Genetic variation in the sensitivity of anther dehiscence to drought stress in rice

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Abstract

Field-based screens for genetic variation in reproductive-stage drought tolerance are often confounded by genetic variation in root depth, flowering date and biomass at flowering. To reduce these confounding effects and to impose drought stress more precisely, we grew contrasting genotypes of rice (Oryza sativa L.) in shallow containers of flooded soil. When water was withheld from the shallow-rooted indica genotype IR64 for 6 days starting at −17, −7, 0, 10 and 20 days after heading (DAH), the impact on grain yield was greatest at −7 and 0DAH. The most sensitive yield component was filled grain percentage (spikelet fertility). Data on yield and spikelet fertility were also obtained for another shallow-rooted lowland indica (BRRI Dhan 31) and two deep-rooted upland tropical japonicas (Azucena and Moroberekan). Compared with well-watered controls, withholding of water for 6 days reduced spikelet fertility by ~80% in IR64, BRRI Dhan 31 and Azucena but by 22% in Moroberekan. In a detailed comparison, water was withheld from IR64 for 5 days and from Moroberekan for 6 days, starting 3 days before the heading date of well-watered controls. Under these stress conditions, heading was delayed for 4 days in IR64 and 6 days in Moroberekan. Spikelet fertility in the top four rachis branches was reduced by 80% in IR64 and 16% in Moroberekan, a difference attributable principally to reduced anther dehiscence and lower stigma pollen density in IR64. Two properties of Moroberekan may contribute to high anther dehiscence after stress/re-watering: (i) constitutively superior development of fibrous structures in the endothecium at the anther apex and base and (ii) better maintenance of pollen size. All ovaries of Moroberekan received pollen—an average of 60 grains in well-watered plants and 42 grains in stressed/re-watered plants. In well-watered IR64, all ovaries received an average of 31 grains, whereas in stressed/re-watered IR64, 67% of ovaries received no pollen and the remainder received an average of eight grains. Moroberekan may, therefore, be a source of reproductive-stage drought tolerance through genes that maintain anther dehiscence during recovery from low water status.

Keywords: Oryza sativa L.; Pollen; Starch; Yield components; Spikelet fertility

1. Introduction

Rice is the staple food for about half of the world’s population and supplies 52–76% of the total calories consumed by the population of such countries as Bangladesh, Cambodia, Indonesia, Myanmar and Vietnam and 30% in India and China (Maclean et al., 2002). Although 75% of the global rough rice production of 600 million tonnes occurs in 100 million ha of irrigated land, the remaining 25% is produced on 55 million ha of rainfed land and is essential for the food security of Asia’s rural poor. Drought is a major threat to rainfed rice production, with over 25 million ha affected in Asia alone (Dey and Upadhyaya, 1996). In Eastern India, a farm family may require 5 years to recover financially from a single season of drought (Pandey et al., 2001).

Breeding strategies for drought-affected areas fall into three categories according to rainfall pattern and ecosystem: drought escape, drought avoidance and drought tolerance...
(Fukai and Cooper, 1995). Progress in breeding for drought escape through short crop duration has been rapid because of considerable genetic variation for crop duration among rice germplasm and the ease with which the trait may be selected in the field. Breeding for drought avoidance through deep or penetrating roots has been slower because these traits are difficult to assess in the field and because constitutively deep roots are commonly associated with low tillering, and hence, low yield. It is even more difficult to select for adaptive components of the trait, such as acceleration of root growth in response to drought stress. Nevertheless, the growth of plants in long cylinders for the analysis of root parameters (Champoux et al., 1995; Sharma et al., 2002) and the use of wax–petrolatum layers for greenhouse measurement of penetration ability (Ray et al., 1996) have led to the identification quantitative trait loci (QTL) that should allow DNA-based marker-aided selection for these traits in field-grown plants (e.g., Sharma et al., 2002).

Breeding for drought tolerance is even more complex because of the need to consider traits operating in three distinct domains (Blum, 1999). These domains are: (i) maintenance of high water status; (ii) maintenance of plant function under low water status; (iii) recovery from low water status. Furthermore, the important traits in each domain may have both constitutive and adaptive components. For example, one genotype may maintain high water status (measured as relative water content) longer than another under identical stress conditions by constitutive mechanisms, such as waxy leaves, deep roots, low biomass and small stomatal aperture, or by adaptive mechanisms, such as drought-induced root growth, osmotic adjustment in leaves, rapid stomatal closure, or leaf rolling (Price et al., 2002).

The four yield components of rice are panicle m⁻², spikelets per panicle, spikelet fertility and 1000-grain weight. In breeding for drought tolerance, it is useful to remember that these yield components are most sensitive to water stress at different stages of plant growth. Any given episode of stress can, therefore, have a greater or lesser impact on yield, depending on the timing of the stress relative to the events of the plant life cycle. In a study of rice by Boonjung and Fukai (1996), the most sensitive yield component was spikelet fertility, and it was most sensitive at about flowering. Among the events known to be drought-sensitive at this stage are anther dehiscence (Ekanayake et al., 1989, 1990) and panicle exsertion (O’Toole and Namuco, 1983).

Of special importance are the irreversible effects of stress. When plant production is reduced only for the duration of the stress and recovers completely following re-watering (e.g., loss of photosynthesis as a result of stomatal closure) (Price et al., 2002), the impact of the stress is limited. By contrast, a greater loss would be expected when a drought-sensitive event is irreversibly disrupted. Examples include drought-induced asynchrony of anthesis and silking in maize (Ribaut et al., 1996) and the above-mentioned inhibition of anther dehiscence (Ekanayake et al., 1989, 1990). Inhibition of panicle exsertion (O’Toole and Namuco, 1983) is an interesting example because it is often only partially reversible on re-watering. We have focused our research on understanding the irreversibly drought-sensitive events that have maximum impact on the yield components, especially on spikelet fertility and on finding genotypes in which these events are relatively drought resistant.

Much research on drought tolerance in rice has compared deep-rooted upland tropical japonicas with shallow-rooted lowland indicas. Mapping populations derived from intersubspecific crosses of such parental lines have revealed QTLs for several drought-related traits, such as deep, thick and penetrating roots (Champoux et al., 1995; Ray et al., 1996; Price et al., 2002; Chandra Babu et al., 2003) and osmotic adjustment (Lilley et al., 1996; Zhang et al., 1999). Here, we compare two indicas (IR64 and BRRI Dhan 31) with two tropical japonicas (Azucena and Moroberekan), both of which are known for deep roots (Sharma et al., 2002). However, we grew the plants in shallow containers of flooded soil specifically to eliminate the drought avoidance conferred on Azucena and Moroberekan by their deep roots. Our objective was to determine whether these cultivars possessed novel drought tolerance traits that might be important in the field but might be overlooked in field screens because of the contribution of deep roots to drought avoidance. By sowing the genotypes at different times in separate trays, we were able to overcome phenological differences and to apply stress at the same time relative to flowering. The shallowness of the containers shortened the time required for evapotranspiration to reduce the soil water content and hence the leaf relative water content to any particular target value. Our target for drought stress was a flag leaf relative water content of 35–50% during the reproductive-stage. Like Boonjung and Fukai (1996), we found that the most drought-sensitive yield component was spikelet fertility and the period of greatest sensitivity was near flowering. However, Moroberekan showed a four-fold higher spikelet fertility after stress than did the other cultivars, and anther dehiscence was the event showing a crucial difference in drought tolerance. To our knowledge, this is the first report of a rice cultivar in which anther dehiscence is tolerant of water deficit.

2. Materials and methods

2.1. Plant growth and drought stress

Seeds of rice (Oryza sativa L.) genotypes IR64, Moroberekan and Azucena were obtained from the gene bank of IRRI; seeds of BRRI Dhan 31 were obtained from Bangladesh Rice Research Institute (BRRI). For experiments conducted in the Phytotron of the International Rice Research Institute (IRRI) in Los Baños, Philippines (14°30’N, 121°15’E), the day/night temperatures were 29/21 °C. The relative humidity was controlled around 70%.
Plastic trays (49 cm × 37 cm × 17 cm) contained 30 kg soil (54% clay, 36% silt and 10% sand). Fertilizer was 0.2 g N, 0.1 g P and 0.2 g K/kg soil and was added 2 days prior to sowing. Twelve pre-germinated seeds of one variety were sown in each tray, and nine plants were retained after 2 weeks. Plants were watered twice every day to maintain standing water above the soil, except during the stress period, when standing water was removed and water was withheld for the indicated periods (3–9 days), starting at the indicated number of days after heading (DAH). Fertilizer (one-quarter of basal) was also added at booting stage. At grain maturity, panicles from individual plants were oven-dried at 80 °C for 72 h. The filled grains and the unfilled grains of each panicle were counted and weighed to determine three yield components: the number of spikelets per panicle, the filled grain percentage and the 1000-grain weight. Moroberekan was sown 17 days earlier than IR64 in order to match heading dates. The interauricle distance for the flag leaf and the penultimate leaf was used to predict the number of days before heading for an individual tiller. For microscopy, plants of IR64 and Moroberekan were grown in pots in the Phytotron using 1 kg of the same soil and fertilizer per pot and the same watering regime.

2.2. Relative water content

For comparisons of IR64 and Moroberekan, the RWC of the flag leaf was monitored every morning during drought stress and the early stages of re-watering. The leaf blades were cut at the base, sealed in glass tubes and quickly transferred on ice to the laboratory. Fresh weights were determined immediately. Leaves were then soaked in distilled water in glass tubes for 24 h at room temperature. After soaking, leaves were blotted dry with tissue paper for determination of turgid weight. Dry weights were obtained after oven-drying the leaf samples for 48 h at 80 °C. RWC was calculated from the equation of Schonfeld et al. (1988): RWC = [fresh weight − dry weight]/[turgid weight − dry weight].

2.3. Anther starch content

Anther starch was visualized by staining with 1% IKI solution and quantified by the anthrone method (Sheoran and Saini, 1996). Three replicates of 50 mg fresh weight of anthers were ground with 200 μl of 80% ethanol using a 2 ml glass tube and a glass rod. The tube and the rod were rinsed twice, each time with 150 μl of 80% ethanol. The pooled extract was heated at 80 °C for 15 min and centrifuged at 16,000 × g for 10 min. The pellet was re-extracted twice in the same manner and the supernatants were combined for total sugar assay with the anthrone reagent (0.15% in H2SO4)( Yemm and Willis, 1954). The washed pellet was hydrolyzed with 500 μl of 30% perchloric acid overnight at room temperature, then incubated at 60 °C for 10 min and centrifuged at 16,000 × g for 10 min; starch content (μ-glucose equivalents) was calculated from total sugar content in the supernatant.

2.4. Microscopy

Spikelets were taken from the top four rachis branches of panicles at the indicated times before, during and after anthesis (spikelet opening) for well-watered and drought stressed/re-watered plants. At least 10 spikelets were examined at each time point for IR64 or Moroberekan. Anthesis in IR64 usually occurred at 09:30–10:30 h, while in Moroberekan it occurred at 10:00–12:00 h. Spikelets were fixed in ethanol (50%), formaldehyde (10%) and acetic acid (5%) under vacuum for 30 min and then at atmospheric pressure overnight. Samples were then dehydrated in a graded ethanol series, and infiltrated and embedded using Paraplast Plus (Sigma Chemical Company, St. Louis). Serial sections of 8 μm thickness were laid on Superfrost microscopic slides (Sigma Chemical Company, Jena) or a Zeiss laser scanning confocal microscope LSM 510.

For examination of pollen tube growth, spikelets were excised and immediately fixed in 3:1 absolute ethanol:glacial acetic acid overnight. Samples were then washed with distilled water and dissected to remove the lemma and palea. Dissected ovaries were transferred to 8N NaOH for 3–5 h and subsequently washed with distilled water. Ovaries were then stained with 0.2% aniline blue (Sigma) in 0.1 M KHPO4, mounted and observed under fluorescent mode using 365 nm excitation filter and barrier filter (LP) 397 nm. Images were taken using the MC 200 chip camera.

Pollen size was determined on sets of five spikelets that were collected and immediately mounted in 3:1 absolute ethanol:glacial acetic acid. The six anthers of each spikelet were dissected, crushed with forceps, stained in 1% IKI solution and covered with glass coverslip. Two fields of about 20 stained pollen grains at each sampling time were examined at 40× magnification using Image-Pro Plus (Media Cybernetics Inc., Singapore) and the Image Browser of LSM510.

3. Results

3.1. Spikelet fertility is the most drought-sensitive yield component in IR64

IR64 was grown to maturity in shallow trays of flooded soil in the IRRI Phytotron (Fig. 1). Drought stress was initiated at various times before and after heading. The earliest stress started 17 days before heading (DBH) (Fig. 1A) and lasted for 6 days, by which time the leaves had rolled as a protective measure (Fig. 1B). The flag leaf relative water content declined to 40%, within our target...
range of 35–50%. Within 1 day after re-watering, leaf rolling was fully reversed (Fig. 1C).

The impact of this stress on yield and yield components was determined at seed maturity (Fig. 2). Yield is usually separated into four yield components (panicles m\(^{-2}\), spikelets per panicle, filled grain percentage and 1000-grain weight). However, in the present experiments, the number of panicles m\(^{-2}\) (panicle per plant) was not altered significantly by the treatments, so yield (measured as filled grain weight per tiller) and the other three yield components are reported here. After 6 days of stress shown in Fig. 1B, initiated at 17DBH, the yield reduction was not statistically significant (Fig. 2A). No yield component was reduced significantly at 17DBH (Fig. 2B–D). Therefore, plants recovered almost completely when this standard stress regime was applied at 17DBH.

By contrast, when the same 6-day stress regime was applied at 7DBH, 0DBH, 10DAH and 20DAH, statistically significant reductions in yield were obtained at all four time points (Fig. 2A). The greatest impact was observed when water was withheld starting at heading itself (80% reduction) and most of this reduction was due to an effect on spikelet
fertility (Fig. 2C). The other yield components were not significantly affected at this time point (Fig. 2B and D).

Sheoran and Saini (1996) and Saini (1997) detected a drought-sensitive event in rice that was concurrent with meiosis in anthers. As anther meiosis occurs 9–10 days before anthesis (Raghavan, 1988) and as anthesis occurs 1–6 days after heading depending on spikelet location in the panicle, all anther meiosis should be completed in a panicle by 3 days before heading. We could, therefore, begin stress at 3 days before heading or later (e.g., 0DBH in Fig. 2) and be sure not to encounter the stress-sensitive event described by Sheoran and Saini (1996) and Saini (1997) at any position in the panicle. However, when stress is begun at 7DBH, we would expect to encounter that event in about 50% of the spikelets. The fact that, in our experiment, yield and spikelet fertility are more sensitive when water is withheld at 0DBH than at 7DBH establishes that in IR64 the drought-sensitive event occurs just prior to anthesis rather than 9–10 days prior to anthesis (further support for this conclusion is provided in Fig. 4, where spikelet fertilities in the top and bottom four rachis branches are compared for water withheld at 3DBH).

Six days of stress starting before or after heading produced small but significant reductions in the other two yield components. The number of spikelets per panicle was reduced by 22% at 7DBH and 1000-grain weight was reduced 15% at 10DAH. These effects are consistent with the results of Boonjung and Fukai (1996).

3.2. Moroberekan shows high spikelet fertility under drought stress

Three other rice genotypes were stressed similarly. They were BRRI Dhan 31, which like IR64, is a shallow-rooted lowland indica, and Azucena and Moroberekan, two deep-rooted upland tropical japonicas. Fig. 3 shows the impact of 3–9 days without watering starting at heading on yield and spikelet fertility in these four genotypes. By either measure, Moroberekan was the most drought-tolerant. After 6 days of stress, yield declined relative to the well-watered control by 32% in Moroberekan but by 75–85% in the other genotypes. The corresponding reductions in spikelet fertility were 22 and 75–85%. The results for BRRI Dhan 31 and Azucena were similar to those for IR64 (Fig. 2A and C). We conclude that Moroberekan possesses a previously unsuspected mechanism of drought tolerance that is additional to its ability to avoid drought in the field through production of deep roots. Furthermore, the mechanism addresses the greatest need for IR64: a source of spikelet fertility under drought stress at heading.

3.3. After stress Moroberekan and IR64 differ markedly in spikelet fertility in the top four rachis branches

We compared IR64 and Moroberekan in detail (Fig. 4). The protocol included determination of the relative water content (RWC) of the flag leaf to monitor the degree of drought stress experienced by the plants. Our target was an RWC of 35–50%. We habitually stressed IR64 (Fig. 4A) for 1 day less than Moroberekan (Fig. 4B) to ensure that the RWC of Moroberekan was equal to or lower than that for IR64, and thus, ensure that the greater yield of Moroberekan was not due to a lesser degree of stress. The longer period of stress was needed because of a slightly lower rate of transpiration per plant in Moroberekan at heading (D.Q. Liao, data not shown).

We noticed a visually striking difference between stressed/re-watered plants of IR64 and Moroberekan at maturity. The many filled grains in Moroberekan were distributed mainly in the four uppermost rachis branches (RB1–RB4 in Fig. 4C). The four lower branches (RB5–RB8) of Moroberekan were not so well-endowed with filled grains. By contrast, the few filled grains of IR64 were distributed more in the lower half of the panicle. Fig. 4D provides quantitative support for these observations. In stressed/re-watered Moroberekan plants, the upper branches showed spikelet fertility of 84% of well-watered control values, while for the same branches in IR64, fertility was only 20%. The corresponding values for spikelet fertility in the bottom four rachis branches were 60% in Moroberekan and 45% in IR64.

As the spikelets of the upper rachis branches are, on average, about 2 days more advanced in development than those of the lower rachis branches, it is clear that Moroberekan and IR64 differ principally in drought tolerance in relation to an event occurring late in anther development rather than an early event concurrent with meiosis.
3.4. Pollination of stigmas is more resilient after stress in Moroberekan than in IR64

We examined ovaries for any drought-induced aberrations that could explain the differences in spikelet fertility reported in Fig. 4D. When drought stress reduced the RWC of flag leaves to 45% in both genotypes, we found marked differences in pollination and pollen tube growth. In stressed/re-watered plants of IR64, pollination failed to occur in 67% of spikelets (Fig. 5A) and, when it did occur, there were on average only 8 pollen grains per stigma, compared with 31 grains per stigma in well-watered controls (Fig. 5B); furthermore, the time taken for the leading pollen tube to have reached the micropyle in every ovule was increased from about 1 to 8 h (Fig. 5C). By contrast, in stressed/re-watered plants of Moroberekan, all ovaries were fertilized (Fig. 5A), and even though the number of pollen grains per stigma was reduced to 42 compared with 60 in well-watered plants, this number for Moroberekan was still higher than in well-watered IR64 (Fig. 5B). Stress/re-watering had no effect on the time (∼3 h) required for the leading pollen tube to reach the micropyle in every ovule (Fig. 5C).
3.5. Anther dehiscence in drought-stressed/re-watered IR64 was inhibited by limited opening of the apical and basal pores and by stickiness of pollen

We extended our examination to the anthers. In well-watered and in stressed/re-watered plants of IR64 and Moroberekan, anthers dehisced within 15 min after the opening of the florets. At this time, the anther filaments were erect and were positioned immediately above the stigmatic surface of the ovary. However, the extent of pollen release differed between IR64 and Moroberekan, in part because of a difference in the opening of the apical and basal pores of the two genotypes. The apical pore opened to a greater extent in Moroberekan than in IR64 and the basal pore opened only in Moroberekan. These differences accounted for most of the difference between IR64 and Moroberekan in stigma pollen density.

At 30 min after floret opening, anther filaments of both genotypes had elongated to such an extent that they drooped outside the floret, as described by Matsui and Kagata (2003). By this time, the basal pore of well-watered IR64 had also opened, but in their inverted state, the anthers were incorrectly located to pollinate the stigma and they tended to release their pollen to the air through the apical pore. By contrast, in the anthers of drought-stressed/re-watered plants of IR64, the basal pore usually failed to open by 30 min, and most of the pollen failed to exit through the apical pores because of stress-induced adherence to one another. The molecular basis of pollen stickiness is under investigation.

3.6. Moroberekan and IR64 differ constitutively in the structure of the endothecium

Anther dehiscence is associated with programmed cell death of the endothecium, the septa and the stomium of the anther wall (Sanders et al., 1999). In particular, death of the endothecium allows fibrous structures contained therein to drive the opening of the locule walls. The opening is preceded by breakage of: (i) the septa that link the locule walls to the central vascular core of the anther and (ii) the stomium that links the walls of neighboring locules within each anther (Matsui et al., 1999b; Sanders et al., 1999). We found that the fibrous structures were concentrated at the apex and the base of each anther, where the apical and basal pores open to allow pollen release. There was no significant difference in the distribution of the fibrous structures between well-watered and drought-stressed/re-watered plants 1 day before anthesis, but there was a significant difference in their distribution in Moroberekan compared with IR64. The fraction of the anther length over which the fibrous structures could be seen was greater in Moroberekan, particularly in regard to the length of the apical pore. Fig. 6 shows transverse sections of the anthers of each genotype at two positions below the apical tip. At the higher position (~180 μm from the apical tip), both genotypes contain fibers within the endothecium (Fig. 6C and D). The fibers are thicker in Moroberekan. At the lower position (~300 μm from the apical tip), only Moroberekan retains fibers in the endothecium (Fig. 6A); the endothecal cells of IR64 show signs of degeneration (Fig. 6B). The endothecal fibers are, thus, a constitutive difference between Moroberekan and IR64 that may contribute to the higher stigma pollen density of well-watered and drought-stressed/re-watered Moroberekan by facilitating greater opening of the apical pore (see Fig. 5).
Section 4 for information on non-dehiscent mutants that lack the fibrous structures of the endothecium).

3.7. Pollen swelling

Matsui et al. (1999a,b, 2000) suggested that rapid pollen swelling after anthesis plays an important role in anther dehiscence in rice and barley by forcing open the locule walls to form the basal (or apical) pore. We have not yet observed this specific event, but we did follow pollen size (cross-sectional area) from 24 to 2 h before anthesis as part of a study on pollen starch content. We found significant differences in pollen size between well-watered and stressed/re-watered plants (Fig. 7). In the case of IR64, by 2 h before anthesis, there was a statistically significant reduction (18%) in average pollen size in stressed/re-watered plants compared with controls. By contrast, in Moroberekan, the reduction was smaller and not statistically significant. The reduction in IR64 pollen size is similar in magnitude to the rapid increase in pollen size reported by Matsui et al., and may, therefore, jeopardize the effectiveness of the rapid increase, if it occurs in stressed/re-watered plants. We suggest that poor dehiscence in stressed/re-watered IR64 plants is due to a
combination of the small size of the basal pore and the failure of pollen to reach a critical average size for cooperative opening of the pore. In both IR64 and Moroberekan, the difference in pollen size between heading in well-watered plants and the end of the stress period is statistically significant. This reduction may reflect a lowered water status in the anthers in both genotypes.

3.8. Chemical assays of starch and sugar contents of anthers

Drought stress delays both heading (through inhibition of peduncle elongation) and anthesis (O’Toole and Namuco, 1983). Since the peduncle and the anthers are distinct tissues, questions arise about how complete is the coordination of this delay between and within the two tissues and how reversible the delay is on re-watering. Questions arise also about undesirable events occurring during the delay. We have used proteomics to address the issue of the completeness of the delay and the effectiveness of re-watering (Liu et al., manuscript in preparation). Here, we address one of the possibly undesirable consequences of the delay.

Limited starch breakdown is known to be one of the latest events in pollen maturation (Raghavan, 1988). Possible functions of this breakdown are: (i) to provide osmoticum for protection of the pollen against desiccation in the period between dehiscence and pollination; (ii) to provide osmoticum to promote water uptake by pollen from the stigmatic surface after pollination; (iii) to provide an energy and carbon source for pollen tube growth; (iv) to provide energy and/or osmoticum for the pollen swelling at dehiscence observed by Matsui et al. (1999a,b, 2000). It is unlikely that starch breakdown provides glucose, sucrose or maltose as osmoticum for rapid pollen swelling (minutes) because such rapid swelling typically involves potassium uptake followed by imbibition (Heslop-Harrison and Heslop-Harrison, 1996; Matsui et al., 2000). However, a little starch breakdown through the action of phosphorylase, glycolysis and the tricarboxylic acid cycle could efficiently provide up to 38 ATP molecules per glucose molecule to drive potassium uptake via coupling to proton-ATPase action.

It seemed possible that starch breakdown might help pollen to retain water under low water status, in which case, it might contribute to the drought tolerance of Moroberekan. On the other hand, if starch breakdown continued unchecked during heading delay, it might leave the pollen deficient in starch for pollen tube elongation and contribute to the slow pollen growth in stressed/re-watered IR64. We decided against assaying starch using IKI staining because of the difficulty of knowing whether unstained areas in pollen represented degradation of starch or redistribution of amyloplasts through attachment to reorganizing cytoskeleton. We assayed starch chemically in whole anthers and assumed that most, if not all, of this starch was located in pollen, as suggested by IKI staining (Fig. 8A). We assayed the starch content (Fig. 8B) and sugar content (Fig. 8C) of anthers in the top four rachis branches and the bottom four rachis branches. In well-watered plants of IR64 and Moroberekan, the anther starch content of the top four rachis branches increased markedly from 3DBH to heading. In drought-stressed plants of Moroberekan, the anther starch content reached about the same level as in the well-watered control and declined by a small and statistically insignificant percentage by heading after re-watering. By contrast, in drought-stressed plants of IR64, the starch content failed to reach the control level and declined significantly during the re-watering period, so that at heading the starch level was ~50% of the control value at heading. The sugar content of anthers was higher at heading after stress/re-watering than at heading in well-watered controls, especially in IR64.

The anthers of the bottom four rachis branches generally contained less starch than those in the top four rachis branches, reflecting the fact that they were on average ~2 days behind the upper four rachis branches in development. There was no sign of starch loss from these anthers at heading after delays of 4 and 6 days for IR64 and Moroberekan, respectively. In both the top and bottom rachis branches of both genotypes, there was a conservation of starch plus sugar content on drought stress, a result suggesting that the overall carbon allocation to the anthers is similar in IR64 and Moroberekan.

We conclude that, in stressed/re-watered IR64, starch content was reduced at heading by 50% through a combination of reduced starch accumulation during stress and accelerated starch breakdown during re-watering. This significant reduction in starch content may affect the ability of IR64 pollen to participate in dehiscence (Fig. 5A), and if dehiscence is successful, to grow through the style to the microyle (Fig. 5C). We are currently attempting to identify the enzymes responsible for this starch breakdown.

4. Discussion

4.1. Moroberekan shows drought tolerance for anther dehiscence and pollination

Although the two upland tropical japonica cultivars, Azucena and Moroberekan, are well-known for drought avoidance through their deep roots (Zheng et al., 2000; Sharma et al., 2002), we showed here that only Moroberekan possesses, in addition, a previously unsuspected drought tolerance mechanism that operates during severe water deficit immediately before heading. Moroberekan recovered from severe water deficit at flowering and gave a higher yield at maturity than Azucena and two lowland indica genotypes, IR64 and BRRI Dhan 31. The higher yield of Moroberekan was due mainly to higher spikelet fertility in the upper four rachis branches, which was in turn attributable to greater resilience of anther dehiscence in these branches. Although
the drought-sensitivity of dehiscence in upland and lowland rice cultivars has been reported previously (Ekanayake et al., 1989, 1990, 1993), this is the first report to our knowledge of genetic variation in the trait for rice. We are now exploring the genetic basis of Moroberekan’s relative drought tolerance at dehiscence to determine whether it is sufficiently simple to be exploited in breeding programs. Zheng and Mackill (1982) found that high temperature (35/27 °C compared with 29/21 °C) affected anther dehiscence and pollination in rice. Although high temperature decreased anther dehiscence in all varieties, there was significant genetic variation in this trait, with IR2006 showing 16% filled grains at high temperature compared with 3.2% for IR52. At normal temperature, the filled grain percentages were 98 and 83, respectively.

4.2. Mechanisms of drought tolerance of dehiscence in Moroberekan

The results presented here already provide some clarification of the mechanism of the drought tolerance of dehiscence in Moroberekan. To maximize pollination, dehiscent anthers should be erect and located directly above the stigma. In well-watered and stressed/re-watered plants of IR64 and Moroberekan, dehiscence occurred in exactly this position, within 15 min after floret opening. The pollen were released through the apical and basal pores of the anthers of Moroberekan but through only the apical pores of the anthers of IR64. Failure of dehiscence cannot be ascribed to failure of floret opening and filament elongation, two processes dependent on potassium-driven uptake of water into lodicules and filaments (Heslop-Harrison and Heslop-Harrison, 1996). It seems instead to be due to failures within the anther itself.

Dehiscence involves several events inside the anther. These include: (i) sequential rupture of the septa and the stomia; (ii) expansion of the locule walls through release of fibrous structures in cells of the endothecium; (iii) swelling of pollen. Interestingly, ruptured septa and stomia could be observed in both genotypes 1 day prior to anthesis (Sanders et al., 1999), are not germane to the improved drought tolerance of dehiscence in Moroberekan. A genotypic difference was
observed, however, in the distribution ofendothecial fibers. These fibers are located near the apex and base of each anther, consistent with their proposed role in creating the apical and basal pores in the anther wall (Hoshikawa, 1989; Sanders et al., 1999). The total anther length is not significantly different between the two genotypes, but the length of the apical and basal pores and the length of the anther over which the endothecial fibers may be observed differ significantly between the two genotypes, especially at the apex. These differences between IR64 and Moroberekan are constitutive; there is no significant change in these traits after drought stress/re-watering. The main difference in anther behavior caused by drought stress/re-watering was an increase in pollen stickiness in IR64, as a result of which pollen tended to stay inside the anthers, even when apical pores were open.

4.3. Anther, stigma and style

Matsui and Kagata (2003) found that in many varieties of rice the length of basal pores on the anthers just after anthesis is strongly correlated both with the percentage of florets receiving adequate pollen and with the number of pollen grains deposited on the stigmas. They considered the size of the basal pores to be an important factor for the reliable self-pollination of rice. They established the equation:

\[ Y = 464.1X_1 + 143.1X_2 - 224.1, \]

where \( Y \) is the number of pollen grains deposited on the stigmas, and \( X_1 \) and \( X_2 \) are the lengths of basal pores and stigmas.

We have examined the stigmas of IR64 and Moroberekan and find that their effective length and the spreading of their branches occurred in well-watered plants during the first 30 min after floret opening. The same schedule of events was observed in drought-stressed Moroberekan. In drought-stressed IR64, however, the schedule was delayed until about 3 h after floret opening (Oane, unpublished data). We suspect that this difference contributed to the low pollen density on stigmas of drought-stressed IR64. Using a segregating population derived from a cross between cold-tolerant and cold-susceptible cultivars, Suzuki (1982) showed that the length of the stigma is correlated with the degree of tolerance to low temperature at the booting stage. Matsui and Kagata (2003) suggested that stigma length may be correlated with the degree of tolerance to low temperature through the number of pollen grains deposited on the stigmas. This may also be a factor in drought tolerance and heat tolerance.

Use of the rolB gene to promote auxin biosynthesis in transgenic plants led Cecchetti et al. (2004) to conclude that auxin plays a key role in the timing of anther dehiscence. They suggested that the dehiscence program is controlled by the somatic tissues of the anther, and that auxin regulates the pistil development needed to facilitate pollen tube growth. Since we find a major change in dehiscence and pollen tube elongation rate in IR64, drought stress may down-regulate auxin biosynthesis in IR64 more than in Moroberekan. We conducted proteomic analysis of the pollen and anther wall of well-watered and drought-stressed IR64 and Moroberekan (Liu et al., manuscript in preparation). We found that a group of anther wall proteins was up-regulated in the last 3 days before heading in well-watered plants of both genotypes. Drought stress, however, reduced the induction of these genes in Moroberekan and actually down-regulated them in IR64. The 50% loss of pollen starch during stress and re-watering of IR64 is also likely to reduce the rate of pollen tube growth and make it more dependent on carbon supplied by the transmitting tissue.

4.4. Pollen swelling

In addition to events that weaken parts of the anther wall (specifically, the septa and the stomia) or cause endothecial fibers to change position, dehiscence is also dependent on rapid swelling of the pollen grains (Matsui et al., 1999a,b). For many years, it was believed (Hoshikawa, 1989) that the opening of the floret at anthesis led to desiccation of the anthers and release of pollen onto the stigmatic surfaces of the ovary. This scenario for dehiscence has now been modified to include active swelling of mature pollen grains just prior to anthesis (Matsui et al., 1999a,b). We have not yet observed this rapid swelling (over minutes) but we reported here slower drought-dependent decreases in pollen cross-sectional area that are comparable in size to those observed during swelling and may, therefore, render swelling ineffective as a mechanism of promoting dehiscence or may prevent the swelling from occurring. After drought stress and re-watering, there is a statistically significant decline in the size of IR64 pollen grains and a smaller and statistically insignificant decline in the size of Moroberekan pollen grains.

Data presented here indicate that the 50% loss of pollen starch at heading in stressed/re-watered IR64 was due to a combination of a shortfall in pollen starch accumulation during stress and breakdown of starch to sugars after re-watering. Neither the shortfall in accumulation under stress nor the later breakdown of starch during re-watering was significant in Moroberekan. A trivial explanation for this result is that drought stress in IR64 anthers is more severe than in Moroberekan anthers in spite of equal relative water contents in flag leaves. This explanation seems untenable because the ABA content per unit dry weight of anthers increases under drought stress equally in IR64 and Moroberekan (data to be published with proteomic analysis). In addition, for both IR64 and Moroberekan pollen, there is a significant decline in pollen size by the end of stress compared with well-watered controls at heading. Both the ABA data and the pollen volume data suggest that anthers in both genotypes suffer similar water deficits and mount similar responses in terms of ABA synthesis. It is
possible that on re-watering Moroberekan can rehydrate its pollen more efficiently than IR64. This conclusion is suggested by the data in Fig. 7, but the changes are not statistically significant. We conclude that delay of heading by drought stress exposes pollen starch to degradation in IR64 but not in Moroberekan.

4.5. Mutants affected in dehiscence

Several mutants of Arabidopsis that are affected in anther dehiscence are deficient in jasmonic acid biosynthesis (Ishiguro et al., 2001; Park et al., 2002; Sanders et al., 2000). It is likely that this plant hormone plays a key role in coordinating the events of anthesis and pollination, possibly in conjunction with ethylene (Rieu et al., 2003). There are also some Arabidopsis mutants that are affected in dehiscence but cannot be returned to full fertility by supply of exogenous jasmonic acid. This mutant group includes non-dehiscence 1, which shows normal filament, anther and pollen development but undergoes unscheduled programmed cell death in the endothecium and connective tissue (Sanders et al., 1999). Programmed cell death appears to prevent formation of the fibrous structures in the endothecium. The gene that is disrupted in non-dehiscence 1 has not been identified.

In mutant ms35 (Dawson et al., 1999), filaments also elongate properly, but functional pollen grains are not released from the anthers, although the stomium is cleaved. This defect is associated with a lack of secondary thickening in the endothecium cells. This mutant lacks the fibrous structures of the endothecium and fails to dehisce. Steiner-Lange et al. (2003) identified an insertion mutant of Arabidopsis in which pollen development was normal but dehiscence was blocked, and the 2R3-MYB transcription factor AtMYB26 (Accession numbers: Z95749, AF175997) was inactivated. This transcription factor is expressed only in inflorescences. AtMYB26 is allelic with ms35. The most closely related OsMYb gene in rice (tBLASTn probability: $1 \times 10^{-58}$) is located at map position 123.2 on chromosome 1 (Accession number: AP002901, bases 29194–30732).

A rice mutant anther indehiscence 1 (aid1) was obtained by Zhu et al. (2004) following insertion mutagenesis. The gene (BAC Accession number: AY429017) is a single domain MYB1R transcription factor located at map position 13.8 on chromosome 6 (BAC Accession number: AP003019, bases 132765–135660). The mutant was recessive and showed partial to complete spikelet sterility, reduced tillering and flowering delay of 10–15 days. Spikelets could be classified into three types based on the viability of pollen grains and the extent of anther dehiscence. Type 1 spikelets (approximately, 25%) were sterile due to a failure in accumulation of starch in pollen grains. Type 2 spikelets (approximately, 55%) had viable pollen grains, but anthers failed to dehisce and/or synchronize with anthesis due to failure in septum degradation and stomium breakage, resulting in sterility. Type 3 spikelets (approximately, 20%) had normal fertility. It is intriguing that the Type 1 spikelets also failed to develop the fibrous structures in the endothecium. Thus, this single mutation was able to disrupt the two apparently unrelated processes that we have suggested here are involved in promoting anther dehiscence and pollen tube growth: (i) formation of endothecial cells containing fibers and (ii) pollen starch deposition.

4.6. Competition or cooperation among pollen?

Each rice floret contains one egg cell and approximately 6000 pollen grains (Raghavan, 1988). The question that arises in relation to stress-induced spikelet sterility is why, given so many pollen grains, none manages to fertilize the egg in many spikelets of stressed plants. This may be a legitimate question for out-crossing plants, where male plants compete to pollinate female plants, but for an inbreeding plant, there is little genetic variation among its pollen, and therefore, little scope for competition. On the contrary, at dehiscence and pollination rice pollen appear to cooperate rather than compete. First, the swelling of all pollen grains at dehiscence would be more effective in opening a weakened anther wall than the swelling of only a fraction of the grains. Second, the report that a minimum of 10 pollen grains must germinate on the stigma to bring about fertilization (Satake and Yoshida, 1978; Matsui and Kagata, 2003) suggests that multiple pollen grains cooperate to initiate certain needed changes in the stigma and the transmitting tissue of the style (Lan et al., 2004).

4.7. Lower rachis branches and inhibition of floret exsertion

Our discussion has focused so far on the upper rachis branches, where the difference in drought tolerance between Moroberekan and IR64 is most marked. In the lower rachis branches, the difference in drought tolerance is less marked, probably, for two major reasons. First, the timing of drought stress in our study is quite precise and on average the florets of the upper rachis branches are about 2 days more mature than those in the lower branches. As a result, the most drought-sensitive process in IR64 (dehiscence) escapes stress and the spikelet fertility of IR64 is higher in the lower branches (45%) than in the upper branches (20%). Second, panicle exsertion depends on the elongation of the peduncle, the uppermost internode of the stem. Peduncle elongation ceased in stressed plants and did not resume until after re-watering, so that heading was delayed relative to the controls: by 4 days in IR64 and 6 days in Moroberekan. After heading in stressed/re-watered plants, peduncle elongation continued but did not achieve the lengths attained in well-watered controls. Although most of the panicle was exerted, the lowest florets were sterile because they did not emerge from the flag leaf sheath or emerged too late for anthesis. This phenomenon occurred in both
cultivars but was more marked in Moroberekan. As a result, spikelet fertility was lower in the lower branches of Moroberekan (60%) than in the upper branches (80%).

5. Conclusion

We have shown that: (a) Moroberekan possesses a potentially useful level of reproductive-stage drought tolerance; (b) this tolerance is related to the resiliency of anther dehiscence under drought stress; (c) two mechanisms that may contribute to this trait in Moroberekan are a greater abundance of fibrous structures in the endothecium and a greater pollen size. A key task for the future is to determine whether the drought tolerance of anther dehiscence in Moroberekan can be transferred to drought-susceptible cultivars, such as IR64. An additional task is to combine resilient dehiscence with improved spikelet exertion under stress. Our results are relevant not only to self-fertilizing plants under drought and other abiotic stresses (Mackill et al., 1982; Matsui and Omasa, 2002; Satake and Yoshida, 1978) but also to out-crossing for hybrid rice production (Virmani, 1996).

The fact that Moroberekan showed greater yield stability under drought stress than Azucena suggests that this trait, as it is expressed in experiments with shallow trays, is not a general feature of upland tropical japonicas. It will be important to extend our observations to other upland tropical japonicas, especially those grown by farmers under drought-prone environments, to see whether efficient anther dehiscence and pollination are widespread among cultivars and landraces that are relatively successful in that ecosystem. Conversely, we plan to extend our study of anther dehiscence to other widely grown lowland indica cultivars beside IR64 and BRRI Dhan 31 to determine whether poor anther dehiscence is a common mechanism for yield reduction under water deficit.

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