Establishment of a survey method to determine rice stigma length from preparation, sampling, and preservation to measurement

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ABSTRACT
The lack of a unified standard for sampling stage, preservation and measurement of stigma length is the main restriction factor for the study of stigma length trait in rice. In this study, stigma lengths were compared among four different developmental stages (T1-T4) of 30 rice materials under two environments (years) and in 10 measurement periods (D1-D10) of 3 rice materials after treatment with 6 fixatives (S1–S6), and finally the results revealed that the T3 were the best sampling stage and the S6 was the most suitable fixative for stigma length. This study thoroughly describes the process for surveying and measuring stigma length in 6 steps: preparation before sampling, flower sampling, focusing, flower dissection and imaging, obtaining measurements, and data analysis. These findings provide an outline of the methodology for the discovery, fine mapping, and functional analysis of the genes that control the stigma length trait.

Introduction
Rice is one of the most important food crops in the world and the most important food crop cultivated in China. Roughly 65% of the population in China consumes rice as a staple food. Due to the growing population and continuously decreasing amount of farmland (hundreds of thousands of hectares per year), drastically increasing the rice yield per unit area is necessary to ensure food security for billions of people (Huang & Han, 2016; Yuan, 2010). Hybrid rice plants, which were first cultivated in China during the 1970s, had a yield increase of 20% compared to conventional rice, which greatly improved the productivity of commercial rice farming (Qian et al., 2016). It has been demonstrated that the application of hybrid vigor in rice is one of the most effective means for increasing rice yield.

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Hybrid rice needs to produce a generation of hybrid seeds every year. China produces and consumes the largest number of hybrid rice seeds, and more than 16.7 million hectares of hybrid rice are planted each year, with 180,000 hectares produced annually (Xia et al., 2019). A previous study revealed that the main limiting factor of hybrid rice seed production is the low outcrossing rate of male sterile lines (Shu et al., 2015). The exerted stigma on the female parent spikelet is the determining factor that affects the outcrossing rate in hybrid rice seed production (Tian et al., 2004). Because the exerted stigma spreads out at a wide angle, the surface area for accepting pollen is increased, and the barrier for transmitting foreign pollen is resolved; thus, rice materials with an exerted stigma can be pollinated the next day until a few days after flowering (Wu et al., 2015). In a study conducted by Cai and Li (1987), the spikelets of the male sterile lines V41A and V20A, were not pollinated on the day of flowering and reached natural outcrossing rates of 35% and 37%, respectively, due to the open pollination of exerted stigmas in the field. This indicated that the higher the percentage of exerted stigma in male sterile lines, the more advantageous the outcrossing (Kato & Namai, 1987). The stigma exertion rate is a quantitative trait. Researchers have detected a total of 104 quantitative trait loci (QTL) that control this trait on 12 rice chromosomes in different mapping populations (Bakti & Tanaka, 2019; Hu et al., 2009; Li et al., 2014; Miyata et al., 2007; Rahman et al., 2016, 2017; Uga et al., 2003; Xiong et al., 1999; Yamamoto et al., 2003; Yan et al., 2009; Yin et al., 2014; Yu et al., 2006; Zhang et al., 2018). However, because the stigma exertion rate is easily affected by the external environment (e.g., weather, humidity, and temperature during the heading and flowering stages), human factors (Liu et al., 2019), and procedures of labor-intensive trait surveys that result in large errors (Beachell et al., 1938), it is difficult to conduct subsequent research after preliminary mapping.

Early in 1973, Virmani and Athwal found that the stigma exertion rate in rice was positively correlated with stigma length. Miyata et al. (2007) and Takano-Kai et al. (2011) found similar results. Stigma development and elongation in rice pistils occur in the spikelets and are minimally affected by the environment. Phenotype surveys on such traits are highly accurate, and the biological process is easy to interpret. Therefore, compared to the stigma exertion rate, stigma length is a more accessible and understandable outcrossing trait index for gene discovery. However, current studies on stigma length remain in the preliminary stages (Chen et al., 2012; Dang et al., 2016; Uga et al., 2003, 2010; Yan et al., 2009). Thus far, Liu et al. (2015) has used a CSSL14/Nilssonare F2 population and fine mapped the major QTL, q57L3.1, which controls stigma length in a 19.8 kb region in the middle of the chromosome 3 short-arm. Previous studies did not describe the survey and sampling methods in detail during the QTL mapping of stigma length, and inconsistencies in these survey and sampling methods may be the limiting factors that have resulted in a lack of in-depth studies on the genes that control stigma length. Chen et al. (2012) surveyed stigma length using spikelets when the anther extended out of the glumes but did not disperse pollen. Wu et al. (2017) performed stigma length surveys of spikelets on the day of flowering at noon or in the afternoon. Chen et al. (2012) and Wu et al. (2017) defined the sampling stage, but such sampling times were not easy to replicate (e.g., spikelets when the anther extended out of the glumes but did not disperse pollen), and this has led to large discrepancies in the phenotypic data. Currently, in order to further study any locus/gene by fine mapping, cloning, or functional analysis, larger populations are needed (Dong et al., 2018; Liu et al., 2015; Liu et al., 2019). Because sampling, dissecting, and measuring stigma lengths require labor-intensive, time-consuming procedures, inappropriate preservation between sampling and obtaining measurements could result in stigma length changes and could impede subsequent studies.

Focusing on the aforementioned problems, in this study, a survey was conducted on the stigma lengths of 30 rice plants with a wide range of origins for 2 years at 4 different developmental stages (i.e., the emergence of the leaf sheath (T1), matured but unopened anther (T2) (Chen et al., 2012), opened spikelets (T3), and panicles on the day of flowering with closed spikelets (T4) (Wu et al., 2017). The effects of different developmental stages on the phenotypic value of stigma length were analyzed and the best sampling stage was determined. Additionally, based on the standard fixative solution (i.e., F.A.A.) used in plant anatomy studies, the preservation of stigma length in 5 fixative solutions and their effects were examined. The significant differences were analyzed, and the fixative solution that could delay the changes in stigma length as much as possible was selected. Moreover, based on these experiments, the survey and measurement procedures of rice stigma length were described to provide theoretical support and methodology for discovering, fine mapping, and analyzing the functions of genes that control the stigma length trait in rice.

Materials and methods

Experimental materials

To study the effects of sampling at different developmental stages on the phenotypic value of stigma length in rice, 30
rice plants were selected, i.e., 18 Indica rice varieties with 9 thermo-sensitive genic male sterile lines and 12 Japonica rice varieties with 1 photo-temperature-sensitive genic male sterile line. In the experiments examining the effects of different fixative solutions on the preservation of rice stigma length, 3 core materials from 2018 were used; Japonica rice variety Nipponbare, the Indica rice variety thermo-sensitive genic male sterile line, Y58S, and the Indica rice variety restorer line, Huazhan. Names and all experimental materials are provided (Supplementary Table S1).

**Field experiments**

In 2018 and 2019, 30 plants were planted at the Lujiang Base of the Rice Research Institute of the Anhui Academy of Agricultural Sciences, China. Seeds were sown on 12 May 2018 and 13 May 2019. Seedlings were transplanted on 17 June 2018 and 15 June 2019. Rice plants were planted in 8 rows with 9 plants in each row and a spacing of 16.7 × 26.7 cm between plants. Plants were routinely managed, that is, the planting pattern was the same as that used by local farmers to grow their rice, and compound fertilizer (750 kg/ha; N:P:K = 18:18:18) was applied within 7 days after rice transplanting. Rice was sprayed pesticide with insecticide 3 times from transplanting to the flowering stage to control Chilo suppressalis, yellow stem borer, Cnaphalocrocis medinalis and rice planthopper. When the rice had sealed, the water in the field was drained, and the plants dried in the sun. After about 10 days, the water was reapplied.

Four stages were set up for examining the effects of sampling at different developmental stages on rice stigma length. The first sampling (T1) was conducted when the leaf sheath emerged on the panicle (7:00–8:00). The second sampling (T2) was conducted when the anther matured but the spikelet was unopened (9:00–10:00 on the day of flowering). The third sampling (T3) was conducted when the spikelets were flowering (11:00–13:00). The fourth sampling (T4) was conducted when the spikelets were on the panicle on the day of flowering (16:00–17:00) (Figure 1(a,b)).

Six fixative solutions and their effects on preserving rice stigma length (S1–S6) were compared; water was used as the control (S0). Detailed recipes of the solutions are provided (Table 1). For the same fixative solution, 10 survey time points were established, including the day of sampling (D1), day 2 (D2), day 4 (D4), day 7 (D7), day 14 (D14), day 21 (D21), day 30 (D30), day 60 (D60), day 90 (D90), and day 120 (D120). Sampling was conducted when the spikelets were open.

For the stigma length survey, 10 panicles were sampled from the main stem ear (i.e., the top ear) of each sample. In the developmental sampling stage of stigma length, the authors stored the stigma in water (S0). The stigma length was measured on the sampling day in order to ensure the accuracy of measurement data. From each panicle, 8 intact pistils were separated and photographed under the light field of an MZ11 microscope with an MDX4 camera (Guangzhou Micro-shot Technology Co., Ltd., Guangzhou, China). Stigma length was measured using the Micro-shot v1.3 image processing system (Guangzhou Micro-shot Technology Co., Ltd., Guangzhou, China). The average of the length of 16 stigmas (2 stigmas on each pistil) was used as the phenotypic value of stigma length at a given stage (Figure 1(c)). Stigma length refers to the distance from the junction of the stigma and ovary to the top of the style. It is the total value of the brush-like and non-brush-like stigma lengths (Figure 1(d)).

**Data analysis**

Statistical data analysis was performed using analysis of variance (Statistix 8.0, Analytical software, Tallahassee, FL, USA), and the means of treatments were compared based on Tukey’s honestly significant difference (HSD) test at the 0.05 and 0.01 probability level. In the analysis of the difference in the stigma length of the same materials in the 4 sampling stages of the same year, a, b, c, and d indicated \( P < 0.05 \), and A, B, C, and D indicated \( P < 0.01 \). When the difference in the stigma length of the same materials was analyzed at the same sampling stage in different years, the stigma lengths of the materials sampled at the 4 sampling stages in 2018 were used as controls to compare the stigma lengths of the materials sampled at the corresponding sampling stages in 2019. ‘*’ and ‘**’ in Table 2 represent \( P < 0.05 \) and \( P < 0.01 \), respectively. When the stigma lengths of materials treated with different fixative solutions at 10 surveyed time points were compared, the stigma length of the material measured on D1 under each treatment condition was used as the control. The stigma lengths of each subsequent surveyed time points were compared with the value of the D1 phenotype of the material. ‘*’ and ‘**’ in Table 3 represent \( P < 0.05 \) and \( P < 0.01 \), respectively.

Heritabilities were calculated using the following formula based on the results described in the ANOVA table (Supplementary Table 2).

\[ h^2(\%) = \frac{\text{VAR}(G)/[\text{VAR}(G)+\text{VAR}(E)]*100\%}{[\text{MSg-MSe}]/(\text{MSg-MSe}+\text{MSe})]*100\%}. \]
Results

Effects of sampling at different developmental stages on the phenotypic value of rice stigma length

The phenotypic values of the stigma length measured on the panicles at 4 different developmental stages during the heading and flowering stages of rice, as well as the significant differences of the same stage between 2 years, are provided (Table 2). In 2018, the stigma length of Indica rice variety C8155 was 2.054 ± 0.087 mm at T1, 2.093 ± 0.092 mm at T2, 2.334 ± 0.080 mm at T3, and 2.363 ± 0.084 mm at T4. In combination with the phenotypic values of stigma length in the other 29 plants at different sampling stages, from T1–T3, all 30 plants exhibited a continuous increase in stigma length between the 2 years, except Malaihong and Yazhan in
Table 1. Fixative solutions used for rice stigma length experimentation.

| No. | Fixing solution (100 ml) |
|-----|--------------------------|
| S0  | water (Control)          |
| S1  | 10% formaldehyde         |
| S2  | 38% formaldehyde 5 ml: acetic acid 5 ml: 70% alcohol 90 ml |
| S3  | 38% formaldehyde 5 ml: acetic acid 5 ml: 50% alcohol 90 ml |
| S4  | 38% formaldehyde 5 ml: propionic acid 5 ml: 50% alcohol 90 ml |
| S5  | 10% formaldehyde 5 ml: acetic acid 5 ml: 50% alcohol 90 ml |
| S6  | 38% formaldehyde 5 ml: acetic acid 5 ml: 50% alcohol 85 ml: glycerol 5 ml |

2018. However, T4 differed from T3. The stigma length of 15 and 11 plants in 2018 and 2019, respectively, was longer at T4 than at T3. These results indicate that the stigma length of rice is a dynamic developmental process, and stigma lengths differ at different developmental stages.

For C815S, the phenotypic values of the stigma length for the same plant in the 2 years at T1 were 2.054 mm (2018) and 1.802 mm (2019), respectively, which were significantly different. The stigma length at T2 was 2.093 mm (2018) and 2.086 mm (2019), and 2.334 mm (2018) and 2.319 mm (2019) at T3, but these differences were not significant (\( P = 0.40 \) and \( P = 0.40 \)). The stigma length at T4 was 2.363 mm (2018) and 2.247 mm (2019), which were significantly different (Table 2). And so is C-Bao (Table 2). Among the 30 plants, 17 exhibited significant differences at T1 between the 2 years, 13 at T2, 16 at T4, and 7 at T3 (Table 2). These results indicate that the phenotypic values of the stigma length at the T3 stage were the most stable. Thus, T3 is the best sampling stage for surveying stigma length.

The analysis of variance results in Table 2 showed that different materials and different sampling stages had a significant impact on the stigma length; however, no significant difference in stigma length was found between different years. As shown in Table 2, the heritability was 98.02% at T1, 98.15% at T2, 99.11% at T3, and 98.59% at T4 in 2018; while the heritability in 2019 ranged from 97.07% (T1) to 98.72% (T3), which is consistent with 2018. The heritabilities at 4 sampling stages in 2 years were all greater than 97%, suggesting that the stigma length was controlled by genes, and the environment had a minimal impact on the stigma length. Heritability was highest at T3 in both 2018 and 2019, which further indicated that T3 was the best sampling stage for stigma length.

The values of stigma length at the T3 stage were used to conduct a variability analysis on all plants used in this study. The phenotypic variance of stigma length among 30 plants was between 1.155 mm (2018) and 1.175 mm (2019) for small grain rice (grain length of 5.3 mm, 1000-grain weight of 11.0 g) and between 3.493 mm (2018) and 3.500 mm (2019) for large grain rice (grain length of 5.3 mm, 1000-grain weight of 66.6 g) (Table 2). The average stigma length between the 2 years was 1.949 ± 0.535 mm (2018) and 1.954 ± 0.518 mm (2019). The coefficient of variation reached 27.43% (2018) and 26.52% (2019), indicating that the plants used in this study came from a wide range of origins and exhibited good representation.

Comparison of the effects of 6 fixative solutions on the preservation of stigma length

In 2018, Nipponbare, Y585, and Huazhan were exposed to 6 fixative solutions with different ratios; water was used as the control. A survey of stigma length was conducted at 10 different stages (D1–D120). Samples from 3 varieties in the control treatment (S0) at D4 were degraded and could not be used for stigma dissection or stigma length measurements. At D1, the phenotypic values of stigma length of the same plant treated with the 6 fixative solutions were not significantly different from that of the control (water).

For the same fixative solution, as the number of days of preservation increased, stigma length decreased to various degrees. For example, the stigma length of Nipponbare in the S1 treatment was 1.691 ± 0.060 mm at D1 and 1.642 ± 0.061 mm at D2, which were significantly different. At D4, stigma length was significantly different from D1. In the S2 treatment, the stigma length between D1 and D7 did not exhibit significant differences, but the stigma length at D14 was significantly different from that at D1. In the S3 treatment, the stigma length at D4 and subsequent stages was significantly different from that at D1. In the S4 and S5 treatments, stigma length at D7 and subsequent stages was significantly different from that at D1. In the S6 treatment, there were no significant differences detected between D1–D30 (Table 3). Y585 and Huazhan exhibited similar changes (Table 3). These results indicate that as preservation time increased, the stigma length in all fixative solutions exhibited a decreasing pattern to various degrees and the effect of preservation in different fixative solutions was significant.

In the S0 control, rice stigmas could only be stored for 2 days (Table 3). Nipponbare, Y585, and Huazhan in the S1 treatment exhibited significant differences at D2, D14, and D7, respectively. Specifically, the rice stigma length in the S1 treatment did not significantly change for 2 days. In the S2 treatment, the 3 plants exhibited significant differences at D14, D7, and D21, respectively,
Table 2. Significant difference, heritability and three-way ANOVA of the stigma length of 30 rice plants grown for 2 years and sampled at 4 different sampling stages.

| No. | varieties    | T1   | T2   | T3   | T4   | 2018) | 2019) |
|-----|--------------|------|------|------|------|-------|-------|
| 1   | C8155        | 2.05b| 2.09b| 2.33a| 2.09b| 2.32a  | 2.25** |
| 2   | Chuang 55    | 2.01b| 2.03c| 2.12c| 1.99b| 2.21b  | 2.17** |
| 3   | Jing 41555   | 1.74c| 1.88b| 2.09a| 1.80c| 1.87b  | 1.94b  |
| 4   | Longke 6385  | 1.90c| 2.13c| 2.65a| 1.57c| 2.24bc | 2.63a  |
| 5   | Shen 08      | 1.61c| 1.72b| 1.94a| 1.76d| 1.94b  | 2.04** |
| 6   | Y58S         | 1.82c| 1.96c| 2.14b| 2.08a| 2.16a  | 2.18a  |
| 7   | Z9135        | 1.87c| 2.09b| 2.69a| 1.74b| 2.22bc | 2.64a  |
| 8   | Zhu 15       | 1.84d| 2.17c| 2.28b| 1.92c| 2.03bc | 2.30a  |
| 9   | Zita 5       | 2.16c| 2.20c| 2.94a| 2.78b| 2.50bc | 2.88a  |
| 10  | Chuangu B    | 2.06c| 2.08bc|2.18a| 1.94b| 2.01bc | 2.18a  |
| 11  | Efengsmiao   | 1.66b| 1.77a| 1.82a| 1.63b| 1.81b  | 1.84a  |
| 12  | Huazhan      | 1.61c| 1.90b| 2.05a| 2.01b| 2.00b  | 1.80** |
| 13  | P143         | 1.91c| 2.03b| 2.19a| 1.85ad|2.10bc | 2.23a  |
| 14  | Wushansmiao  | 1.76c| 1.79b| 1.99a| 2.00a| 1.67c  | 1.91** |
| 15  | Yazhan       | 1.46b| 1.46b| 1.65a| 1.50b| 1.61a  | 1.69a  |
| 16  | Dalidao      | 2.65d| 2.77c| 3.49a| 3.33b| 2.58b  | 2.79c  |
| 17  | Kasalath     | 1.54c| 1.64b| 2.21b| 2.35c| 1.47bc | 1.68b  |
| 18  | Malaihong    | 1.48b| 1.48b| 1.60a| 1.41bc|1.52b  | 1.58ab |
| 19  | 70015        | 1.46c| 1.50b| 1.63a| 1.40c| 1.49b  | 1.71** |
| 20  | C Bao        | 1.33c| 1.52b| 1.77a |1.23b| 1.60ab | 1.77a  |
| 21  | Huaida No.10 | 1.39a| 1.41b| 1.43a| 1.36b| 1.46ab | 1.46a  |
| 22  | Huajing No.6 | 1.13c| 1.28b| 1.42a| 1.22a| 1.33b  | 1.44a  |
| 23  | Jia 159      | 1.09c| 1.24b| 1.39a| 1.18bc|1.23b  | 1.44** |
| 24  | Ningjing No.1| 1.06a| 1.13b| 1.27a| 1.21a| 1.18c  | 1.34** |
| 25  | R254         | 1.17c| 1.27b| 1.33ab|1.36a|1.15c  | 1.32** |
| 26  | Zhendao 11   | 1.12c| 1.21b| 1.35a| 1.17c| 1.22b  | 1.39a  |
| 27  | A7444        | 1.69c| 1.80b| 1.86a| 1.94c| 1.70b  | 1.88a  |
| 28  | Baojizijing  | 1.29c| 1.73b| 1.79a| 1.40b| 1.48bc | 1.80a  |
| 29  | Nipponbare   | 1.25c| 1.52b| 1.70a| 1.19c| 1.48b  | 1.72** |
| 30  | Xiaolidao    | 1.12a| 1.15a| 1.16a| 1.13a| 1.14a  | 1.18a  |

| h² (%) | 98.02 | 98.15 | 98.19 | 98.20 | 98.59 | 98.72 | 98.72 |

Three-way ANOVA

| G   | T   | Y   | G×T  | G×Y  | T×Y  | G×T×Y |
|-----|-----|-----|------|------|------|-------|
| **  | **  | ns  | **  | **  | **  | **    |

Data are presented as the mean ± standard deviation. * and ** represent significant differences in stigma length of the same rice plant at a given sampling stage between the 2 years; * P < 0.05, ** P < 0.01. G: Genotype; T: Sampling stage; Y: Year. G × T, interaction between Genotype and Sampling stage; G × Y, interaction between Genotype and Year; T × Y, interaction between Sampling stage and Year; G × T × Y, interaction among Genotype, Sampling stage and Year; ns, no significant differences.
indicating that S2 treatment for 7 days did not affect the measurements. In the S3 treatment, the 3 plants exhibited significant differences at D4, D30, and D7, respectively; the stigma length of plants treated with S3 did not significantly change for 4 days. In the S4 treatment, the 3 plants exhibited significant differences at D7, D30, and D7, respectively; thus, S4 could preserve stigma length without any significant changes after 7 days. In the S5 treatment, the 3 plants exhibited significant differences at D7, D30, and D4, respectively; panicles treated with S5 could preserve rice stigma length without any significant changes for 4 days. In the S6 treatments, Nipponbare, YS85, and Huazhan at D60, D120, and D60, respectively, exhibited significant differences from D1, indicating that there were no significant changes in stigma length on the panicles in the S6 treatment within 30 days of sampling (Table 3).

### Discussion

This study surveyed the phenotypic value of stigma length at 4 different developmental stages of the panicle in 30 rice plants harvested in 2 years. Results revealed that stigma length at different developmental stages exhibited significant differences, and the opened spikelet (T3) stage was the best sampling stage for measuring rice stigma length. The methods of this study differ from those of Chen et al. (2012), who conducted their study when the rice anther was mature, but pollen had not yet dispersed (T2 in this study). The methods of this study also differed from those of Wu et al. (2017), who used the panicle on the day of flowering with a closed spikelet (T4 in this study) to survey stigma length. In Chen et al. (2012) and Wu et al. (2017), the technology needed for sampling at the T2 and T4 stages was insufficient. Specifically, sampling from large populations, which requires many workers, was difficult to maintain, and errors are thus inevitable in the data analysis.

In this study, after comparing the phenotypic data of stigma length between the T4 and T3 stages based on 60 data sets, 26 sets showed that stigma length at T4 was larger than at T3, while the other 34 sets exhibited the opposite results. This may be due to the fact that when spikelets are closed, the fertilization process has already been completed. At this time, the ovary is enlarged and the stigmas have degenerated (Figure 1(c-t4)), which resulted in errors in the data. The heritability of T3 was higher than that of T4 in both years. These results indicate that T3 is more suitable than T4 as the optimal sampling period. Additionally, this study used the stigma length of 8 intact spikelets (i.e., 16 stigmas) as the phenotypic value of one rice plant. The sample size in this study was 60% larger than the 5 spikelets used in Chen et al. (2012) and Wu et al. (2017); thus, the obtained data in this study were more reliable. Overall, based on a comparative study using 30 plants from a wide range of origins, it is proposed that the open spikelet (T3) is the best sampling stage for measuring rice stigma length. This developmental stage is easy to identity and therefore research assistants without high expertise can conduct measurement based on the developed protocol. Moreover, 8 spikelets can fit into the same visual field; thus, the entire procedure, including sampling and flower dissection, is easy and efficient.

This study was based on the standard fixative solution, F.A.A. (formalin 38% formaldehyde) (5 ml), acetic

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**Table 3. The effects of 7 treatments and 10 survey stages on stigma length in 3 rice plants.**

| Varieties    | Treatment | D1 | D2 | D4 | D7 | D14 | D21 | D30 | D60 | D90 | D120 |
|--------------|-----------|----|----|----|----|-----|-----|-----|-----|-----|------|
| Nipponbare   | S0        | 1.71| 1.68|   |    |     |     |     |     |     |      |
|              | S1        | 1.69| 1.64**| 1.62**| 1.58**| 1.55**| 1.57**| 1.58**| 1.52**| 1.53**| 1.53** |
|              | S2        | 1.71| 1.69| 1.68| 1.67| 1.65**| 1.65**| 1.65**| 1.63**| 1.61**| 1.41** |
|              | S3        | 1.71| 1.67| 1.65*| 1.60**| 1.62**| 1.62**| 1.62**| 1.57**| 1.60**| 1.53** |
|              | S4        | 1.69| 1.70| 1.71| 1.64*| 1.62| 1.60**| 1.59**| 1.58**| 1.55**| 1.58** |
|              | S5        | 1.71| 1.70| 1.72| 1.63**| 1.64**| 1.55**| 1.58**| 1.49**| 1.50**| 1.47** |
|              | S6        | 1.71| 1.69| 1.70| 1.68| 1.70| 1.69| 1.69| 1.56**| 1.54**| 1.55** |
| YS85         | S0        | 2.14| 2.10|   |    |     |     |     |     |     |      |
|              | S1        | 2.14| 2.14| 2.13| 2.12| 2.07**| 2.08*| 2.08| 2.06**| 2.00**| 1.99** |
|              | S2        | 2.15| 2.14| 2.12| 2.02**| 2.01**| 1.98**| 2.01**| 1.97**| 1.94**| 1.90** |
|              | S3        | 2.15| 2.14| 2.13| 2.13| 2.12| 2.10| 2.04**| 2.07**| 1.98**| 1.89** |
|              | S4        | 2.14| 2.13| 2.15| 2.14| 2.11| 2.10| 2.04**| 2.06**| 2.05**| 1.99** |
|              | S5        | 2.14| 2.14| 2.14| 2.14| 2.13| 2.12| 2.07**| 2.05**| 1.94**| 1.98** |
|              | S6        | 2.14| 2.14| 2.14| 2.14| 2.14| 2.14| 2.14| 2.13| 2.11| 1.97** |
| Huazhan      | S0        | 2.03| 2.06|   |    |     |     |     |     |     |      |
|              | S1        | 2.03| 2.03| 2.00| 1.95**| 1.99| 1.94**| 1.95| 1.92**| 1.91**| 1.89** |
|              | S2        | 2.00| 2.00| 1.98| 2.00| 1.96| 1.85**| 1.89**| 1.84**| 1.86**| 1.77** |
|              | S3        | 2.04| 2.01| 1.98| 1.97**| 1.95**| 1.94**| 1.95**| 1.94**| 1.93**| 1.85** |
|              | S4        | 2.02| 2.00| 1.97| 1.95*| 1.97| 1.91**| 1.89**| 1.90**| 1.87**| 1.80** |
|              | S5        | 2.04| 2.03| 1.97**| 1.98**| 1.95**| 1.93**| 1.89**| 1.82**| 1.80**| 1.74** |
|              | S6        | 2.01| 2.02| 2.00| 2.01| 1.98| 2.01| 1.98| 1.84**| 1.81**| 1.82** |

* and ** represent significant differences in stigma length at a given survey stage and D1 under the same treatment condition; * P < 0.05, ** P < 0.01.
acid (5 mL), 70% ethanol (90 mL) (S2)), and 5 different fixative solutions with different ratios or concentrations were established; water was used as the control. By comparing the stigma length of 3 representative plants over 1–120 days at 10 observational stages, the control (S0) and 5 fixative solutions (S1–S5) preserved rice stigma length without significant changes for only 2–7 days. Additionally, S6 (38% formaldehyde (5 mL); acetic acid (5 mL); 50% ethanol (85 mL); glycerol (5 mL)) maintained rice stigma length without significant changes after 30 days. Thus, S6 is a suitable fixative solution for the survey of rice stigma length using large populations.

Based on the aforementioned sampling stages and preservation methods used for measuring stigma length, the survey and measurement procedures for rice stigma length are described as follows:

Step 1. Prepare the fixative solution. To prepare 1 L fixative solution, measure 38% formaldehyde (50 mL), acetic acid (50 mL), glycerol (50 mL), 100% ethanol 425 (mL), and distilled H2O (425 mL), and mix thoroughly in a volumetric flask. Dispense 1.5 mL fixative solution into pre-numbered 2-mL centrifuge tubes with a pipette (Figure 2(a)).

Step 2. Flower sampling. When the plants in the rice experimental field are at the full-bloom stage, use ophthalmic forceps (with teeth) to clip the base of the flowering spikelet on the main stem ear (i.e., the top ear) without touching the glume to prevent damaging the stigma, remove it, and place it into the centrifuge tubes pre-filled with fixative solution. Collect 10 open spikelets from each rice variety and transfer the samples to the laboratory for measurements (Figure 2(b)).

Step 3. Focusing. Turn on the MZ11 microscope equipped with a MDX4 camera that is connected to a computer with Micro-shot v1.3.10.8 software. Place a ruler with a scale on the stage of the microscope and adjust the focal length until the scale of the ruler is clearly visible under the microscope. Capture an image.
and save. The focus does not need to be changed again after this step (Figure 2(c)).

Step 4. Flower dissection and imaging. Take 10 spikelets preserved in the centrifuge tubes and place them on a 2.5 × 7.5 cm slide. Pick one spikelet, press down on the edge of the inner glume with Vetus forceps (18 cm), remove the outer glume (14 cm), and expose the stigmas. Use the forceps to break apart the stigma and glume at the style, then straighten the 2 stigmas so that there is an angle between the stigma and style (~150°). Repeat this step for the other spikelets. Place 8 intact spikelets with 16 stigmas in 2 rows on a slide (4 spikelets in each row) and place the slide on the stage of the microscope for imaging. Capture an image and save (Figure 2(d)).

Step 5. Measurements. Open the Micro-shot digital imaging system, click on ‘file’ – open image for measurement – open “ruler” – insert “unit ruler” – set up parameters – capture image and save. Then, click on ‘file’ – open image for measurement – open images for stigma length measurement – insert line – measure each stigma length – export as EXCEL file – save’ to complete the measurements (Figure 2(e,f)).

Step 6. Data analysis. Export the average of the 16 measurements to an Excel spreadsheet to use as the phenotypic value of stigma length for each measured rice plant (Figure 2(f)).

Fine mapping and the subsequent study of rice quantitative traits often require large genetic populations (Dong et al., 2018; Liu et al., 2015). However, the procedures for sampling, dissecting, and measuring stigma length were cumbersome (Figure 2), which resulted in difficulties in completing the sample measurements in a time-efficient manner. The obtained data may be more reliable when using the sampling stage, preservation method, and routine specimen dissection and measurement outlined in this study, the obtained data may be more reliable. This study provides a method for the discovery, fine mapping, and functional analysis of the genes that control rice stigma length and also provides important theoretical support for uncovering the genetic basis of stigma-related traits and for breeding male sterile lines with a high outcrossing rate.

Conclusions

Spikelet flowering (T3) is the best sampling stage for measuring stigma length, and 56 (38% formaldehyde (5 mL); acetic acid (5 mL); 50% ethanol (85 mL); glycerol (5 mL)) is the most suitable fixative solution to maintain rice stigma length without significant changes up to 30 days. This study thoroughly describes the process for surveying and measuring stigma length in six steps: preparation before sampling, flower sampling, focusing, flower dissection and imaging, obtaining measurements, and data analysis. These findings provide outline the methodology for the discovery, fine mapping, and functional analysis of the genes that control the stigma length trait.

Disclosure statement

No potential conflict of interest was reported by the authors.

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