

Identification of Metabolomic Biomarkers of Seed Vigor and Aging in Hybrid Rice

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Research Article

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Abstract

Background

Seed deterioration during rice seed storage will lead to seed vigor loss, which adversely affects agricultural production, the long-term preservation of germplasm resources, and the conservation of species diversity. However, the mechanisms underlying seed vigor maintenance remain largely unknown.

Results

In this study, 16 hybrid rice combinations were selected from four sterile lines and four restorer lines. Following artificial aging and natural aging treatments, the metabolite markers that could accurately reflect the aging degree of the hybrid rice seeds were identified based on the germination percentage and metabolomics analysis by gas chromatography-mass spectrometry. Significant differences in the degree of seed deterioration were observed among the 16 hybrid rice combinations tested, with each restorer and sterile lines after storage having the different germination percentage. The hybrid rice combination with the storage-resistant restorer line Guanghui122 exhibited the highest germination percentage under both natural and artificial storage. A total of 89 metabolic peaks and 56 metabolites were identified, most of which were related to primary metabolism. Interestingly, the content of galactose, gluconic acid, fructose and glycerol in the seeds increased significantly during the aging process. Absolute quantification indicated that galactose and gluconic acid were very significantly negatively correlated with the germination percentage of the seeds under the different aging treatments. The galactose content was significantly positively correlated with gluconic acid content. Additionally, while the relative content of raffinose did not change much during storage, a significant positive correlation between raffinose and the germination rate of the artificially aged seeds before storage was detected.

Conclusion

Based on the metabolomics, metabolite markers which could accurately reflect the aging degree of hybrid rice seeds were identified. Galactose and gluconic acid were very significantly negatively correlated with the germination percentage of the seeds which suggested that these metabolites could constitute potential metabolic markers of seed aging. These findings are of great significance for the rapid and accurate evaluation of seed aging, the determination of seed quality, and the development of molecular breeding approaches for high-vigor rice seeds.

Background

Rice (*Oryza sativa* L.) is one of the most important food crops for human beings, serving as a major staple for over half of the world's population. Rice production is crucial in ensuring China's food security. Seed vigor refers to the sum of the properties that determine seed performance, such as the performance under field and storage conditions (Hampton and TeKRONY 1995). Generally, rice seed vigor tends to decline under the high temperature and high humidity conditions in the south of China (Fu 1985). The

germination percentage of hybrid rice seeds in the rice cultivation region of southern China can be reduced to less than 70% after one year of storage, thus having significant loss on production. Seed vigor is also related to the long-term storage of germplasm resources and the preservation of species diversity. Therefore, improving seed storability by maintaining seed vigor during storage is important in rice production (Aibara et al. 1986).

Multiple “omics” studies have demonstrated that many physiological, cellular, biochemical, and metabolic alterations occur during seed storage, and a number of genes related to the storability of rice seeds have been identified, such as aldehyde dehydrogenase 7 (*OsALDH7*) (Shin et al. 2009), three rice lipoxygenase (*LOX*) isozymes (Zhang et al. 2007), and rice protein repair enzyme L-isoaspartyl methyltransferase PIMT1 (Aibara et al. 1986). However, the mechanisms regulating the maintenance of vigor during seed storage are still largely unclear. Differences in genes or proteins during seed storage lead to alterations in metabolites, ultimately resulting in the production and metabolism of small molecules (Suzuki and Matsukura 1997). Thus, metabolomics, as a comprehensive, nonbiased, high-throughput analysis of complex metabolite mixtures in target organisms, has been applied in several seed studies (Hall et al. 2002).

Metabolites are essential for plant development and human nutrition, providing energy for the seed, acting as defense signaling molecules, and serving as nutrients for humans and animals (Hu et al. 2015). It is generally believed that seed vigor relies on the accumulation of storage substances. As the seed matures, proteins, starches, and other substances gradually accumulate, and the seed germination ability and vigor gradually increase, reaching a peak at the physiological maturity stage (Bewley et al. 2012). At the later stage of seed development, stress resistance-related substances such as storage proteins, Late Embryo Abundance (LEA) proteins, oligosaccharides, abscisic acid (ABA), and tocopherols gradually accumulate, which contribute to seed vigor and enhance the adaptability of seeds to environmental stress (Sun et al. 2007). During storage, the seeds undergo aging and deterioration, showing a decline in the total amount of storage substances and degradation of storage proteins (Fu 1985; Wang et al. 2015).

Rice varieties vary in seed storability, which depends to some extent on the chemical composition of the mature seeds. Inositol galactosides and raffinose family oligosaccharides accumulate at the ripening stage in the seeds of some crops such as legumes, indicating their role in the desiccation tolerance and longevity of seeds (Sengupta et al. 2015; Salvi et al. 2016). The content of inositol galactosides is considered to be a marker of seed vigor in *Arabidopsis*, cabbage, and tomato (de Souza Vidigal et al. 2016). Through peroxidation, some polyunsaturated fatty acids generate end-products such as malondialdehyde, hydroxyl radicals, keto fatty acids, and other reactive oxygen molecules, which accumulate in the cells. Once they reach a very high level, they cause damage to the cells by reacting with macromolecular substances (Pisoschi and Pop 2015). The peroxidation of polyunsaturated fatty acids is a major factor affecting seed storability (Xu et al. 2015). Alterations in the content of soluble sugars in seeds affect seed vigor (Bernal-Lugo and Leopold 1995). Raffinose, trehalose, and other oligosaccharides have been found to be related to seed desiccation tolerance (Bernal-Lugo and Leopold 1992; Nishizawa et al. 2008).

We previously compared the proteomic and metabolomic differences between control and hybrid combinations with poor storability and found that the seeds with poor storability exhibited differential increases in proteins and metabolite changes during natural aging. We also suggested that raffinose might be related to hybrid rice seed vigor (Gao et al. 2016; Yan et al. 2018). Therefore, in the current study, we used a greater number of varieties and additional aging treatments to determine the most reliable metabolic markers for assessing the storability and aging of hybrid rice. In this study, four sterile lines and four restorer lines were used to form 16 hybrid combinations. Through germination tests and metabolomics analysis using gas chromatography-tandem mass spectrometry (GC-MS/MS) of the seeds after artificial and natural aging treatments, we found that the seeds of restorer line Guanghui122 (tolerant to storage)-derived hybrid combinations were highly resistant to deterioration, the relative content of raffinose had a significant positive correlation with seed storability. Importantly, we also discovered that the contents of galactose, glycerol, and gluconic acid had a significant negative correlation with the seed germination percentage and thus could be used as metabolic markers for seed aging. The results of the study guide the molecular breeding of storage-tolerant rice and can inform the quality control of rice seeds during production.

Results

The seed germination of the different varieties (combinations) following aging treatment differed significantly

Using 16 hybrid rice variety combinations as experimental materials, the harvested seeds were subjected to natural aging and artificial aging treatments, and the seed germination before and after aging was calculated respectively (Fig. 1). There was no significant difference in the seed germination of the different varieties prior to the treatment. After two years of aging, the germination percentage of all varieties, regardless of the aging treatment, demonstrated a significant downward trend. There was also a significant difference in the degree of the decline in seed germination among the different varieties, indicating that the seed storage tolerance or seed vigor among the tested 16 hybrid rice varieties (combinations) differed (Fig. 1).

After two years of natural aging, the seed germination of BIIIY-122, TY-122, IY-122, QY-122, and TY-3618 was greater than 80%, and these five varieties could thus be regarded as being resistant to storage under natural conditions. However, the germination percentage of IY 998 and IY 3618 were only 45% and 40%; that of QY 3618 was 50%; that of BIIIY3618 was 52%; and that of IY 368 was 53%. These five combinations can thus be regarded as less resistant to natural aging (Fig. 1).

The germination dynamics of the artificially aged seeds were similar to the naturally aged seeds. The germination percentage of the above five varieties, i.e. BIIIY-122, TY-122, IY-122, QY-122 and TY-3618, which are relatively resistant to natural storage, was higher than 80% after artificial aging, which is

consistent with the natural storage aging results, and thus these can be considered as high-vigor seeds. For IY-998, BIIY-3618, IY-3618 and IY-368 which are not resistant to natural storage, the seed germination under artificial aging was also lower than 80%, and thus these can be considered as relatively low-vigor seeds. The other combinations were considered to be moderately vigorous.

The maintenance of vigor during seed aging varies according to the parental sterile line and restorer line

Before storage or aging, no difference in the average germination percentage of seeds among the sterile lines was detected. Conversely, the germination percentage differed significantly following natural aging or artificial aging, indicating that the sterile line could affect the seed vigor retention ability or life span (Fig. 2A). The hybrid rice combinations (varieties) of the II-32A sterile line had poor storage resistance except IY-122, being lower than the average value of the other sterile lines after natural aging (Fig. 1).

The overall trend of the restorer lines was similar to that of the sterile lines. There was no difference in the average germination percentage of seeds of the four restorer lines before aging, but there was a significant difference after aging (Fig. 2B). Restorer line Guanghui 122 showed the highest germination percentage under both artificial and natural aging treatments, being significantly higher than that of the other three restorer lines, thus demonstrating good storability, its germination after natural aging and artificial aging was 91% and 88%, respectively (Fig. 2B). As Guanghui122 is a storage-resistant restorer line, the germination percentage of the hybridized combinations, e. t. BXY-122, TY-122, IY-122 and QY-122 were 87%, 87%, 88%, and 87%, respectively (Fig. 1). However, the seeds of Guanghui 998 and Guanghui 368 showed only moderate storability, and their derived hybrid combinations presented weak storability (Fig. 2B, Fig. 1).

Comparative analysis of the metabolomes of hybrid rice seeds before and after natural aging

We assessed if the chemical composition of the seeds was associated with seed vigor and storability using a GC-MS-based metabolomics approach before (0 months) and after the 24-month storage period. The intensities of the compound peaks in the total ion chromatogram (TIC) differed remarkably among the 32 samples (16 before storage and 16 after storage), especially for peaks between the retention times of 17 and 26 min (Fig. S1, Table S2). A total of 89 metabolite peaks were detected, and 61 peaks (representing 56 metabolites) were identified based on authentic standards, except for two compounds that were identified by matching the MS spectra to those in the NIST11 library. Among the 56 identified metabolites, 24 were identified as sugar-related compounds, 20 were amino acid-related compounds, two were free fatty acids, six were tricarboxylic acid (TCA) cycle-related intermediates, and four were other compounds. The retention times and fragmentation patterns of these compounds are provided in Fig. S2.

Most metabolites detected using GC-MS are generally primary metabolites, which may reflect the physiological states of the seeds.

The relative intensities of the 89 metabolite peaks were normalized based on the intensities of the internal standards and subjected to multivariate statistical analyses. The supervised pattern-recognition tool PLS-DA was used to generate an overview of the metabolic patterns of the four samples. The results indicated that the non-aged and aged seed samples were distributed in separate groups, thus indicating distinct metabolic differences (Fig. 3 A–D).

Pairwise comparisons of the seed metabolome using orthogonal (O) PLS-DA were performed to identify metabolites that differed significantly in each comparison group. Following model diagnosis, the S-plot visualization allowed us to identify metabolites that differed significantly between the samples, as shown in Fig. 3 E–F.

Changes in metabolites related to seed aging during storage

The differential metabolites were analyzed using univariate Student's *t*-tests. Although we did not find any differently abundant amino acids and lipids related to seed aging or seed storage tolerance, we did detect differential soluble sugars and their derivatives.

As shown in Fig. 4, the relative contents of galactose, fructose, gluconic acid, and glycerol increased significantly following 24 months of natural aging (Fig. 4 A–D). Conversely, the levels of glucopyranoside and oxaloacetic acid decreased significantly after 24 months of natural aging in all samples (Fig. 4 E, F).

Among these six sugar-related metabolites, both the levels of galactose and gluconic acid were significantly greater in the seeds after 24 months of natural storage, ranging between 4 and 50 times more for the former, and between 3 and 100 times more for the latter across the different varieties (Fig. 4C, D). Glycerol and fructose were two times higher in the seeds after 24 months of natural storage (Fig. 4A, B), while glucopyranoside and oxaloacetic acid decreased after the 24-month natural storage period (Fig. 4 E, F). These results suggested that these six metabolites might constitute candidate markers for seed aging.

Among the other identified sugar-related metabolites, the levels of raffinose, galactinol, sucrose and myo-inositol remained relatively constant in all the 16 tested seeds during the 24-month natural storage period (Table S3), which is similar to the findings in our previous study (Yan et al. 2018). Furthermore, we found that there was no significant correlation between the relative content of galactinol, sucrose, trehalose, and myo-inositol and seed germination (Table S4). However, before storage, the correlation coefficient between seed raffinose content and seed germination under artificial aging ($r=0.5111$, $P< 0.05$) was significant (Table S4). This confirmed our previous research that detected a positive correlation between raffinose content and seed vigor (Yan et al. 2018). In addition, the relative content of fructose was

significantly negatively correlated with seed germination (correlation coefficient $r = -0.6030$). These two metabolites could thus potentially constitute candidate markers for seed vigor.

Galactose, gluconic acid, and glycerol are markers of seed aging

To verify the potential role of these metabolites as markers of seed aging, we first selected two combinations, namely G8Y2156 and G8Y169, and subjected them to artificial aging treatment for 6 d and 15 d, respectively, to obtain seeds with different aging degrees. The candidate metabolites of the above-mentioned six kinds of seed aging degree marker, including galactose and raffinose, were determined respectively in the seed embryo and endosperm, and galactinol was used as the control (Fig. 5).

The results showed that as seed germination decreased with the increase in aging treatment duration (Fig. 5 A, B), the content of fructose, glycerol, galactose, and gluconic acid in the endosperm also increased (Fig. 5 C, E). The change trend of these substances in the endosperm was consistent with the above results in the seeds under natural aging. However, the metabolite changes in the embryo were not completely consistent with the above trend, and only the fructose content gradually increased (Fig. 5 D, F). These results also suggested that the contents of these sugars should be determined only in the endosperm, which can provide a semi non-destructive means of testing, with potential broad application prospect. The other two candidates, oxaloacetic acid and glucopyranoside, exhibited little relationship with seed aging. However, for galactinol that was selected as a control (previous study showed that it related to seed longevity), there was no significant correlation between galactinol content and seed aging degree, and the opposite trend was observed.

Regression equations predict the seed germination percentage during storage

We then used an absolute quantitative method to measured the galactose, gluconic acid, fructose, glycerol, oxaloacetic acid, glucopyranoside, and raffinose contents in the four other rice varieties (combinations, G8Y165, NXRZ, NYZ, G8B) stored at room temperature for different durations, comparing them against a galactinol control. The seed germination of the different varieties under natural storage for different durations ranged between 5% and 95% (Table S5), indicating that the seeds differed in their aging degree and vigor. Among these metabolites, the correlation coefficient between galactose and gluconic acid and seed germination was greater than -0.95 , indicating a significant negative correlation, and the correlation coefficient between the two metabolites was as high as 0.99 (Table S5). In addition, glycerol was negatively correlated with seed germination. The correlation coefficient between the sum of the absolute content of galactose, gluconic acid, and glycerol and seed germination reached -0.96 , indicating an extremely significant negative correlation (Table S5).

The changes in oxaloacetic acid, glucopyranoside, and fructose content were not significantly correlated with seed aging (Table S5), which contrasts with the previous relative quantitation results. Interestingly, although only the raffinose level was positively correlated with seed germination, this correlation was not

significant. Moreover, the content of galactinol (although it has been reported that it is related to the storage capacity of seeds), which we used as the control, exhibited no significant correlation with the degree of natural aging in the rice seeds.

We calculated the regression equation of galactose level, gluconic acid level and seed germination in order to predict the germination rate of the older seeds during storage. The regression equation between the absolute content of galactose and the seed germination was: $y = 90.636 - 0.1912x$, and the determination coefficient was 0.9229 (Fig. 6A). The regression equation between the absolute content of gluconic acid and the seed germination was: $y = 103.49 - 7.5943x$, and the determination coefficient was 0.9131 (Fig. 6B). On this basis, we also determined a three-variable linear regression equation of seed germination (y) and gluconic acid level (x_1), galactose (x_2), glycerol (x_3), where $y = 99.0279 - 0.1539x_1 - 0.3852x_2 - 3.3263x_3$, with a determination coefficient of 0.94. The three-variable regression equation was considered to be more accurate than the single-variable equations.

Discussion

Seed storability in different hybrid combinations is related to the male parent restorer line

The production of high-quality seeds is the primary aim of the seed industry; however, seed deterioration during storage is a considerable problem that increases crop production costs. Eliminating or minimizing losses from seed deterioration under storage is thus a priority of the seed industry. Seed losses are typically believed to be related to the harvesting and drying processes, together with the storage conditions (Nishizawa et al. 2008). Various engineering and technological approaches, such as low temperature and low humidity technology, have been explored for maintaining seed integrity under long-term storage. However, low-temperature storage is costly and is not completely effective, and the safe storage of germplasm resources and food poses another challenge (Towill 2002). A study from the U.S. National Plant Germplasm System assessed the seed vigor of 42,000 samples stored in gene banks for 16–81 years and found that the average seed germination percentage decreased from the initial 91% to 58% (Wang et al. 2011; Chen et al. 2013). Improving seed storability and prolonging seed vigor have thus become increasingly relevant. Rice varieties vary in storability, and seed storability is affected by genetic factors in addition to environmental factors. Employing a genetics approach provides a cost-effective means of addressing the decline in seed germination ability during storage.

A large number of studies have reported that the storage tolerance of inbred rice seeds is better than that of hybrid rice seeds (Ellis 1993; Chen 1994; Gao et al. 2016). It is believed that the physiological advantage of easy germination in hybrid rice seeds is a primary factor in poor storage tolerance (Chen 1994). Some experiments have also demonstrated that the storage tolerance of different hybrid combinations is closely related to their parents; for example, the restorer line Guanghui122 was found to be associated with high storability (Liu et al. 2005).

Our study confirmed a close association between the restorer lines and the storage tolerance of the combinations, which could provide a reference for the breeding of rice storage tolerance. For example, combinations from the high-storability restorer line Guanghui122 demonstrated high storage resistance, with an average seed germination after two years of natural aging of 87%, which was significantly higher than the average value of the other restorers (Fig. 1). Guanghui998 and Guanghui368 have moderate storability, and their derived hybrid combinations showed weak storability (Fig. 1).

Notably, Guanghui 3618 has strong storage resistance, and its germination was very high following both natural and artificial storage. However, the storage capacities of the four cross combinations of Guanghui3618 differed significantly. After storage, the germination percentage of TY 998 was 81%, indicating that its storage capacity was high, while that of ILY3618 was the lowest at 40%, and those of BY3618 and QY3618 were 52 and 50%, respectively, which is also poor. It is unclear if this is directly due to the effect of the restorer line on storability, or whether the interaction between the restorer and sterile lines may affect the function of the storage tolerance genes. These questions are worthy of further study.

The screening of these long-life hybrid rice combinations has provided an excellent foundation for further exploring the associated storability genes and clarifying the mechanism of seed storage resistance, which is of great theoretical and practical significance to seed biology.

The content of galactose and gluconic acid could accurately reflect the aging degree of each variety

During the rice seed sale and transportation process, it is common for seeds to encounter high temperature and humid conditions, causing the seeds to undergo inevitable and irrevocable changes in vigor, mean seed differentiation, or seed aging (Hampton and Tekrony 1995; McDonald 1999). The germination speed of aged seeds is slow, and the resulting seedlings will typically be non-uniform or will fail to emerge in stressful environment (Demir et al. 2008; Khajeh-Hosseini et al. 2009; Mavi et al. 2010). The rapid identification of seeds suitable for long-term storage or that need to go to the market immediately would help improve the decision-making process of the industry and avoid losses due to storage.

Several biochemical tests have been used as indicators of seed quality, including the redox indicator tetrazolium (Hampton and Tekrony 1995), enzyme activity assays (Ramiro 1995), and determination of volatile compounds (Zhang and Roos 1997). Metabolites have great potential use as biomarkers, as it is relatively easy to develop diagnostic tests for their detection. A recent study showed that the galactinol content of mature dry seeds can be used as a biomarker for seed longevity in Brassicaceae and tomato (de Souza Vidigal et al. 2016). However, our study found no relationship between galactinol level and rice seed vigor and/or longevity. We discovered that the relative raffinose level in the seeds before aging was significantly positively correlated with seed germination after artificial aging, while the relative content of fructose was significantly negatively correlated with seed germination after artificial aging. As seed

germination after artificial aging is the index used to measure seed vigor, the relative content of raffinose and fructose in the new seeds could be used to evaluate the vigor among different varieties. This result confirms previous conclusions (Yan et al. 2018), but differs from others (Bentsink et al. 2000).

In the present study, the relative contents of galactose, fructose, gluconic acid, and glycerol in all the samples increased significantly following natural aging for 24 months (Fig. 4 A–D). Among these, galactose and gluconic acid have the most significant changes between aged and unaged seeds. Furthermore, the absolute content of galactose and gluconic acid was negatively correlated with seed germination rate, and thus these could be used as candidate markers for seed aging.

We used regression equations to predict the seed germination corresponding to different metabolite contents (Fig. 6). In comparison with the conventional germination test, this method greatly saved time, used fewer seeds, and improved the testing efficiency. Future studies should improve the regression curves based on the seed characteristics of different combinations of varieties to increase the reliability of rapid testing.

Notably, the correlation between galactose and gluconic acid was highly significant (0.99), with both metabolites exhibiting nearly the same trend. Galactose is an important intermediate substance for ascorbic acid synthesis, while gluconic acid has anti-aging effects (Katagata 2011; Wang et al. 2013), which might explain their association with seed aging. A more interesting speculation is that galactose and gluconic acid form lactobionic acid (4-O- β -galactopyranosyl-D-gluconic acid), and lactobionic acid can prevent oxidative damage to the cell membrane. Lactobionic acid has roles in anti-aging, anti-oxidation, and promoting metabolism (Bai et al. 2012), which may provide insight into the subsequent clarification of related mechanisms.

Conclusion

There were significant differences in the deterioration for seeds of different hybrid rice combinations during natural and artificial aging. The combination crossbred by Guanghui 122, a storage-resistant restorer, showed the highest germination rate under both natural and artificial storage. A total of 89 metabolites and 56 metabolites were identified in both storage-tolerant and storage-intolerant hybrid rice combinations, most of which were related to primary metabolism. During the aging process, the content of galactose, fructose, glycerol, gluconic acid and other substances increased significantly, whether it was storability or sensitive hybrid combination. Absolute quantification indicated that galactose and gluconic acid were very significantly negatively correlated with the germination percentage of the seeds under the different aging treatments, which suggested that these metabolites could constitute potential metabolic markers of seed aging. These findings are of great significance for the rapid and accurate evaluation of seed aging, the determination of seed quality, and the development of molecular breeding approaches for high-vigor rice seeds.

Methods

Plant materials

The restorer lines Guanghui 122, Guanghui 998, Guanghui 3618, and Guanghui 368, the sterile line Qiu A and Tianfeng A were obtained from Guangdong Golden Rice Seed Industry Co. Ltd (Guangdong, China), and the sterile lines Il32A and Bol11A were obtained from the Guangxi Academy of Agricultural Sciences (Guangxi, China). The four restorer lines were combined respectively with the four sterile lines to generate 16 hybrid rice combinations (Table S1).

Artificial aging treatment

The artificial aging approach was based on the methods of Liu et al. and Chen et al. with slight modifications (Jun et al. 2000; Chen et al. 2012). The harvested rice seeds were divided into control group and treatment group. The seeds in the treatment group were artificially aged as follows: rice seeds were pre-treated at 15°C under 85% relative humidity (RH) for 3 d, and then transferred to an incubator at 43°C under 85% RH for 8 d, following which they were dried at 25°C under 32% RH for 3 d. The seeds of the control group did not undergo any aging treatment.

Natural aging treatment

The natural aging treatment of the seeds followed the method of Gao et al. (2016). Briefly, the seeds were harvested, dried, and stored for 12 months or 24 months under natural conditions (below 28°C and 65% humidity using an air conditioner). The germination percentage were then determined.

Seed germination test

Fifteen grams of each treatment of the dried seeds was weighed and fully absorbed water for 12 h that called pre-soaking. One-hundred seeds from each treatment were assessed for germination percentage. The seeds were placed in a germination dish with two layers of moist paper at the bottom. During germination, the dish was kept in a 15000 lux light incubator (12 h light/12 h dark). The germination potential after 4 d and the germination rate after 7 d were calculated. The experiment included three biological repeats.

Metabolite profiling by GC-MS/MS

Metabolites were extracted from 50 mg of the hybrid rice seeds using the protocol described by Yan et al. (2018). The 300- μ L extract solutions were dried in a vacuum concentrator for GC-MS/MS analysis. The dried extracts were derivatized with N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) as described previously Yan et al. (2018) and transferred to glass vials for GC-MS/MS (7890A-5975C, Agilent Technologies, Santa Clara, CA, USA) analysis. One μ L from each sample was injected into the GC-MS

(7890A-5975C, Agilent) at 270°C in split mode (50:1) with the helium carrier gas (>99.999% purity) flow set to 1 mL/min. A DB-35 MS (30 m × 0.25 mm, 0.25 μm) capillary column was used for separation. The temperature was isothermal for 5 min at 85°C, followed by an 8°C per min ramp up to 205°C, where it was held for 5 min, and then finally ramped up at 8°C per min to 300°C and then held for 5 min. The transfer line temperature was set to 280°C, and the ion source temperature was set to 230°C. The mass range analyzed was from m/z 60 to 1000.

Qualitative analysis of sugar metabolites in rice seeds

Sugar metabolites were extracted from 100 mg of hybrid rice seeds using the protocol described by Yan et al. (2018), and a 200-μL extract solution was dried in a vacuum concentrator for GC-MS analysis. The dried extract was derivatized with MSTFA as described previously and then transferred into glass vials. One μL from each sample was injected into the GC-MS with the same parameters as described above. The temperature was isothermal for 4 min at 90°C, followed by an 8°C per min ramp up to 205°C, where it was held for 2 min, and then finally ramped up at 15°C per min to 310°C and maintained for 5 min. The transfer line temperature was set to 300°C, and the ion source temperature was set to 230°C. The mass range analyzed was from m/z 85 to 700. The MS analysis was conducted in selected ion monitoring (SIM) mode, and the quantitative ions are listed in [Table S2](#).

Data analysis

Chroma TOF 4.3X software (LECO Corporation) was used for GC-MS data analyses including peaks extracting, data baseline filtering, calibration of the baseline, peak alignment, deconvolution analysis, and comparison of the peak area. LECO-Fiehn Rtx5 database and the NIST library were used for peak identification (Kind et al. 2009). First, missing values of raw data were filled by half of the minimum value, and then detected peaks were retained using the interquartile range denoising method. In addition, an overall normalization method was employed in this data analysis. The resulting three-dimensional data involving the peak number, sample name, and normalized peak area were fed to the SIMCA software package (V14, Umetrics AB, Umea, Sweden) for multivariate statistical analyses, including principal component analysis (PCA), orthogonal partial least squares-discriminant analysis (OPLS-DA), and permutation analysis of different models. To refine this statistical analysis for significantly changed metabolites, the first principal component of variable importance projection (VIP) was obtained.

Declarations

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Author Contributions

J. Liu and H. Fu designed the research., J.-D. Gao, S.-J. Yan, H. Fu, B.-X. Chen, Y.-X. Zhang and Q. Zhang performed the experiments. J. Liu, H. Fu, S.-J. Yan and B. X. Chen analyzed the data, J. Liu and B.-X. Chen wrote the manuscript.

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Availability of data and materials

All data generated or analyzed in this study are included in this published article and its additional information files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

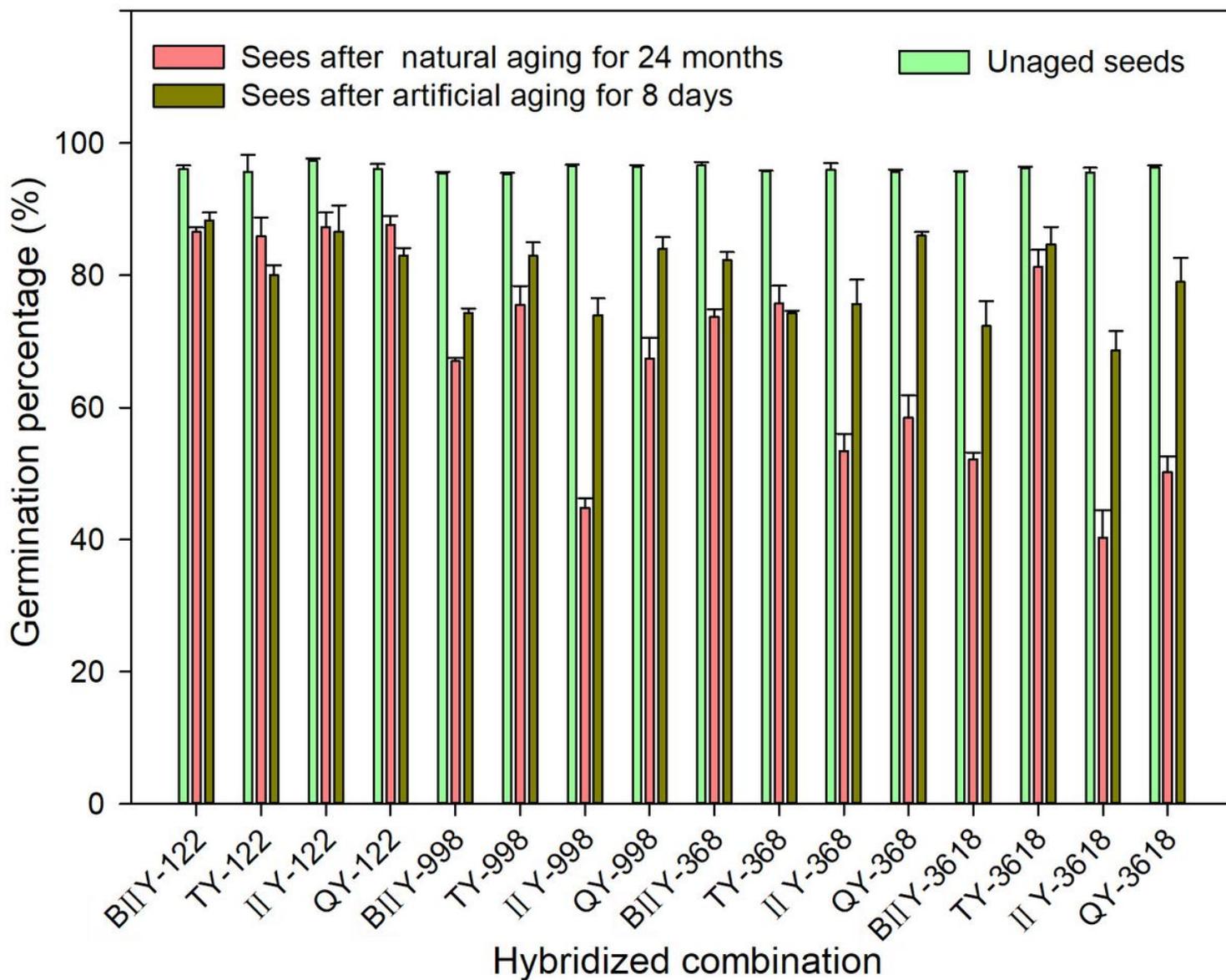


Figure 1

Changes in seed germination of 16 hybrid rice combinations composed of 4 sterile lines (BIIIY, TY, IIY, QY) and 4 restorer lines (122,998,368,3618) after 24 months of natural aging or 8 days of artificial aging.

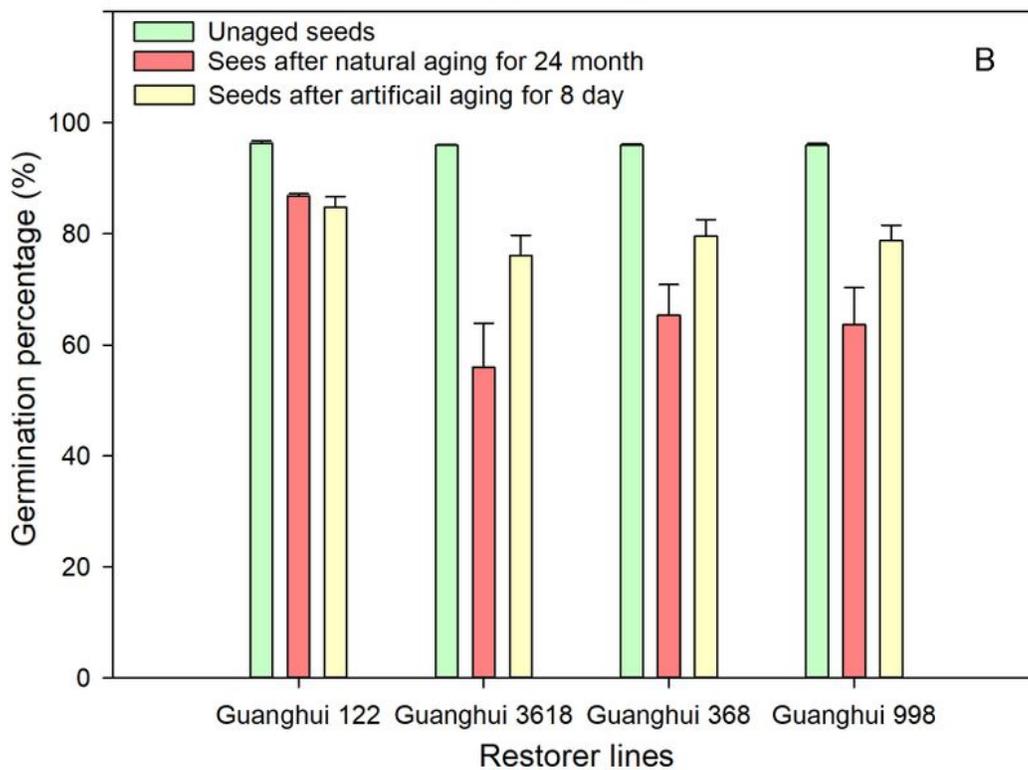
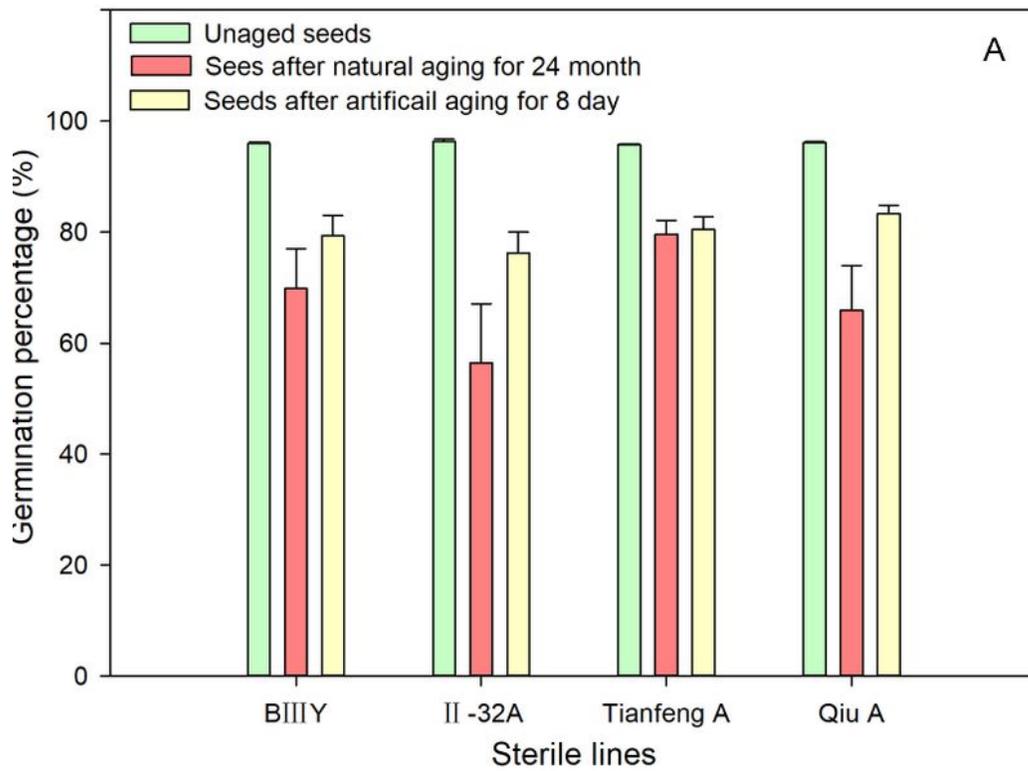


Figure 2

Changes in rice seed germination of 4 sterile lines (BIIIY, II-32A, Tianfeng A, Qiu A) (A) and 4 restorer lines (Guanghui 122, Guanghui 3618, Guanghui 368, Guanghui 998) (B) after 24 months of natural aging or 8 days of artificial aging.

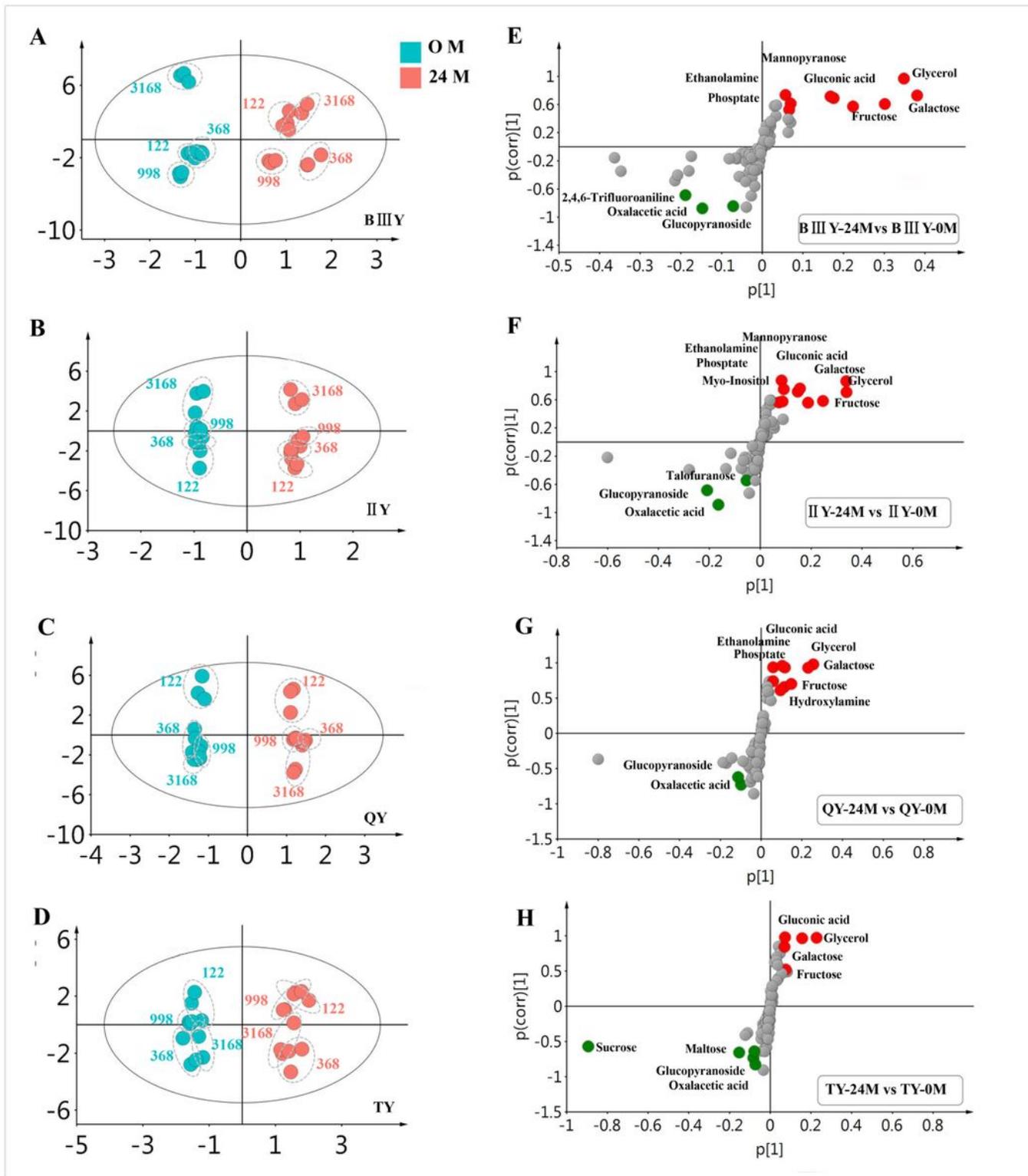


Figure 3

The score plots (A-D) and S-plot of the different metabolites (E-H) generated from OPLS-DA of pairwise GC-MS data comparison showed the distinct metabolome change among the BIIIY-122, BIIIY-998, BIIIY-368, BIIIY-3618 (A, E), theIIY-122, IIY-998, IIY-368, IIY-3618 (B, F), theQY-122, QY-998, QY-368, QY-3618 (C, G), theTY-122, TY-998, TY-368, TY-3618 (D, H). The significantly different metabolites with $VIP \geq 1$ and $p(\text{corr}) \geq 0.5$ were labeled in the S-plot (B, D, F, H).

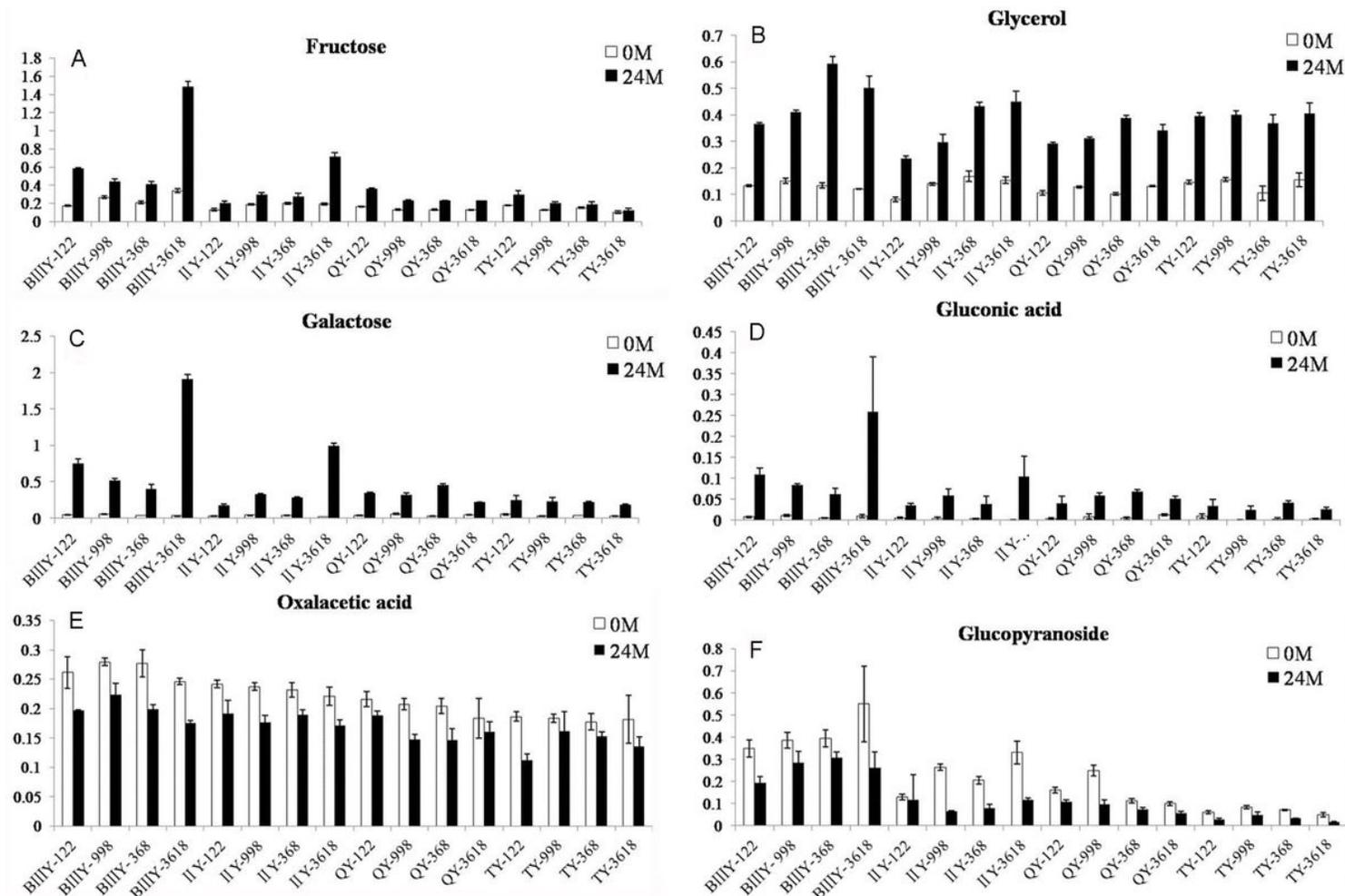


Figure 4

Changes of relative contents of fructose (A), glycerol (B), galactose (C), gluconic acid (D), oxaloacetic acid (E) and glucopyranoside (F) in seeds of 16 hybrid rice combinations stored for 24 months.

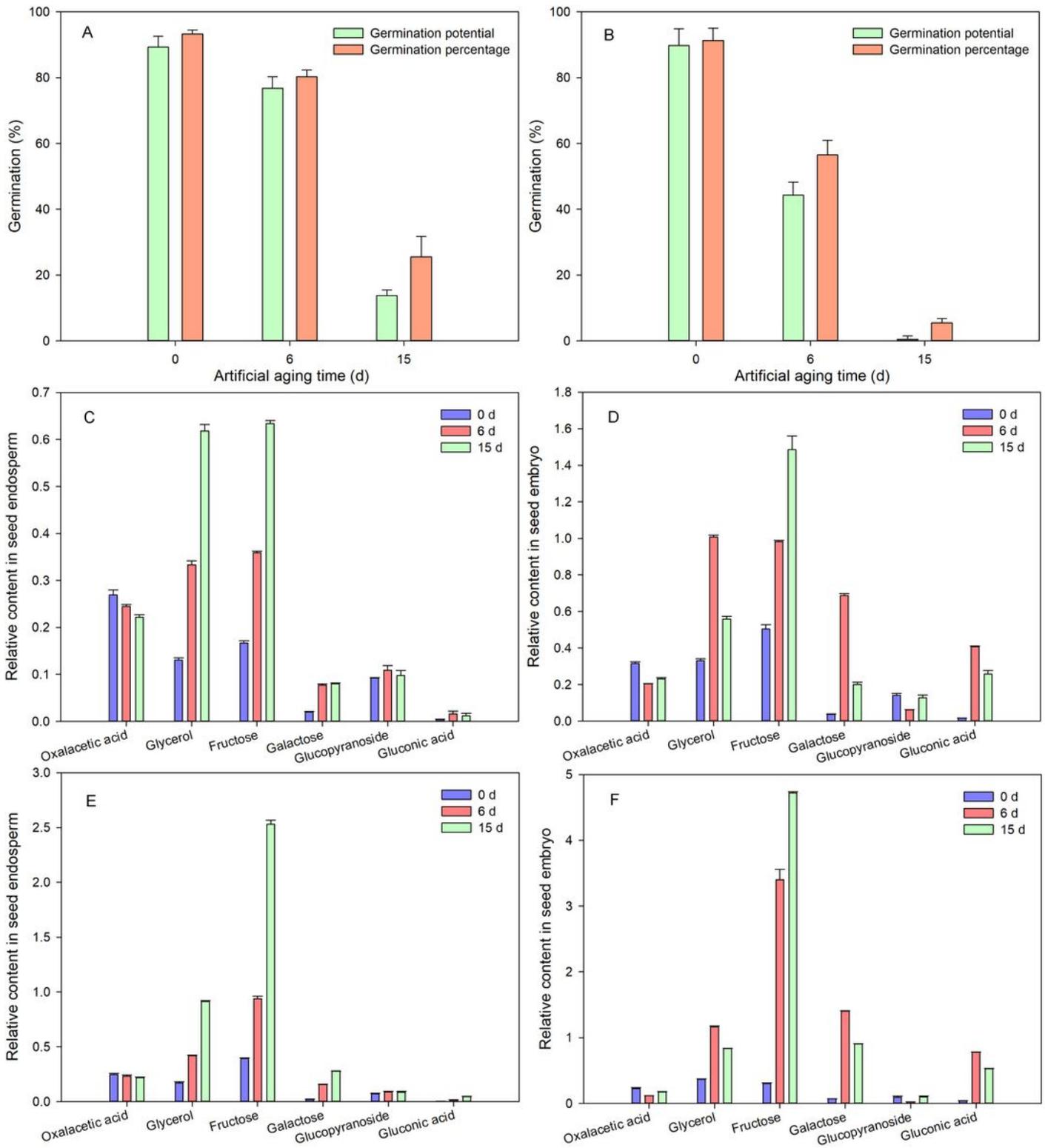


Figure 5

Germination percentage of rice seeds G8Y2156 (A, C, D) and G8Y169 (B, E, F) artificially aged for 6 days and 15 days and changes of major sugars in endosperm (C, D) and embryo (D, F).

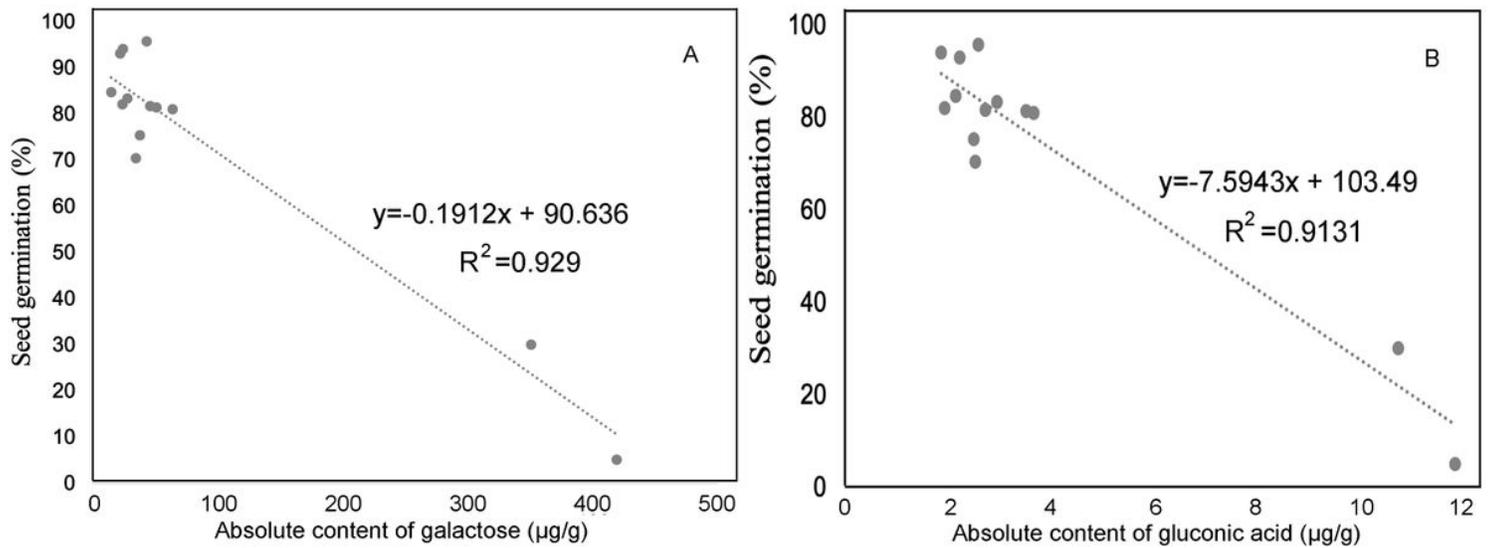


Figure 6

Regression equation curve between the content of galactose (A) or gluconic acid (B) and germination percentage of 16 hybrid rice seeds after 24 months storage.

Supplementary Files

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