same amino acid substitution (Lys79Asn) was shown to lead to reduced shattering in this cross (Lin et al., 2007). Another study also identified the involvement of an *SH4* allele, named *SHAT2*, in which a frameshift mutation from a single nucleotide insertion in the second exon of the gene led to reduced shattering in *indica* (Zhou et al., 2012). Despite the importance of this locus in reduction of shattering in rice cultivars, several studies have shown that the *SH4* gene has little or no relevance for the regulation of shattering in most weedy rice (Thurber et al., 2010; Zhu et al., 2012; Nunes et al., 2014; Huang et al., 2018; Hoyos et al., 2020; Li et al., 2022). Many weedy rice accessions carry the Lys79Asn mutation associated with rice domestication, as expected given their de-domestication origins; however, despite the mutation, weedy rice still exhibits a high level of shattering.

Also among the first loci identified as playing a role in reduction of shattering during rice domestication is *qSH1*, initially mapped to chromosome 1 in a cross

between Kasalath, an *aus* rice cultivar that has high shattering levels, and Nipponbare, a *japonica* rice cultivar that does not shatter (Konishi et al., 2006). The *qSH1* locus, which explained 68.6% of the observed variation in this cross, was found to correspond to single nucleotide polymorphism (SNP) in the 5' regulatory region of a rice ortholog of the *A. thaliana* *REPLUMLESS* (*RPL*) gene, which encodes a BEL1-type transcription factor gene. A G to T substitution in *qSH1* was found to prevent formation of the AL and cause the loss of shattering in *japonica* (Konishi et al., 2006). Through examining genotypes at this locus in wild rice and cultivated rice varieties, the non-shattering mutation at *qSH1* was found to be limited to only a subset of *japonica* accessions and relevant to reduction of shattering only in these cultivars (Zhang et al., 2009). A study of relative expression and nucleotide sequence of this genomic region in 14 weedy rice accessions and 18 rice cultivars further confirmed that there is no variation at this locus in *indica* cultivars nor weedy rice, indicating a lack of impact of this gene on shattering variation in these groups (Nunes et al., 2015).

More recently, the *OsSh1* locus, located to the *qSH3* QTL, was found to play a role in the shattering phenotype in an *indica* cultivar (Li et al., 2020). A 13 base-pair deletion in the third exon gives rise to alternative splicing of *OsSh1* and results in a decrease of shattering. The *OsSh1* gene acts downstream to the *qSH1* gene (Li et al., 2020) and encodes a transcription factor from the YABBY family (Lin et al., 2012). In another study, the substitution of a cytosine for a thymine at position 70 in the first exon of *OsSh1* was associated with a low-shattering phenotype (Ishikawa et al., 2022). This mutation is highly conserved in the *indica* and *japonica* groups, but not frequently observed in *aus* cultivars or in wild rice (Ishikawa et al., 2022; Li et al., 2020). However, introducing domestication mutations at *OsSh1* or *SH4* alone in the *O. rufipogon* genetic background did not result in loss of shattering, suggesting that it is the interaction between these two genes that has been fundamental for the changes in shattering during rice domestication (Ishikawa et al., 2022).

4.1.2 Shattering loci with no known role in rice domestication

The roles in rice domestication for other loci regulating the rice shattering trait, particularly those identified through mutagenesis screens, are less clear. For instance, treatment of a shattering chromosome segment substitution lines (CSSL) containing *O. rufipogon* introgression in an *indica* background with gamma rays, gave rise two mutant strains with no shattering, named *shat1* and *shat2* (Zhou et al., 2012). The *SHAT1* gene was identified and defined as the ortholog of the *APETALA2* (*AP2*), a gene coding for a transcription factor associated with flower development in *A. thaliana* (Chen, 2004). RNAi silencing of the wild type allele of *SHAT1* in the *aus* cultivar, Kasalath, which presents some degree of

Table 1 - QTLs reported to be related with seed shattering in weedy rice

QTL	Crosses	Chr.	Marker interval or nearest one	%PV ^a	Ref.
SHT	<i>japonica</i> cultivar x weedy rice collected from France	1	RM212	30.2	1
sh1	<i>indica</i> cultivar x weedy rice collected from Korea	1	RM315-RM319	8.0	2
sh2		2	RM526-RM318	17.3	
sh5		5	RM163-RM7568	8.9	
sh6		6	RM527-RM539	15.2	
sh11		11	RM3701-RM3137	7.5	
qSH3	<i>indica</i> cultivar x weedy rice collected from Thailand	3	RM486	9.0	3
qSH4		4	RM471	12.0	
qSH7		7	RM118	10.0	
qSH8		8	RM135B	8.0	
qSH3	<i>indica</i> cultivar x weedy rice collected from US	3	RM1334	8.0	4
qSH7		7	RM6403	2.0	
qSH9		9	RM5515	2.0	
qSS1b	<i>indica</i> cultivar x weedy rice collected from US	1	RM104	-	5
qSS2s	<i>indica</i> cultivar x weedy rice collected from US	2	RM236	-	5
qSS11s		11	RM332	-	
qSS12s		12	RM3246	-	
qSH2 ^{BR}	<i>japonica</i> cultivar x weedy rice collected from US	2	RM13910-RM13938	8.9	6
qSH3 ^{BR}		3	RM3203-RM3372	4.4	
qSH4 ^{BR}		4	RM5506-RM127	22.3	
qSH7 ^{BR}		7	RM429-RM3555	3.6	
qSH10 ^{BR}		10	RM6100-RM1146	5.3	
qSH4 ^{CR}	<i>japonica</i> cultivar x weedy rice collected from US	4	RM5506-RM127	30.3	6
qSH6 ^{CR}		6	RM8225-RM4924	3.2	
qSH10 ^{CR}		10	RM171-RM1146	4.0	
wd-qsh1	<i>indica</i> cultivar x weedy rice collected from South Korea	1	RM323-RM283	11.6	7
wd-qsh3		3	RM5626-RM7097	31.4	
wd-qsh5		5	RM169-RM548	16.8	
qSH2S	<i>indica</i> cultivar x weedy rice collected from US	2	4352643 ^b	51.7	8
qSH11S		11	4320500 ^b	2.0	
qSH1Bb	<i>indica</i> cultivar x weedy rice collected from US	1	38361220 ^b	7.2	8
qSH3Bb		3	25000637 ^b	14.1	
qSH5Bb		5	1786217 ^b	6.9	
qSH6Bb		6	20058587 ^b	1.9	
qSH8Bb		8	20478068 ^b	4.6	
qSH12Bb		12	4712192 ^b	5.5	
QWDHS1	<i>indica</i> cultivar x weedy rice at Asian high latitudes	1	M241803-M241239	10.2	9
QWDHS2		1	M289867-M297534	12.3	
QWDHS3		8	M1775129-M1777170	10.9	
QWDHS4		9	M1937927-M1942126	10.5	

QTL: quantitative trait loci; Chr: chromosome; Ref.: original article reference. ^aPercentage of phenotypic variation explained by QTL. ^bThe genomic location of the nearest marker in the MSU6.0 rice reference genome

1. Bres-Patry et al., 2001; 2. Oh et al., 2004; 3. Gu et al., 2005; 4. Mispan et al., 2013; 5. Thurber et al., 2013; 6. Subudhi et al., 2014; 7. Yao et al., 2015; 8. Qi et al., 2015; 9. Sun et al., 2019

shattering, gave rise to plants with reduced shattering phenotype (Zhou et al., 2012). This study also compared the expression of *SH4*, *qSH1* and *SHAT1* genes at different developmental stages, proposing a model where *SH4* acts before *SHAT1* and *qSH1*, and *SHAT1* is responsible for the activation of *qSH1*; and both of the latter loci are related to the maintenance of *SH4* expression by some unexplained feedback process, promoting the differentiation of the abscission layer (Zhou et al., 2012).

The *SH5* gene located on chromosome 5 was detected through in-silico analysis, due to its high similarity to *qSH1*. This gene also encodes a BEL1-type transcription factor. Silencing of *SH5* in the easy-shattering cultivar Kasalath minimized the development of the abscission layer and reduced the shattering; overexpression of *SH5* in the non-shattering cultivar Dongjin accelerated the development of the abscission layer and resulted in moderate shattering (Yoon et al., 2014). While there is no known role of *SH5* in rice domestication, the expression of *SH5* is conditioned to the expression of *qSH1* and upregulates the expression of the *SH4* and *SHAT1* genes. *SH5* has been proposed to be involved in inhibition of deposition of lignin at the base of the grains, which favors shattering (Yoon et al., 2014). Another study identified the interaction of *SH5* with the *OSH15* gene (Yoon et al., 2017), which encodes a KNOX protein that acts in interaction with BELL homeobox genes, such as *qSH1* and *SH5*. The KNOX:BELL complex is associated with lignin synthesis and the knockout of the *OSH15* gene in the *japonica* cultivar Dongjin reduced the lignin deposition in the AZ which elevated the shattering level (Yoon et al., 2017).

The *OsCPL1* gene in the *sh-h* QTL on chromosome 7 was another locus identified through the analysis of the F_2 progeny from a cross of an easy-to-shatter mutant strain with a hard-to-shatter rice cultivar, both from *japonica* subspecies (Ji et al., 2010). *OsCPL1* encodes the C-terminal domain of a phosphatase-like enzyme. By silencing the *OsCPL1* gene, it was shown that a 15-base pair (five amino acids) deletion from the C-terminal domain in the mutant resulted in a shattering phenotype (Ji et al., 2010). There are indications that the phosphatase related to the *OsCPL1* gene is involved in cell differentiation and inhibits the formation of the abscission layer. Consequently, when the gene is inactivated through deletion, it promotes the development of AL and results in an increase in shattering. Curiously, another study found that a higher expression of this gene was observed in the high shattering weedy rice than in the low shattering cultivated rice (Nunes et al., 2014).

Through a screening of mutagenized wild rice introgression lines with high shattering, the rice accession with loss-of-shattering phenotype, named *ssh1*, was identified (Jiang et al., 2019). The *SSH1* gene was considered as an allele of the *SUPERNUMERARY BRACT* (*SNB*) gene, which encodes a domain of the AP2 transcription factor. *SSH1* has a recessive character, it is located on chromosome 7 and the replacement of a cytosine by an adenine in the

ninth intron of this gene results in the loss of functionality and less shattering (Jiang et al., 2019).

Other genes may be indirectly involved in the shattering process. The genes *OsXTH8* (synonym *OsXRT5*) and *OsCel9D* (synonym *OsGLU1*), related to cell wall development (Kikuchi et al., 2003), were associated with seed shattering in weedy rice based on relative expression studies (Nunes et al., 2014). The *OsXTH8* gene encodes the enzyme xyloglucan endotransglycosylase-hydrolase catalyzing the breakdown or formation of β -1,4-xyloglucan. This gene is highly expressed when elongation and differentiation processes are active and gibberellin levels are high (Jan et al., 2004). In weedy rice accessions, the expression of the *OsXTH8* gene is directly related to the level of shattering, that is, the higher the expression, the lower the BTS (Nunes et al., 2014). The *OsCel9D* gene encodes an endo-1,4- β -glucanase involved with internode elongation and cell wall composition, mainly cellulose and pectin contents (Zhou et al., 2006). In weedy rice, the expression of this gene was inversely proportional to the shattering, indicating that the changes in the cell wall resulted from the high expression of this gene may hinder the formation of the abscission layer and thus reduce the shattering (Nunes et al., 2014). The *OsLG1* gene encodes a squamosa promoter-binding protein and correlated with panicle shape by controlling the ligule development and laminar joint (Lee et al., 2007). The higher the expression of *OsLG1*, the more open the panicle and the higher the shattering level in plants with *japonica* background (Ishii et al., 2013). Similarly, the *OsGRF4* gene encodes a growth-regulation transcription factor and regulates precursors of cytokinin dehydrogenase, and higher expression levels of *OsGRF4*, resulted in fewer shattered seeds (Sun et al., 2016). Likewise, the silencing of *NPC1* increased the silicon content and mechanical strength in rice nodes, and resulted in a hard-to-shatter phenotype (Cao et al., 2016).

4.1.3 Loci involved in shattering in weedy rice

Compared to cultivated rice, no shattering gene has yet been cloned in weedy rice. However, several QTL mapping studies have identified at least 40 chromosomal regions (Table 1), a few overlapping, with some influence on the shattering trait (Bres-Patry et al., 2001; Gu et al., 2005; Subudhi et al., 2014; Thurber et al., 2013; Yao et al., 2015; Qi et al., 2015; Mispan et al., 2013; Oh et al., 2004; Sun et al., 2019). The cross between a *japonica* cultivar and a weedy rice collected from France identified the QTL *sh-2*, which explains 30.2% of the observed variation for shattering in this cross (Bres-Patry et al., 2001). *sh-2* is located on chromosome 1, and close to the *qSH1* locus (Yao et al., 2015). A backcross population involving a weedy rice from Thailand and an *indica* rice cultivar identified the QTLs on chromosomes 3, 4, 7 and 8, which collectively account for less than 33% of the observed phenotypic

variability in shattering in this population (Gu et al., 2005). Crosses of an indica cultivar with two distinct weedy rice ecotypes from the US, SH and BHA, identified one QTL of large effect (51.7 % of variation in *indica* x SH cross) on chromosomes 2 and multiple QTLs of small effect (2 in *indica* x SH cross; 6 in *indica* x BHA cross) on chromosomes 1, 3, 5, 6, 8, 11, and 12; this study also demonstrated that the loci underlying the evolution of shattering in different weedy rice populations differ from each other, suggesting independent acquisition of the shattering trait QTLs (Qi et al., 2015; Thurber et al., 2013). In another study, two recombinant inbred lines developed through crosses between a *japonica* cultivar Bengal and a US weedy rice accession (BR population), and between the *japonica* cultivar Cypress and the same weedy rice accession (CR population) were used to identify QTLs. Together, these QTLs explained 44.5 and 37.5% of the observed variation of shattering in BR and CR populations, respectively (Subudhi et al., 2014). The *qSH4^{BR}* and *qSH4^{CR}* loci overlap each other and overlap the *SH4* locus (Subudhi et al., 2014). However, the presence of the non-shattering allele of the *SH4* gene in the weedy rice accession implied the possible involvement of a linked locus or an alternative molecular mechanism within or near the *SH4*. In another study, the analysis of the F₂ generation from the cross between a low-shattering rice *indica* cultivar, and a high shattering weedy rice accession collected from South Korea identified QTLs located on chromosomes 1, 3 and 5, respectively (Yao et al., 2015). The *wd-qsh3* locus was responsible for 31.4% of the phenotypic variation in relation to shattering (Yao et al., 2015). *wd-qsh3* overlaps the *qSH3* QTL, where the *OsSh1* gene is located. This discovery opens new possibilities for the evaluation of this locus, which was considered secondary until the beginning of the 2020 s.

4.2 Molecular analyses

While much progress has been made in the last two decades in discovering some of the loci involved in seed shattering in *Oryza*, there are still multiple gaps in knowledge. These include understanding the gene networks regulating formation of the AL, acquisition of cellular competence for the perception of abscission signals, and initiation of the abscission process. Additionally, given the multiple domestication events of cultivated rice (Choi et al., 2017; Civián et al., 2015; Jing et al., 2023) and independent improvement efforts on different rice varieties, identifying which genes have been critical for loss of shattering in each of these evolutionary events is necessary. Because of the multiple losses and gains of seed shattering in the cultivated and weedy rice group, all gene discovery studies need to take into account the genetic background. Despite this, various existing methodologies are available for molecular analyses of seed shattering.

4.2.1 Gene discovery

4.2.1.1 QTL mapping

QTL mapping has been widely used to identify the loci responsible for seed shattering in cultivated and weedy rice (Konishi et al., 2006; Li et al., 2006a; Thurber et al., 2013; Subudhi et al., 2014). The choice of parental lines is a pivotal decision when conducting QTL mapping for shattering. Depending on the objectives, which may involve identifying the loci associated with variation of seed shattering within a given group or identifying the loci contributing to loss or gain of shattering during cultivated or weedy rice evolution, the evolutionary relationship between crossing parents must be considered. For instance, in a QTL study that employed a cross between wild rice and an *indica* cultivar, the *SH4* gene was successfully pinpointed (Li et al., 2006a). This choice of parents, with a well-defined ancestor-descendant evolutionary relatedness, highlighted the likelihood of discovering loci involved in the reduced seed shattering that has occurred during the domestication of *indica* cultivars. Notably, the mutation in the *SH4* gene associated with the reduced shattering phenotype has been under strong selection in *indica* cultivars. A follow-up study found this locus was also involved in the domestication of *japonica* cultivars (Zhang et al., 2009). Another example of a cross involving an ancestor-descendant relationship was the crossing of an *indica* cultivated rice with its weedy-derived counterpart, SH, a major weedy rice group in the US (Qi et al., 2015; Thurber et al., 2013). One shattering QTL on chromosome 2, explaining more than 50% of the observed phenotypic variation, has been identified from this cross (Qi et al., 2015; Thurber et al., 2013) and overlaps with a genomic region that has undergone strong selective pressure during weedy rice evolution (Li et al., 2017), suggesting the importance of this locus in the re-gaining of the shattering trait in this weedy group.

BTS, currently the most precise quantitative method for evaluating seed shattering phenotypes, is the predominant trait employed in shattering QTL mapping studies. However, there is an opportunity to explore alternative quantitative phenotypic traits to assess seed shattering levels and perform QTL mapping. One potential avenue is the characterization of the morphology of AL itself. For example, certain rice accessions exhibit AL while others do not. By quantitatively assessing the AL morphology, such as its length, in mapping populations from the crosses between the rice varieties with and without fully developed AL, we may identify loci responsible for AL development, which could ultimately impact seed shattering levels.

4.2.1.2 DNA sequencing

Genome-wide association studies (GWAS) are another method used in plants to identify loci associated with quantitative traits. GWAS panels typically exhibit greater genetic diversity compared to the genetic diversity between

the two parental lines in a QTL mapping study. This diversity advantage allows for simultaneous detection of numerous natural allelic variations in a single study (Hamblin et al., 2011; Lipka et al., 2015; Myles et al., 2009). However, two significant challenges when applying GWAS in *Oryza* groups are the strong population structure that occurs among cultivated rice varieties and among weedy rice groups, and the extensive linkage disequilibrium (Yano et al., 2016). The crucial factor for the success of GWAS lies in selecting populations that exhibit a high degree of phenotypic diversity but are not genetically highly structured (Yano et al., 2016).

To date, relatively few GWAS have been conducted for seed shattering in rice. One study carried out using 587 rice accessions identified over 50 QTLs related to seed shattering, but it is unclear how the accessions were chosen and whether the impact of population structure on QTL identification was considered (Wu et al., 2022). Additionally, distinct patterns of BTS distribution were observed for shattering phenotypes across two seasons, consistent with the known environmental influence on the shattering trait but leading to variation in the GWAS results across years.

Although substantial phenotypic variation in shattering has been observed within *indica* or *japonica* cultivars, it is important to recognize that seed shattering levels are heavily influenced by the genetic background (Thurber et al., 2010). For instance, as a general trend, *japonica* cultivars tend to exhibit lower levels of shattering compared to *indica* and *aus* varieties (Thurber et al., 2010). This inherent background dependency can result in challenges when identifying loci through GWAS, potentially yielding a limited number of loci or yielding loci specific to certain genetic backgrounds. One potential approach to enhance GWAS effectiveness is to combine cultivated rice and weedy rice with similar genetic backgrounds in the study. This strategy can increase phenotypic diversity while mitigating the influence of genetic structure.

DNA sequencing also offers a valuable tool for investigating the evolutionary processes affecting known shattering genes or QTLs. For instance, researchers have sequenced the *SH4* and *qSH1* gene in different rice populations to better understand their roles in rice domestication (Zhang et al., 2009; Zhu et al., 2012) and in weedy rice evolution (Thurber et al., 2010; Li et al., 2022). Additionally, the increasing availability of genomic resources has streamlined the application of genome selection scans. One example is that the *SH4* gene has consistently been identified in genomic regions showing robust selective signals detected (Wang et al., 2018).

4.2.1.3 RNA sequencing

RNA-seq has been commonly used for characterizing gene expression in a given biological sample. Because the AL is a crucial structure for seed shattering, understanding gene expression within the AL at different developmental

stages is essential for unraveling the potential genetic mechanisms underlying the shattering process. For example, such profiling could help identify the genes responsible for initiating AL differentiation during the early stage and those involved in facilitating the separation of AL from its surrounding cells.

Tissue-specific RNA seq on weedy rice through dissection of the AL and its upper and lower surrounding regions was performed at two developmental stages (Yu et al., 2020). The study employed Weighted Gene Co-expression Network Analysis (WGCNA) to identify co-expression modules enriched in the AL. These modules consisted of genes exclusively or highly expressed in the AL compared to surrounding regions, suggesting their potential roles in AL development. This approach proved effective as it led to the identification of several known shattering genes, such as *SH4*, *qSH1*, *AP2* genes within these AL-specific modules. Moreover, the analysis also unveiled the presence of additional genes, like *MYB26*, whose expression in the AL was confirmed through *in-situ* hybridization. Wu et al. (2022) also performed RNA seq to identify potential genes responsible for seed shattering in rice. This process entailed comparing genes expressed between AL and non-AL tissue from the remainder of the pedicel, followed by a comparison of these AZ-specific genes between two rice accessions exhibiting the extreme shattering phenotypes. Plant hormone signal transduction was enriched in the differentially expressed genes (DEGs) of the high shattering rice accession, while phenylpropanoid biosynthesis showed enrichment in the DEGs of the low shattering accession.

Obtaining a comprehensive profile of genes exclusively expressed in the AL presents one of the most challenging aspects of this research. Previous studies (Wu et al., 2022; Yu et al., 2020) used non-AL tissues as a reference. However, the presence of contamination from other tissues within the AL is inevitable, due to tissue heterogeneity, which represents an inherent limitation of bulked RNA-seq. A more promising and practical alternative could be the use of single-cell RNA seq to gain a better understanding of the genes expressed within this thin layer, particularly considering that some shattering genes exclusively expressed in the AL could serve as AZ-specific marker genes. Furthermore, for a comprehensive insight into the genes involved in seed shattering, it is crucial to investigate multiple developmental stages and a diverse range of rice genotypes.

4.2.2 Gene validation

4.2.2.1 Relative gene expression

Many of the known shattering genes exhibit a specific expression pattern: some are exclusively expressed in the AL, such as *SH4* (Li et al., 2006a), *qSH1* (Konishi et al., 2006) and *AP2* (Simons et al., 2006; Zhou et al., 2012), while others are expressed in the AL and certain other floret tissues, such as *YB2* (Lin et al., 2012; Lv et al., 2018) in the

AL and floral bracts. Thus, examining the expression of candidate genes is essential for validating their involvement in AL development and potential effects on seed shattering.

While RNA-seq can provide a comprehensive gene expression profile, this approach demands higher cost and more lab-intensive efforts for library preparation and sequencing. For analyzing the expression patterns of a specific gene, the most commonly used methods have been RT-qPCR, which quantifies gene expression level, and *in situ* hybridization, which is particularly valuable to determine the spatial localization of an expressed gene.

Examining and comparing gene expression across multiple tissues, including the AL and its surrounding tissues using RT-qPCR could also suggest where the gene is expressed. For example, the *SH4* gene was only expressed in the flower and pedicel junction, but not in any other remaining parts of flower or pedicel, suggesting its potential role in controlling AL development. Additionally, monitoring the expression of the candidate genes throughout various developmental stages can offer clues about their potential roles in impacting the shattering process. For example, the *SHAT1* gene (Zhou et al., 2012), associated with AL differentiation, was expressed in early developmental stages.

4.2.2.2 Gene silencing/overexpression

Proof of gene function ultimately must involve alteration of the gene candidate and demonstration of the consequential phenotypic effects. The most common approaches have been through gene silencing and gene overexpression. Gene silencing have been used for seed shattering genotypic and phenotypic characterization through RNA interference (Cao et al., 2016) and CRISPR-Cas9 (Li et al., 2020; Sheng et al., 2020; Ning et al., 2023), and proposed as strategy for seed shattering mitigation (Yan et al., 2017) in *Oryza*. Furthermore, virus-induced gene silencing (VIGS) presents an untapped potential for enhancing our understanding of gene function in seed shattering studies as its proven effectiveness in various gene function investigations (MacGregor, 2020), and the presence of established VIGS methods for rice (Kant, Dasgupta, 2017). There are also examples of using overexpression as a tool for seed shattering analysis in rice through plant transformation (Yoon et al., 2014; Cao et al., 2016).

While these methods have been used to validate the function of certain candidate shattering genes, some of these approaches are limited by the fact that successful transformation can only be carried out in a subset of rice accessions. In rice, transformations are most easily carried out in *japonica* accessions. As previously mentioned, the mechanisms explaining the loss or gain of seed shattering are different for different genomic backgrounds. Thus, functional validation is often restricted to genes that are of relevance in the *japonica* background, and it is much harder to validate

loci that have been involved in loss or regaining of shattering in *indica* or other lineages. The presence of different AL phenotypes in different rice cultivars, which suggests that the morphological basis for loss of shattering is not common to all cultivated rice, illustrates some of difficulties involved. For example, an *indica* candidate gene that may affect the acquisition of shattering competence of the AL, cannot have its function easily verified in a *japonica* accession that does not ever form an AL. Using an incorrect genotype for functional validation may lead to unexpected phenotypic outcomes. Eventual development of transformation protocols that work for a wide variety of rice genotypes is likely to have a strong positive impact on our ability to determine the genetic factors underlying seed shattering in all rice groups.

5. Conclusions

In this review, we aimed to provide valuable suggestions and precautions for the study of seed shattering in cultivated and weedy rice, considering both phenotypical and genotypical aspects. Given the substantial variation observed in this trait, it is imperative to employ an adequate number of replications and carefully select appropriate developmental stages, environments, and methodologies to ensure consistent and meaningful phenotypical comparisons. When designing experiments to unravel the genetics underlying seed shattering, the genetic background is a critical factor that cannot be overlooked.

Author's contributions

All authors were involved in the conceptualization, writing and editing of this paper. All of them read and agreed to the published version of the manuscript.

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