A Novel Gene, ROA, Is Required for Normal Morphogenesis and Discharge of Ascospores in Gibberella zeae

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Head blight, caused by Gibberella zeae, is a significant disease among cereal crops, including wheat, barley, and rice, due to contamination of grain with mycotoxins. G. zeae is spread by ascospores forcibly discharged from sexual fruiting bodies forming on crop residues. In this study, we characterized a novel gene, ROA, which is required for normal sexual development. Deletion of ROA (Δroa) resulted in an abnormal size and shape of asci and ascospores but did not affect vegetative growth. The Δroa mutation triggered round ascospores and insufficient cell division after spore delimitation. The ascospore of the Δroa strain discharged fewer ascospores from the perithecia but achieved a greater dispersal distance than those of the wild-type strain. Turgor pressure within the asci was calculated through the analysis of osmolytes in the epispasms liquid. Deletion of the ROA gene appeared to increase turgor pressure in the mutant asci. The higher turgor pressure of the Δroa mutant asci and the mutant spore shape contributed to the longer distance dispersal. When the Δroa mutant was outcrossed with a smal1-2 mutant, a strain that contains a green fluorescence protein (GFP) marker in place of the MAT1-2 gene, unusual phenotypic segregation occurred. The ratio of GFP to non-GFP segregation was 1:1; however, all eight spores had the same shape. Taken together, the results of this study suggest that ROA plays multiple roles in maintaining the proper morphology and discharge of ascospores in G. zeae.

Gibberella zeae (anamorph: Fusarium graminearum) causes Fusarium head blight in wheat, barley, and rice, as well as ear rot and stalk rot in maize (20, 23). The infected grains are frequently contaminated by mycotoxins, such as trichotheccenes and zearalenone, which are harmful to humans and animals (6). The fungus overwinters in crop debris in the form of perithecia (38). Trail et al. (41) estimated that the acceleration of ascospore discharge is spread by ascospores forcibly discharged from sexual fruiting bodies forming on crop residues. In this study, we characterized a novel gene, ROA, which is required for normal sexual development. Deletion of ROA (Δroa) resulted in an abnormal size and shape of asci and ascospores but did not affect vegetative growth. The Δroa mutation triggered round ascospores and insufficient cell division after spore delimitation. The asci of the Δroa strain discharged fewer ascospores from the perithecia but achieved a greater dispersal distance than those of the wild-type strain. Turgor pressure within the asci was calculated through the analysis of osmolytes in the epispasms liquid. Deletion of the ROA gene appeared to increase turgor pressure in the mutant asci. The higher turgor pressure of the Δroa mutant asci and the mutant spore shape contributed to the longer distance dispersal. When the Δroa mutant was outcrossed with a smal1-2 mutant, a strain that contains a green fluorescence protein (GFP) marker in place of the MAT1-2 gene, unusual phenotypic segregation occurred. The ratio of GFP to non-GFP segregation was 1:1; however, all eight spores had the same shape. Taken together, the results of this study suggest that ROA plays multiple roles in maintaining the proper morphology and discharge of ascospores in G. zeae.

Although recent research has shed light on the physiological basis of ascospore discharge, the genetic basis remains largely unknown (38). The main force responsible for the observed shooting is turgor pressure within the extended asci. In G. zeae, a buildup of K⁺ and Cl⁻ ions drives the influx of water and causes turgor pressure that stretches the asci (41). Asci can accumulate polyols as well as ions. In a previous study, it was shown that the polyols are comprised mainly of mannitol and glucose; however, the concentration of these polyols is too low to make a significant contribution to turgor pressure (42). When the turgor pressure exceeds the threshold of the asci, apical pores rupture and ascospores are forcibly discharged (38). Trail et al. (41) estimated that the acceleration of asco-
spores in G. zeae is 8,500,000 m s⁻² using an iterative model to predict initial velocity. Recently, Yafetto et al. (44) used high-speed video photography to examine several large-spore fungi, including Ascolobus immites, and to predict acceleration during dispersal. The asci of A. immites are more than 12-fold larger in diameter than the asci of G. zeae (38). The size difference between these fungi greatly affects the behavior of their projectiles and results in an initial speed for G. zeae that is too great for application of the video photography method (for further discussion, see the supplemental material).

To date, only one gene from G. zeae, the calcium ion channel gene cch1, has been shown to be involved in ascospore discharge (12). Deletion of this gene was shown to arrest ascospore discharge without affecting spore and ascus morphology. Since the genomic sequence of G. zeae is now available, the functional analysis of genes involved in sexual development has been accelerated. Random insertional mutagenesis is one strategy that has been used to identify novel genes associated with sexual development (13, 34). Previously, we produced a collection of more than 20,000 mutants from G. zeae by using the restriction enzyme-mediated integration (REMI) transformation procedure (13). In this study, the G. zeae mutant Z43R9901, which was isolated from a screening of REMI transformants, showed an unusual phenotype during sexual development. Further analysis demonstrated that the novel gene ROA is involved in ascospore morphogenesis and discharge in G. zeae. The results of this study increase our understanding of sexual development in the fungus.

**MATERIALS AND METHODS**

**Strains and culture conditions.** The wild-type strain Z03643 of G. zeae (3) and mutants derived from this strain were used for this study (see Table S1 in the supplemental material). The mutant strain Z43R9901 was generated by REMI mutagenesis (13). All strains were stored as mycelia and conidia in a 20% glycerol solution at -80°C. Standard laboratory methods and culture media for the Fusarium species were used (23). For conidial production, the strains were inoculated in carboxymethyl cellulose medium (4).

**Nucleic acid manipulations, primers, and PCR conditions.** Fungal genomic DNA was extracted as previously described (23). Total RNA was isolated from mycelia that were ground in liquid nitrogen using an Easy-Spin total RNA isolation kit (Intron Biotech, Seongnam, Republic of Korea). Standard procedures were used for restriction endonuclease digestion, agarose gel electrophoresis, and Southern and Northern analysis (33). The PCR primers (see Table S2 in the supplemental material) for this study were synthesized by the Bionics oligonucleotide synthesis facility (Seoul, Republic of Korea). General PCR was performed, following the manufacturer’s instructions (Takara Bio Inc., Otsu, Japan). Plasmid DNA was purified from Escherichia coli grown in 3 ml of LB medium at 37°C with shaking at 220 rpm; the column temperature was 80°C for 4 min and then was increased to 220°C at a rate of 10°C/min; the injector temperature was 250°C; the interface temperature, and 20 ml of cold water containing ice was added. The aqueous layer was extracted twice with 5 ml of diethyl ether. The combined extracts were then dried over anhydrous MgSO₄ and evaporated until completely dry. The residue by the acetylation method as previously described (17). Briefly, each extract was dissolved in dry pyridine (1.2 ml) followed by acetic anhydride (3 ml), and the mixture was stirred at 100°C. After 1 h, the mixture was cooled at room temperature, and 20 ml of cold water containing ice was added. The aqueous layer was extracted twice with 5 ml of diethyl ether. The combined extracts were then dried with anhydrous MgSO₄, separated under reduced pressure, and the crude products were dissolved in 500 µl of ethyl acetate, and 1 µl of the solution was subsequently injected into a capillary Shimadzu QP-5050 GC-MS (Shimadzu, Kyoto, Japan). The mobile phase was a 0.48 M NaOH solution at a flow rate of 0.4 ml/min. Confirmation of the sugar alcohols was performed by gas chromatography-mass spectrometry (GC-MS). Sugar alcohols in the epiplastic fluid were derivatized by the acetylation method as previously described (17). Briefly, each extract was dissolved in dry pyridine (1.2 ml) followed by acetic anhydride (3 ml), and the mixture was stirred at 100°C. After 1 h, the mixture was cooled at room temperature, and 20 ml of cold water containing ice was added. The aqueous layer was extracted twice with 5 ml of diethyl ether. The combined extracts were then dried over anhydrous MgSO₄, separated under reduced pressure, and the crude products were dissolved in 500 µl of ethyl acetate, and 1 µl of the solution was subsequently injected into a capillary Shimadzu QP-5050 GC-MS (Shimadzu, Kyoto, Japan). The analytical conditions were as follows: a DB-5 fused silica column was used (30 m by 0.25 mm [inside diameter], 0.25-µm film; J&W Scientific, Folsom, CA); the column temperature was 80°C for 4 min and then was increased to 220°C at a rate of 10°C/min; the injector temperature was 250°C; the interface temperature was 250°C; the ionizing voltage was 70 eV; and the ionizing current was 300 µA. The K⁺ and Na⁺ contents were determined on an inductively coupled plasma-atomic emission spectrometer (ICP-AES) at an emission-line wavelength of 766.490 nm using the Jobin-Yvon Model 170 ICP emission spectrometer Ultracore (Jobin-Yvon Ultima, Longjumeau, France). The Cl⁻ content was quantified by ion chromatography (IC) using a Metrohm model 761 Compact IC (Metrohm, Herisau, Switzerland) with a suppressor module and equipped with an ICSep AN2 analytical column (4.6 by 250 mm) and an ICSep AN2 guard column (4.6 by 50 mm). The ions were detected using a suppressed conductivity detector that had a full scale of 250 µS/cm, which was optimized with respect to the maximum signal-to-noise ratio for the ions being analyzed.

**Mathematical model for predicting initial velocity and turgor pressure.** Previously, we used a computer program which predicted initial velocity based on successive approximations, working back from the mean distance traveled (41). However, to account for the altered launch speed and velocity, a new model was developed. The model used the launch speed of the spore emerging from the ascus as an essential step in predicting the turgor pressure. We used two methods for this prediction here, modifying the iterative program to use Stokes’ law and a “full-model” approach. The program was the same for both except in the way it approximated drag, varying drag coefficients for the “full model” and using the
standard 24/Re drag coefficient reflected in Stokes’ law. The effective size was calculated using predictions of drag at two extremes (spore flying lengthwise and spore flying crosswise with respect to the trajectory). The results were averaged, and then the sphere diameter that gives an equivalent result was used (41). The diameter for the wild type and revertant was 11.03 μm; for the mutant, it was 11.40 μm; density was taken as 1,200 kg m⁻³. The iterative interval was 20 ms, which produced about 1,000 steps, and the launch pitch angle was 2 degrees. Launch speeds were determined from calculated trajectories that produced the observed horizontal ranges, as done previously (41).

The following formulas were used to approximate the drag (D) of spore shapes, cylinders or long ellipsoids, with their long axes normal and parallel to flow, respectively: \[ D = (4μμa)/(ln(l/a) + 0.193) \] and \[ D = (2μμa)/(ln(l/a) - 9.087) \] where l and a are length and radius, respectively, of the cylinder or ellipsoid. An earlier publication with these formulas contained an error in the second equation (41).

Staining of asci and ascospores. Nuclei and chromosomes of G. zeae were stained with acriflavine following the procedure described by Raju (29). Discharged ascospores and perithecia were hydrolyzed in 4 M HCl at 30°C for 15 to 20 min. The samples were washed twice with distilled water and stained in acriflavine solution (100 μg of acriflavine and 5 mg of K₂S₂O₃ in 1 ml of 0.1 M HCl) for 20 min. The stained samples were washed three times (2 min each) in an HCl-70% ethanol mixture (2-98 [vol/vol]) and then twice in distilled water.

Specimen fixation and preparation for light microscopy. The perithecia were collected from the carrot agar surface by gently scraping with a scalpel. The collected perithecia were fixed, dehydrated, and embedded in Spurr’s resin (36) as previously described (19). The resin blocks were sectioned at 1 μm with a glass knife on an MT-X ultramicrotome (RMC, Tucson, AZ) and stained with 1% toluidine blue for light microscopy (40).

GFP tagging. To check the cellular localization of ROA, the ROA open reading frame (ORF), which included its own terminator region, was amplified from the wild-type genomic DNA with ROA-AUG(−) and ROA-3R primers. The 5′ flanking region of ROA, which included its own promoter, was amplified with the ROA-promoter and ROA-GFP 5R primers. The GFP was amplified from pGFP-P (15) with the EGFP-M and EGFP-p1 primers and fused to the N terminus and 5′ flanking region of ROA by DJ-PCR. The fused construct was then amplified with the nested primers ROA-promoter N and ROA-3N. The fusion construct was cloned into the pGEM-T easy vector (Promega Corp., Madison, WI) and subcloned into pUCHI (43). The clone was transformed into Δroa or the wild-type strain, and the phenotypes of the transformants were compared with those of the wild-type strain.

Microscopy. The perithecia were dissected on glass slides in a drop of 20% glycerol, and the ascii were flattened under the cover glass. Both GFP-tagged and acriflavine-stained nuclei were examined with the 488-nm excitation and 515/530-nm emission wavelength filters. Differential interference contrast (DIC) and fluorescence images were captured on a DE/Axio Imager A1 microscope (Carl Zeiss) with a CCD camera. The sizes of the ascii and ascospores were measured using the AxioVision release 4.7 software program (Carl Zeiss).

RESULTS
Phenotype of REMI mutant Z43R9901. Following self-fer-tilization, the wild-type Z03643 strain produced eight normal, spindle-shaped ascospores containing two to four cells each, while the asci produced by the mutant Z43R9901 contained eight ascospores that were round or oval and contained one or two cells each. The numbers of perithecia and ascospores per ascus were 23 and 7.0 μm, respectively, while those of the wild-type ascs were 23 μm and 4.5 μm, respectively. Therefore, ascospores from the Δroa strain were shorter and wider than wild-type ascospores (P < 0.01). The ascus shape is partially determined by the ascospores, and the shorter ascus may be due to the wider, shorter ascospores. We estimated a cylindrical shape for the volume calculation of the wild-type ascospores and an ellipsoid shape for the Δroa ascospores. A cy-
lindrical shape overestimates volume, since the ascospores taper at their end, and the ascospore volume was divided by 1.2 to adjust the overestimation (41). Based on these measurement criteria, the average volumes of the wild-type and Δroa ascospores were by chance the same and were estimated to be $2.94 \times 10^{-16}$ m$^3$.

Approximately 300 perithecia from the wild-type and Δroa strains were fixed and sectioned for microscopic observation. The number and shape of perithecia from Δroa strain were similar to those of the wild-type strain (Fig. 3) despite having a reduced number of discharged ascospores. In contrast to the tightly packed asci in wild-type perithecia, the arrangement of Δroa asci was loose and sparse. Intact ascus rosettes were rarely observed in the old perithecia from Δroa at the 9-DAI time point, while perithecia from the wild-type strain at the same time point exhibited intact asci (Fig. 3). When mature perithecia from the Δroa strain were dissected with needles under a microscope, the ascospores were scattered from the perithecia without forming the typical rosettes of asci. However, wild-type perithecia at the 14-DAI time point still contained intact asci that were ready for the release of ascospores.

**Ascospore discharge.** The wild-type strain discharged ascospores for more than 9 days after 5 DAI (maturation point), while the Δroa strain discharged ascospores for only 3 days (from 6 to 8 DAI). Approximately 67% of the ascospores from the Δroa strain were discharged at 6 DAI. We collected ascospores from the lid of the culture plate until 14 DAI and estimated the total number of discharged ascospores per perithecium. The wild-type strain discharged approximately 3,800 ascospores per perithecium on average, while the Δroa strain discharged 630 ascospores per perithecium ($P < 0.01$).

The shooting distance of more than 1,000 ascospores from each strain was measured in still air. The mean distances of discharged ascospores were 3.6 mm, 5.0 mm, and 4.0 mm in the

![FIG. 1. Targeted deletion and complementation of ROA.](image)

![FIG. 2. Morphology of asci rosettes and ascospores.](image)
wild type, Δroa, and Δroa::ROA strains, respectively (Fig. 4 and 5). The Δroa strain discharged ascospores farther than the wild-type strain and showed a broader range of shooting distance. The discharge pattern of the ascospores from the Δroa::ROA strain was similar to that of the wild type.

The launch speed of the ascospores was estimated from the discharge distance. In addition, the turgor pressure required for discharge was estimated from the launch speed (Table 1). The discharge distance was set to a range where 95% of the ascospores dispersed from the mean distance. Ascospores from the Δroa strain were launched at a higher speed than wild-type ascospores, suggesting that they possessed a higher turgor pressure in the asci. The estimated launch speed and

![Fig. 3](image-url) Light microscopy of developing asci and ascospores. Perithecia of wild-type (A, B, and C) or Δroa (D, E, and F) strains were stained with toluidine blue. Perithecia were collected from carrot agar 5, 7, and 9 days after sexual induction (DAI). Scale bar = 20 μm.

![Fig. 4](image-url) Forcible ascospore discharge of the wild-type (WT), Δroa, and Δroa::ROA strains. Photographs were taken 48 h after the assay was initiated. A semicircular agar block (arrowhead) covered with perithecia was placed on a coverslip in the chamber. This placement allowed for ascospores (arrow) to be discharged horizontally down the length of the chamber onto the coverslip.

![Fig. 5](image-url) Number of ascospores discharged at indicated distances in still air. Wild-type (black bar), Δroa (dashed bar), and complement Δroa::ROA strains (white bar) are shown. One thousand spores from each strain were assessed.
turgor pressure of the Δroa::ROA strain were similar to those of the wild type.

**Epiplasmic fluid analysis.** Based on estimates of epiplasmic fluid volume, the Δroa strain contained a higher concentration of ions (K⁺, Na⁺, and Cl⁻) and sugar alcohols in the fluid per ascus than the wild-type strain (Fig. 6). The asci from the Δroa strain contained approximately four times more ions than the wild-type asci (Fig. 6A). Glycerol, arabitol, mannitol, and glucose were found in the epiplasmic fluids from both the Δroa and wild-type strains (see Fig. S3 in the supplemental material). Among the polyols, glycerol was the major component of the epiplasmic fluid of both strains (Fig. 6B), and both the wild-type and Δroa strains had similar patterns of polyol production (Fig. 7). The concentration of polyols in the mutant ascus was also markedly higher than that in the wild-type ascus. The total concentration of the identified sugar alcohols per ascus was approximately 20 times higher in the Δroa strain than in the wild-type strain.

The volume of epiplasmic fluid was calculated as the volume of extended asci minus the measured volume of spores (41). Since the ascospore volumes were equal in the wild-type, Δroa, and Δroa::ROA strains, the volume of the epiplasmic fluid was set as the same (5.77 × 10⁻¹⁵ m³). The volume of epiplasmic fluid in the unextended mature ascus was calculated previously (2.1 × 10⁻¹⁵ m³) (41). The concentration of osmolytes in the extended asci was calculated from the mass of the osmolytes and the volume of epiplasmic fluid. The turgor pressure in the extended ascus was estimated from the concentration of osmolytes using the concentration of osmolytes in the epiplasmic fluid. The turgor pressure of the Δroa::ROA strain showed a significantly higher predicted turgor pressure than the wild-type strain (P < 0.01). In contrast, the turgor pressures of the Δroa::ROA and wild-type strains were similar.

**Effects of ROA in outcrossing.** The outcrossing between the female Δmat1 strain tagged with hH1-GFP and the male Δroa strain showed that the GFP and non-GFP ascospores within an individual ascus had a 1:1 segregation ratio (Fig. 8). However, the shapes of eight ascospores in each individual ascus were identical. Approximately 10% of ascis contained eight round ascospores (5 to 14 μm in length), similar to case with the Δroa strain, while ~60% had eight spores with wild-type shapes (20 to 30 μm in length). In addition, ~30% of ascospores had a shape that resembled an intermediate form (15 to 19 μm in length). The progeny of the cross between the female Δmat2

### TABLE 1. Estimation of launch speed and turgor pressure in *G. zeae*

| Parameter                      | Value in model for genotype | Stokes’ law       |
|--------------------------------|-----------------------------|-------------------|
|                                | Full                        | Δroa              | Δroa::ROA         |
| Measured distance (mean, range) (mm) | 3.6 (1.4–5.5) 5 (1.1–8.7) 4 (1.7–6.2) | 12.0 (3.9–20.6) 16.9 (2.7–36.0) 12.5 (4.9–24.2) | 8.4 (3.2–12.9) 11.0 (2.4–19.0) 8.8 (3.9–14.5) |
| Estimated mean launch speed (m s⁻¹) | 12.0 (3.9–20.6) | 16.9 (2.7–36.0) | 12.5 (4.9–24.2) | 8.4 (3.2–12.9) 11.0 (2.4–19.0) 8.8 (3.9–14.5) |
| Estimated pressure (MPa)¹       | 0.57                        | 1.13              | 0.62              | 0.27 0.69 0.31 |
| Measured turgor pressure (MPa)² | 0.41                        | 1.6               | 0.40              | 1.13 0.62 0.31 |

¹ Launch speed was estimated from measured distance.
² Estimated pressure was the pressure that is required for discharging ascospores calculated from the launch speed.
³ Measured pressure was the osmotic pressure calculated using the concentration of osmolytes in the epiplasmic fluid.
⁴ WT, wild type.

![FIG. 6. Mass of epiplasmic fluid components per ascus. Wild-type (black bar), Δroa (dashed bar), and complement Δroa::ROA (white bar) strains are shown.](image)

![FIG. 7. High-performance liquid chromatography (HPLC) chromatogram of polyol standards (A), polyols in the epiplasmic fluid of the wild-type strain (B), or polyols in the epiplasmic fluid of the Δroa strain (C). Peaks 1 to 5 are glycerol, erythritol, arabitol, mannitol, and glucose, respectively.](image)
and male Δroa strains and the female Δroa Δmat2 and male wild-type strains showed the same phenotype (data not shown). When we randomly isolated ascospores from the three outcrossing sets, cultured them, and induced sexual development, they produced their respective wild-type or round ascospores regardless of the initial shape. When ascospores that discharged from the outcrossing between the female Δmat2 (genotype mat1-2 ROA) strain and the male Δroa (genotype MAT1-2 roa) strain were randomly isolated, the segregation ratio of the genotypes was 25:7:10:24 (mat1-2 ROA/mat1-2 roa/MAT1-2 ROA/MAT1-2 roa, respectively). The genetic distance of the two linked genes (MAT1-2 and ROA) was estimated to be 19 cM based on chromosome maps of G. zaeae (21), and therefore, the segregation ratio was consistent with the expected number (χ² = 2.8).

**GFP localization.** We tried complementing the Δroa strain by introducing a plasmid that contained the ROA gene fused to GFP. We obtained 60 transformants from three independent transformation trials, and GFP expression was detected in 24 transformants, of which only one strain fully restored the wild-type phenotype. However, this complemented strain carried multiple copies of the construct, and other strains that carried a single copy of the construct did not restore the wild-type phenotype. We amplified the construct from each transformant for sequencing and found each construct carried several point mutations resulting in nonsynonymous substitution. Regardless of the patterns of point mutation and integrated copy number, GFP in the 24 transformants was localized in the cytoplasm in all stages of the life cycle, including ascospores, conidia, and mycelia, but was not detected in young asci before spore delimitation. We could not determine GFP expression in perithecia of the transformants, because perithecia produced by the wild-type strain had strong autofluorescence. We also introduced the construct into the wild-type strains and selected 30 transformants. Southern blot analyses showed that five independent mutants carried a single integration of the construct in their genome and that two of them carried an integration at the 5′ flanking region of ROA (see Fig. S4 in the supplemental material). In both strains, GFP was also localized in the cytoplasm in all fungal stages except for young asci (Fig. 9).

**DISCUSSION**

In this study, we identified a novel gene, ROA, from the screening of a REMI mutant collection. The Δroa mutant showed unique characteristics of ascospore morphology and
discharge without any defects in vegetative growth, conidiation, and conidia germination. Despite their abnormal shape, germination of Δroa ascospores occurred normally. These results suggest that ROA has a specific role in ascospore morphology and discharge in G. zeae. The most striking phenotypes of the Δroa mutant are the reduction in the number of ascospores discharged from the mature perithecium and the increased distance that these spores are fired.

We hypothesized that the mutant had a defect in the shooting of ascospores, since the total number of ascospores per perithecium was the same in the Δroa and wild-type strains. The turgor pressure in asc is known to be the predominant driving force behind the discharge of ascospores from asc (32, 38, 42). In addition, components of the ascus epiplasmic fluid have been identified and quantified in G. zeae (39). Therefore, we analyzed the osmolyte components of the ascus fluid. Assuming the volumes of the epiplasmic fluid are equal in asc of the mutant and the wild type, the measured turgor pressure of the Δroa asc is four times higher than that of the wild-type strain.

The difference between the estimated and measured pressures (Table 1) for both the mutant and the wild type is relatively small, and the full model did a better job of predicting the measured pressure for the mutant. If our assumption that the epiplasmic fluid volume is equal in the mutant and wild-type is incorrect, and there is greater fluid in the mutant, then the measured turgor pressure would be lower and may align more closely with the estimated pressure (Table 1). Wild-type spores appear to nest against each other in the ascus, whereas the shape of the mutants prevents this (Fig. 2A and D), which may increase the fluid in the discharging mutant ascus. For further discussion of the model, see the supplemental material.

The change in spore shape may also contribute to the distance fired. Recent work (32) weighed the evolutionary pressures on spore shape from friction at the ascus pore to reduction of drag and suggests the latter as the driving force for spores without appendages or multiple cells. Our data from a previous publication (41) were used to develop this model, although G. zeae spores have multiple cells. Additionally, one of the assumptions of the model is that spores exit the ascus singly and without accompanying ascus fluid, an assumption, which was based on our previous results (42). The figure in question, however, shows accompanying fluid clinging to 7 of 8 discharged spores. However, for the Δroa mutants, the aspect ratio (length divided by width) plotted against Reynold’s number (Re) fits within 1% of that predicted by the model for minimum drag, whereas the wild-type spores fall outside this region. The study also predicts that the rounder shape of the mutant would minimize friction during exit of the ascus pore, resulting in a longer shooting distance.

The asci of G. zeae were shown previously to accumulate predominantly mannitol and glucose (42). In this study, we identified several other sugar alcohols in the epiplasmic fluid of G. zeae asc, including glycerol and arabitol (Fig. 6). The polyol profile of epiplasmic fluid is consistent with several reports of other fungi (16). Polyol profiles may vary according to conditions under which the fungi are grown. Magnaporthe oryzae was shown to use glycerol to drive turgor pressure in the appressoria (5). However, the concentration of the glycerol (4.6 mM) in G. zeae asc is too low to reach the required turgor pressure (0.57 MPa) for the release of ascospores. In contrast, M. grisea accumulates a large amount of glycerol (3.3 M) in the appressorium that creates a sufficient force (5.8 MPa) for penetration (5). Therefore, G. zeae uses ions (predominantly K+ and Cl−) instead of polyols to increase the turgor pressure in asc, as previously reported (41). An analysis of ion components has not been published for the M. oryzae system.

Although the morphologies of the ascospores from the Δroa and wild-type strains were quite different, acrihin staining showed that the Δroa strain maintained normal meiosis and a third division of the nuclei. Three nuclear divisions (2 meiotic and 1 mitotic) that generated eight nuclei occurred normally in the young Δroa asc before spore delimitation; however, further cell division in these ascospores did not accompany these divisions as expected. Ascospores are formed from the compartmentalization of the ascus cytoplasm during ascospore delimitation (2), and the shape of the young ascus may govern the shape of ascospores produced within it (30). Therefore, insufficient cell division after spore delimitation in the Δroa strain may be a consequence of abnormal ascus physiology.

Unusual phenotype segregation occurred in the outcross between the Δmat1 mutant tagged with hH1-GFP and the Δroa mutant (Fig. 8). All eight spores from one ascus had the same shape; however, the GFP/non-GFP segregation ratio was 1:1. Genotypes of F1 progeny ascospores had an expected segregation ratio and confirmed that the outcross underwent normal sexual recombination. This result showed that the initial shape of F1 ascospores from the outcross was not determined by the spore genotype. This unusual phenomenon may be caused by a diffusible factor, such as a protein or RNA that gets captured by asc at various concentrations, since the asc produced by the outcross have haploinsufficiency in ROA. This phenomenon also can result independently of haploinsufficiency. Genetic studies of N. crassa showed a phenomenon similar to the unusual phenotypic segregation observed in this study, but this phenomenon in N. crassa is not related to haploinsufficiency. Crosses of the N. crassa wild-type strain with the Round spore mutant (R) strain produced 100% round-spore asc, suggesting that R is ascus dominant (24). Shi et al. (35) hypothesized that this phenomenon was a result of meiotic silencing by unpaired DNA (MSUD) and involved a molecular mechanism that was similar to RNA silencing: when DNA from the parental cells is unpaired in early stages of meiosis, both the unpaired DNA and its homologue were silenced. When SAD-1, an important gene involved in MSUD, is self-silenced, the ascus dominance of R becomes suppressed (semidominance). A cross of sad-1 UV− with R produced 35% round-spore asc in Neurospora (35). In this regard, MSUD in G. zeae may be partially functioning, since this semidominance is similar to the outcross results of Δmat1 and Δroa in G. zeae in our study and major genes of MSUD are conserved in G. zeae (25).

In conclusion, the novel gene ROA is required for proper sexual development in G. zeae. Interestingly, the round-spore mutant has a possible selective advantage in that it shoots farther, which would help in distribution, but it does not shoot as many spores. The round-spore mutant has not been previously identified in nature, which may reflect the balance of propague number versus firing distance. Deletion of the ROA gene suggests an increase in turgor pressure in the ascus through the accumulation of ions. In addition, this mutation
triggered the formation of round ascospores and caused insufficient cell divisions after spore delimitation. In this study, we have shown a close relationship between ascospore morphology, turgor pressure, and ascospore discharge and have identified additional polyol components in ascii. Future work will explore the mechanisms by which ROA affects turgor pressure. These studies will also help explain how the physiology and structure of ascii affects turgor pressure and the morphogenesis of ascospores.

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