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Lower anther culture response in indica than in japonica rice is associated with a callus- induction bottleneck and early browning of sporophytic anther tissues

Christabell Nachilima
Hokkaido University
Nanami Kudo
Hokkaido University
Yuka Konno
Hokkaido University
Meilan Xu
Hokkaido University
Kosuke Miyamoto
Hokkaido University
Kana Takashima
Hokkaido University
Yoshiki Tokuyama
Hokkaido University
Yohei Koide
Hokkaido University
Yoshihiro Okamoto
Rakuno Gakuen University
Yuji Kishima
kishima@agr.hokudai.ac.jp

Hokkaido University

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Abstract

The response to anther culture in rice is strongly influenced by genetic background. Japonica cultivars generally have a higher anther culture efficiency than indica cultivars. A comparison of three japonica and two indica cultivars showed that the difference occurred mainly at the callus induction stage, whereas regeneration efficiency and the proportions of green and albino plants formed were not significantly different. To examine the relationships among successive components of the anther culture response process, we used 54 chromosome segment substitution lines (CSSLs) carrying Kasalath (indica) segments in a Nipponbare (japonica) background. These CSSLs exhibited broad variation in callus induction, regeneration, and green plant formation. Callus induction was not significantly correlated with regeneration or green/albino plant formation, indicating that these successive components varied largely independently across the CSSL population. Nevertheless, callus induction constituted the initial numerical bottleneck because only induced calli could contribute to subsequent plant recovery. Comparison of Nipponbare and Kasalath further revealed early anther browning as a key feature associated with poor callus induction. Browning began near the filament-attachment region, progressed through the sporophytic tissues and extended towards the microspores. No visible callus emerged from any of the completely browned anthers examined. Browning also spread faster in Kasalath than in Nipponbare during the induction stage. These results suggest that the early browning of sporophytic anther tissues may contribute to poor callus formation.

Key Message

Lower indica anther-culture response is primarily linked to poor callus induction, while early, progressive browning in Kasalath is associated with failure of callus formation.

Introduction

Anther culture has emerged as a valuable tool for rice improvement (Chen et al. 2025; Feng et al. 2025). Through the anther culture process, it is possible to generate haploid plants that can be rapidly converted to doubled haploids (DHs), allowing complete homozygosity to be achieved within a single generation (Forster et al. 2007; Germanà 2011; Hale et al. 2022; Choi et al. 2026). This shortens the breeding cycle and improves selection efficiency. Although anther culture has great potential for rice improvement, its practical use is still limited by several physiological challenges. The overall efficiency of the system depends on sequential steps, starting with callus induction, followed by plant regeneration, and finally the development of either green or albino plants (He et al. 1998; Mishra and Rao 2016; Chen et al. 2025). Each of these stages demonstrates a marked dependence on genetic background, with substantial differences observed between rice subspecies (He et al. 2006; Mishra and Rao 2016).

Japonica genotypes typically show greater responsiveness to anther culture, whereas indica varieties are often poor, with reduced embryogenic competence and lower regeneration efficiency (He et al. 1998; Mayakaduwa and Silva 2023; Gao et al. 2024; Guo et al. 2024). This pronounced genotype dependency

presents a limitation in the application of anther culture in many indica cultivars. Callus induction, regeneration, and differentiation between green and albino plants do not always happen with the same efficiency, and problems early on can limit the system's overall results (Mishra and Rao 2016).

Chromosome segment substitution lines (CSSLs) provide an effective genetic resource for dissecting complex quantitative traits because individual chromosomal segments derived from a donor parent can be evaluated in a nearly uniform genetic background. In rice, CSSLs developed from japonica and indica lines have been widely used for high-resolution mapping of agronomically important traits (Kubo et al. 2002; Ebitani et al. 2005; Furuta et al. 2014; Shan et al. 2025). Therefore, CSSLs offer an ideal system to evaluate the genetic basis of anther culturability and to compare the efficiencies of successive anther-culture stages within a largely uniform genetic background.

Tissue browning is a common physiological problem in plant tissue culture and often reduces culture efficiency. In rice, excessive browning has been associated mainly with reduced regeneration capacity, largely through oxidative damage caused by phenolic compound oxidation (Wu et al. 2022; Liu et al. 2024; Permadi et al. 2024; Wang et al. 2024). Although anther browning has been associated with reduced anther culture efficiency, its spatial progression within the anther and its relationship with microspore-derived callus formation remain poorly understood. Moreover, the timing and extent of anther browning have rarely been compared between japonica and indica cultivars.

In this study, we first compared three components of anther culture response—callus induction, plant regeneration, and green/albino plant formation—between japonica and indica cultivars. We then evaluated 54 CSSLs carrying Kasalath chromosome segments in the Nipponbare genetic background to determine whether variation in these successive components was correlated. Subsequently, we compared the temporal and spatial progression of anther browning in Nipponbare and Kasalath. Our results indicate that callus induction represents the principal bottleneck in the indica cultivars and indica-derived materials examined and that rapid browning of sporophytic anther tissues is associated with poor callus formation.

Materials and methods

Plant materials

Baseline relationships among callus induction, plant regeneration, and green/albino plant formation during anther culture were investigated using three japonica cultivars (Nipponbare, Kitaake, and Kitakaori) and two indica cultivars (Kasalath and Supa). To examine genetic variation in these three components found in anther culture response, we evaluated a panel of 54 chromosome segment substitution lines (CSSLs) derived from a Nipponbare × Kasalath cross, which were kindly provided by the Rice Genome Resource Center (RGRC) in National Agriculture and Food Research Organization (NARO) in Japan (Mizuta et al 2005). Each CSSL contains one or a few defined chromosomal segments from Kasalath within an otherwise uniform Nipponbare genetic background (Suralta et al. 2008). The

substituted chromosomal regions were previously characterized using 214 restriction fragment length polymorphism (RFLP) markers distributed across the 12 rice chromosomes (Fig. S1).

Panicle sampling and cold pretreatment

Plant materials were cultivated under greenhouse conditions according to standard agronomic management practices until panicle emergence (12 h day length and 30°C day/20°C night). Panicles were sampled when microspores were estimated to be at the late uninucleate stage, which is optimal for callus induction in rice anther culture. The developmental stage for sample collection was determined based on auricle distance of 2.5–7 cm. To enhance embryogenic competence, harvested panicles were cold pretreated at 10°C for 10 days in darkness. After cold pretreatment, to confirm the nuclear stage of the microspores, representative spikelets from anther were fixed in FAA solution, and microspores were stained with acetocarmine using a light microscope. Microspore developmental stages were classified according to Kinoshita et al. (2000). For anther culture, only anthers containing predominantly uninucleate microspores were selected.

Anther culture

Harvested, cold-treated panicles were surface-sterilized in 70% ethanol for 1 minute under sterile conditions prior to anther plating. Immature spikelets were removed. Anthers were excised with care, omitting the top two spikelets of each primary branch to reduce developmental variability. To control for potential positional effects within the panicle, spikelets from the upper, middle, and lower sections of each panicle were harvested and processed using a standardized protocol across all samples.

Isolated anthers were cultured on N_6 callus induction medium according to the protocol described by Chu (1978). Anthers were distributed evenly across 90 mm × 15 mm Petri dishes, with no more than 52 per dish to prevent overcrowding. Cultures were incubated in complete darkness at 25°C for approximately 32–60 days. Callus formation was monitored at regular weekly intervals. The callus induction frequency was calculated as the percentage of cultured anthers that produced callus. Calli that reached approximately 3 mm or more in diameter between 32 and 60 days after anther plating were transferred to N_6 regeneration medium (Chu 1978), with a maximum of 12 calli per dish, and cultured under continuous light (24-hour photoperiod) at a constant temperature of 25°C. After culturing for about 60 days on the regeneration medium, plants were observed to have formed in the culture dish. Regeneration efficiency was assessed by the proportion of calli that generated shoots, while the frequency of green/albino plant formation was determined as the proportion of green/albino plantlets among all regenerated plants. These metrics were used to evaluate genotypic variation in anther culture response and to provide a baseline for subsequent genetic analyses.

Observation of anther browning

To investigate anther browning during rice anther culture, the parental lines Nipponbare and Kasalath were selected. These genotypes offered a clear contrast for comparing browning behavior and callus formation under in vitro conditions. Panicles were collected at the uninucleate microspore stage and processed using the standard anther culture protocols described above. Anthers showing clearly visible brown pigmentation in any part of the tissue were scored as browned by visual inspection. Each week, the number of browned anthers for each genotype was recorded, and representative images were captured. Observations continued until callus formation was detected.

Histological observation of browning progression

The temporal progression of anther browning during in vitro culture was investigated by collecting anthers immediately after cold treatment (0 weeks) and after plating 1, 2, 3, and 4 weeks of culture on callus induction medium. Samples were embedded in optimal cutting temperature compound and rapidly frozen in liquid nitrogen. The frozen samples were sectioned into 25-μm-thick slices using a cryostat maintained at − 20°C. Sections were examined under a light microscope. The morphology of the anther wall and microspores, as well as the location and progression of browning, were compared across the different culture periods to assess histological changes associated with anther browning.

Statistical analysis

All analyses were conducted using R version 4.6.1. Callus induction, regeneration, and green/albino plant formation were calculated relative to cultured anthers, calli, and regenerated plants, respectively. Regeneration rate was calculated as the proportion of calli that regenerated relative to the total number of calli formed. Data from japonica and indica cultivars are presented as mean ± standard deviation (SD) from three independent experiments, except for Supa (n = 2), and anther-browning data are presented as mean ± SD from three independent replicates. Differences between japonica and indica groups were evaluated using a two-sided, two-sample Student's *t*-test assuming equal variances, with cultivar means treated as independent observations. Differences among individual genotypes were evaluated using one-way ANOVA followed by Tukey's HSD test at $P < 0.05$. Relationships among callus induction, regeneration, and green/albino plant formation in the 54 CSSLs were evaluated using Pearson's correlation with pairwise-complete observations. For lines that produced no regenerated plants, green and albino plant proportions could not be calculated and were treated as missing values in correlations involving these traits. Low and high responses were defined by the 25th and 75th percentiles, respectively. Anther browning was recorded cumulatively, and newly browned anthers during each weekly interval were calculated from consecutive counts. Browning progression was analysed using a grouped binomial discrete-time model with genotype, culture week, and their interaction as fixed effects and experimental replicate as a block. Likelihood-ratio tests were used to evaluate the genotype × culture-week interaction, with significance determined at $P < 0.05$.

Results

Differences in anther culture responses between japonica and indica cultivars

Three japonica cultivars (Kitaake, Kitakaori, and Nipponbare) and two indica cultivars (Kasalath and Supa) were evaluated for callus induction, plant regeneration, and the proportions of green and albino regenerants (Figs. 1 and 2a; Table S1). Mean callus induction was significantly higher in japonica (38.47%) than in indica cultivars (3.35%; P = 0.0475). Among the japonica cultivars, callus induction was highest in Kitakaori (53.67 ± 10.41%), followed by Nipponbare (36.90 ± 1.56%) and Kitaake (24.83 ± 10.05%). In contrast, callus induction was 2.70 ± 1.15% in Kasalath and 4.00% in Supa (Fig. 1; Tables 1 and S1).

Because regeneration rate was calculated relative to the number of induced calli, it did not directly reflect differences in callus-induction frequency. Regeneration rates did not differ significantly between the japonica and indica groups (44.11% and 37.85%, respectively; P = 0.7553) (Table 1). Nipponbare had the highest regeneration rate (72.23 ± 34.76%), whereas the remaining cultivars ranged from 26.67% to 38.50% (Table S1). **The proportions of green and albino regenerants also did not differ significantly between japonica and indica cultivars (*P*= 0.1451 and*P*= 0.1450, respectively) (Table 1).** The mean proportion of green regenerants was numerically much higher in japonica than in indica cultivars (59.46% vs. 5.25%), but this difference was not statistically significant (P = 0.145), reflecting large variation among japonica cultivars (Table 1). Green plants accounted for 82.08 ± 6.54% and 79.62 ± 9.13% of regenerants in Kitaake and Nipponbare, respectively, whereas Kitakaori produced mainly albino plants, with only 16.67 ± 3.79% green regenerants. In the indica cultivars, green plants accounted for only 3.00 ± 2.65% of regenerants in Kasalath and 7.50% in Supa (mean of two experiments) (Table S1). Despite its high callus induction, Kitakaori produced predominantly albino regenerants (83.33 ± 3.79%), with green plants accounting for only 16.67 ± 3.79% of the regenerants (Table S1). Supa was evaluated in two experimental replicates because sufficient plant material at the appropriate developmental stage was unavailable for the third replicate; therefore, variability was not reported for this cultivar (Tables 1 and S1).

CSSLs exhibit distinct patterns of callus induction and regeneration

A set of 54 chromosome segment substitution lines (CSSLs) was developed by introducing defined Kasalath chromosome segments into the Nipponbare genetic background (Fig. S1) (Mizuta et al. 2005). These lines share a largely uniform Nipponbare but differ in the chromosome segments inherited from Kasalath, thereby providing a genetically diverse population in which relationships among successive components of anther culture response could be evaluated (Figs. 2a and b). The CSSL population was used to determine whether variation in callus induction was associated with variation in plant regeneration and green/albino plant formation.

The CSSLs showed broad variation in callus induction and plant regeneration. Callus induction rates ranged from nearly 1% to 63% (Fig. 2b; Table S2). Kasalath, CSSL9, CSSL6, and CSSL11 were among the least responsive lines, whereas CSSL1, CSSL29, and CSSL54 exceeded Nipponbare (Fig. 2b). CSSL1 had the highest callus induction (approximately 63%), followed by CSSL54 (58%) and CSSL29 (52%**)** (Fig. 2b;

Table S2)**.** Kasalath, CSSL30 and CSSL47 produced only albino regenerants (Fig. 2c; Table S2). Despite their varying callus induction rates, CSSL5, CSSL9 and CSSL6 showed relatively high regeneration rates around 62.1%, 54.5% and 46.2%, respectively (Fig. 2c; Table S2). CSSL29 showed the highest regeneration rate at 67.6% and all its regenerated plants were green (Fig. 2c; Table S2).

For comparison among traits, the genotype order established by increasing callus induction was retained in the regeneration panel (Fig. 2c). Regeneration, however, did not follow the same ordered pattern. CSSL9, for example, had very low callus induction rate but relatively high regeneration rate, whereas CSSL1 had the highest callus induction but low regeneration (Fig. 2; Table S2). Among the lines with high callus induction rate, CSSL29 and CSSL54 also showed high regeneration rate, while CSSL51 showed little regeneration (Fig. 2; Table S2). These contrasting response patterns suggested that callus induction and regeneration varied largely independently across the CSSLs.

Callus induction was not significantly correlated with regeneration

Correlation analysis showed that callus induction did not explain variation in regeneration or in the proportions of green and albino regenerants (Figs. 3 and S2). Callus induction was not significantly correlated with regeneration (r = −0.17, P = 0.230), green plant formation (r = −0.07, P = 0.633) (Fig. 3), or albino plant formation (r = 0.07, P = 0.633) (Figs. 3 and S2). Lines with similar callus induction frequently differed markedly in downstream responses, and both high and low regeneration values occurred across the callus-induction range.

Quartile-based classification separated the CSSLs according to callus induction and regeneration (Fig. 4). The broad distribution of lines across the four quadrants showed that callus induction did not clearly explain regeneration performance. CSSL9, for example, combined low callus induction with high regeneration, whereas Nipponbare showed relatively high callus induction but low regeneration (Fig. 4). Kasalath had very low callus-induction rate but moderate regeneration (Fig. 4). These contrasting responses further indicated that callus induction rate was not associated with the proportion of calli that subsequently regenerated. Although callus induction was not correlated with regeneration efficiency, it represents an initial numerical bottleneck because only induced calli can contribute to subsequent green plant recovery.

Regeneration rate is positively associated with green plant production

Regeneration and green/albino plant production varied considerably among the 54 CSSLs and their parental lines (Fig. 2). Regeneration rates ranged from 0% in CSSL20, CSSL33 and CSSL39 to 67.65% in CSSL29. Several CSSLs, including CSSL3, CSSL4, CSSL5, CSSL6, CSSL9, CSSL10, CSSL11, CSSL18, CSSL29, CSSL49, CSSL52 and CSSL54, showed relatively high regeneration rates more than 40% (Fig. 2; Table S2). CSSL29 had the highest regeneration rate and produced only green plants. CSSL4, CSSL6 and CSSL18 also combined high regeneration with 100% green plant production. The proportions of green/albino plants differed markedly among the regenerated CSSLs. Most lines produced predominantly green plants, whereas CSSL30 and CSSL47 produced only albino plants. Nipponbare and Kasalath had similar regeneration rates of 18.81% and 20.00%, respectively, but differed substantially in

the type of regenerants produced (Fig. 2; Table S2). Nipponbare produced 73.68% green and 26.32% albino plants, whereas all plants regenerated from Kasalath were albino (Fig. 2; Table S2). Correlation analysis showed a moderate positive association between regeneration rate and green plant rate (r = 0.41, P = 0.003) (Figs. 3 and S2). A corresponding negative association was observed between regeneration rate and albino plant rate (r = −0.41, P = 0.003) (Fig. S2). Thus, CSSLs with higher regeneration rates generally tended to produce a greater proportion of green plants, although considerable variation remained among individual lines.

Anther browning progresses more rapidly in Kasalath than in Nipponbare

Sequential observation of representative anthers revealed contrasting browning patterns between Kasalath and Nipponbare (Fig. 5a). Kasalath anthers were initially pale but showed rapid discoloration during culture, with extensive browning already visible by Week 2 and completely or nearly completely browned by Week 4 (Figs. 5a and S4). Nipponbare anthers browned more slowly and generally retained substantial pale tissue. By Week 4, several Nipponbare anthers had produced visible callus, whereas the monitored Kasalath anthers remained brown without visible callus formation (Figs. 5a and S4). Because this initial observation involved only four representative anthers per genotype, it was used to characterize the appearance and progression of browning rather than to estimate its frequency.

Browning frequency was subsequently quantified using three independent replicates of 55 anthers per genotype (Figs. 5b and S3). No browned anthers were detected at Week 0. By Week 1, browning had increased sharply to 56.36% ± 8.33 in Kasalath, compared with 18.18% ± 1.82 in Nipponbare. Kasalath remained highly affected at Week 2 (62.42% ± 8.59) and showed a further increase to 87.27% ± 3.64 at Week 3 and 93.94% ± 1.05 at Week 4. In contrast, browning in Nipponbare increased gradually from 21.82% ± 1.82 at Week 2 to 24.24% ± 1.05 at Week 3 and 28.48% ± 1.05 at Week 4 (Figs. 5b and S3). The temporal progression of browning differed significantly between the two genotypes, with a significant genotype × culture-week interaction (likelihood-ratio χ^2 = 22.98, df = 3, P < 0.001) (Fig. 5b; Table S5). Thus, the qualitative difference observed among the representative anthers was confirmed quantitatively: Kasalath showed an early and rapid increase in browning, whereas browning in Nipponbare remained comparatively limited throughout culture.

Browning initiates in the sporophytic tissue and progresses toward the gametophytic microspores

Whole-anther imaging of Kasalath and Nipponbare revealed a defined spatial and temporal progression of browning during culture. The earliest visible pigmentation was observed in Kasalath at Week 1 at the filament-attachment region, where the anther had been detached from the donor plant during excision (Fig. 6a). Browning was initially restricted to a small lesion at this site. The lesion subsequently enlarged and extended longitudinally through the anther, producing progressively broader and darker regions by Week 4. Callus emerged from anthers that remained predominantly non-browned in Nipponbare, whereas completely browned anthers showed no visible callus formation. The same contrasting outcomes were observed in independent anthers (Figs. S5 and S6).

The initial browning site was located within the sporophytic tissues of the anther (Figs. 6a, S5 and S6), whereas callus originates from the gametophytic microspores enclosed within the locules. Representative cut sections captured an intermediate stage in which the central sporophytic tissue was already pigmented, while the enclosed microspores remained distinguishable and showed only initial signs of browning (Fig. 6b). At the advanced stage, pigmentation extended throughout the surrounding sporophytic tissues and was also evident within the microspores.

Cryostat sections further resolved the internal distribution of browning in Nipponbare and Kasalath. As shown in Fig. 6c, at Week 0, both genotypes showed four clearly defined locules surrounded by pale sporophytic tissues, with no visible pigmentation in the enclosed microspores. The earliest internal browning appeared in the central connective region between the locules, near the filament-attachment site. By Week 2, pigmentation had extended from this region along the inner locular boundaries and into the surrounding sporophytic tissues. The pigmented regions broadened during Weeks 3 and 4, progressively involving a larger proportion of the locule-enclosing tissues. This progression was particularly pronounced in Kasalath, whereas Nipponbare retained largely non-browned tissues at most time points. The progression was reproduced in independent cryostat sections (Fig. S7). These observations showed that browning was first detected in the sporophytic tissues, and pigmentation subsequently became visible in the enclosed gametophytic microspores. Collectively, the whole-anther observations, cut sections and cryostat sections documented a consistent spatial sequence: browning first appeared at the filament-attachment and central connective region, expanded through the sporophytic tissues enclosing the locules and was subsequently detected in the gametophytic microspores. This progression was more extensive in Kasalath than in Nipponbare and coincided with the absence of visible callus emergence from completely browned anthers.

Discussion

Callus induction is the primary bottleneck in indica anther culture

Genetic background strongly influenced callus induction in this study. Callus induction was substantially higher in the japonica cultivars than in the indica cultivars examined, whereas regeneration rates and the proportions of green/albino regenerants did not differ significantly between the two groups (Fig. 1; Tables 1 and S1). The generally higher anther culture response of japonica cultivars than of indica cultivars is consistent with previous reports (He et al. 1998; Mishra and Rao 2016; Feng et al. 2025). Our stage-specific analysis further showed that among the cultivars examined, the difference between japonica and indica rice was most clearly evident at the callus-induction stage rather than during subsequent plant regeneration. Improved induction media have also been reported to increase callus formation in Nipponbare and indica × japonica hybrids but not in the indica cultivar NSIC Rc480, further illustrating the strong genotype dependence of callus induction (Ali et al. 2021). Our results further refine

this genotype-dependent response by showing that the major japonica–indica difference is expressed primarily at the callus-induction stage.

The stage-specific nature of anther culture response was also evident in the CSSL population. This population provided broad variation in anther culture responses within a largely uniform genetic background, allowing relationships among successive stages of anther culture to be evaluated. The CSSLs showed broad variation in callus induction, regeneration, and green/albino plant formation, but callus induction was not significantly correlated with any of the downstream traits (Figs. 2 and 3). For example, CSSL9 combined very low callus induction with relatively high regeneration, whereas CSSL1 showed the highest callus induction but relatively low regeneration. These contrasting responses indicate that callus induction and regeneration represent largely distinct components of anther culture performance. This interpretation is consistent with previous studies showing that genetic factors affecting callus induction, plant regeneration, and green plant production can differ among successive stages of rice anther culture (He et al. 1998; Yamagishi et al. 1998; Kwon et al. 2002; Sun et al. 2023; Wu et al. 2022). Nevertheless, callus induction constitutes the initial numerical bottleneck because only induced calli can contribute to subsequent plant recovery. Consequently, even a high regeneration rate results in limited green plant production when few calli are induced. Efficient anther culture therefore requires sufficient callus induction followed by effective regeneration and green plant formation.

Regeneration rate was moderately associated with the proportion of green regenerants, although considerable variation remained among the CSSLs (Fig. 3). This suggests that regeneration capacity and green plant formation are related but not identical components of anther culture response. Some CSSLs combined high regeneration with predominantly green regenerants, whereas others regenerated less efficiently or produced mainly albino plants (Fig. 2). Thus, callus induction establishes the number of calli available for subsequent plant recovery, whereas regeneration and green/albino differentiation influence the efficiency and outcome of the regenerated plants.

Rapid anther browning is associated with a low callus-induction rate

Our findings show that rapid anther browning is an early event associated with poor callus induction in Kasalath (Fig. S4). The contrast between Kasalath and Nipponbare raises the possibility that the timing and extent of browning contribute to their different callus-induction responses. Because browning was first detected in sporophytic tissues near the filament-attachment site and subsequently became visible in the enclosed microspores, failure of callus induction may be linked, at least partly, to earlier deterioration of the surrounding sporophytic tissues. However, the present observations do not establish a direct causal relationship between browning and the loss of microspore embryogenic competence.

The location of the initial lesion suggests that tissue injury during anther excision may contribute to the onset of browning (Fig. 6a; Figs. S5–S7). Detachment of the anther damages tissues at the filament-attachment region, including the associated vascular and connective tissues. Wounding can induce phenolic accumulation, while oxidation of phenolic substrates can generate quinones and polymerized

compounds associated with visible tissue browning (Lagrimini 1991; Jones and Saxena 2013). ROS production is also a recognized component of plant wound responses and can participate in peroxidase-dependent oxidation and cross-linking of phenolic compounds (Lagrimini 1991; Razem and Bernards 2003). Although these processes were not directly measured in the present study, they provide a plausible biological explanation for the initiation of browning near the excision site. Direct measurements of ROS accumulation, phenolic content, and oxidative enzyme activity, together with experiments that suppress browning, will be required to determine whether early browning directly restricts callus induction. Such studies may provide strategies for improving callus induction and haploid plant production in indica rice.

Collectively, our results identify callus induction as the principal numerical bottleneck in the lower anther culture response of the indica materials examined. The spatial and temporal patterns of browning further suggest that early deterioration of sporophytic anther tissues may contribute to this bottleneck, providing a testable basis for improving indica anther culture.

Abbreviations

AC, anther culture; CSSL, chromosome segment substitution line; DH, doubled haploid; FAA, formalin–acetic acid–alcohol; RFLP, restriction fragment length polymorphism; ROS, reactive oxygen species; SD, standard deviation.

Statements and Declarations

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CRediT authorship contribution statement

C.N. and Y.K. conceived the study and designed the experiments. C.N. and N.K. conducted the experiments on anther culture components. C.N., Y.Kon., and M.X. conducted the anther-browning experiments. Y.O. provided guidance on anther culture. K.M. and K.T. supported the anther-culture experiments and maintained the plant materials. Y.T., Y.Koi., Y.O., and Y.K. supervised the research. C.N. prepared the original draft of the manuscript, and Y.K. reviewed and edited the manuscript. All authors read and approved the final manuscript.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Data availability

All relevant data can be found within this article and supporting materials.

Ethics approval

This study involved rice plants and did not involve human participants or animals. Ethical

approval was not applicable.

Consent to participate

Not applicable.

Consent to publish

Not applicable.

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Table

Table 1 Comparison of anther culture traits between japonica and indica rice cultivars

Trait[a]	Japonica mean (%)[b]	Indica mean (%)[b]	*P* value[c]
Callus induction	38.47	3.35	0.048*
Regeneration	44.11	37.85	0.755
Green plants	59.46	5.25	0.145
Albino plants	40.54	94.75	0.145

 Callus induction is the percentage of cultured anthers producing callus; regeneration is the percentage of calli producing shoots; green and albino plants are expressed as percentages of regenerated plants.

 Values are means of cultivar means for three japonica cultivars (Nipponbare, Kitaake, and Kitakaori) and two indica cultivars (Kasalath and Supa).

 P values were obtained using a two-sided two-sample Student's *t*-test assuming equal variances. * $P < 0.05$.

Figures

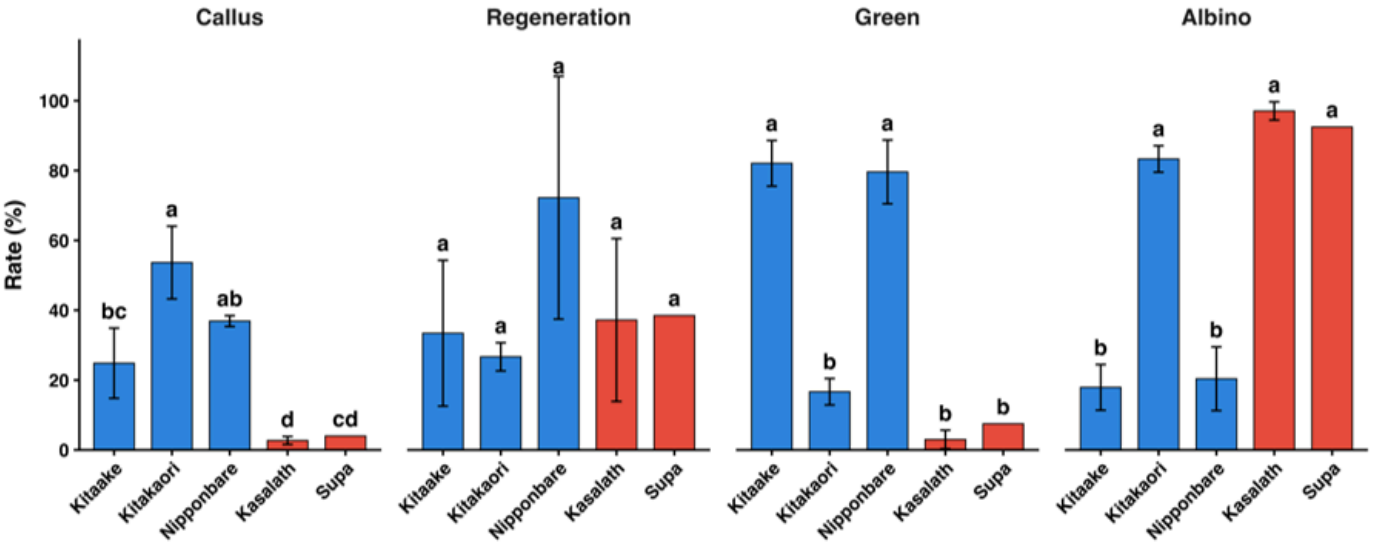

Figure 1

Anther culture responses of japonica and indica rice genotypes. Callus induction, regeneration, green plant, and albino plant rates are shown for three japonica genotypes (Kitaake, Kitakaori, and Nipponbare) and two indica genotypes (Kasalath and Supa). Blue and red bars represent japonica and indica genotypes, respectively. Bars represent mean ± SD, except for Supa (n = 2). Different lowercase letters indicate significant differences among genotypes.

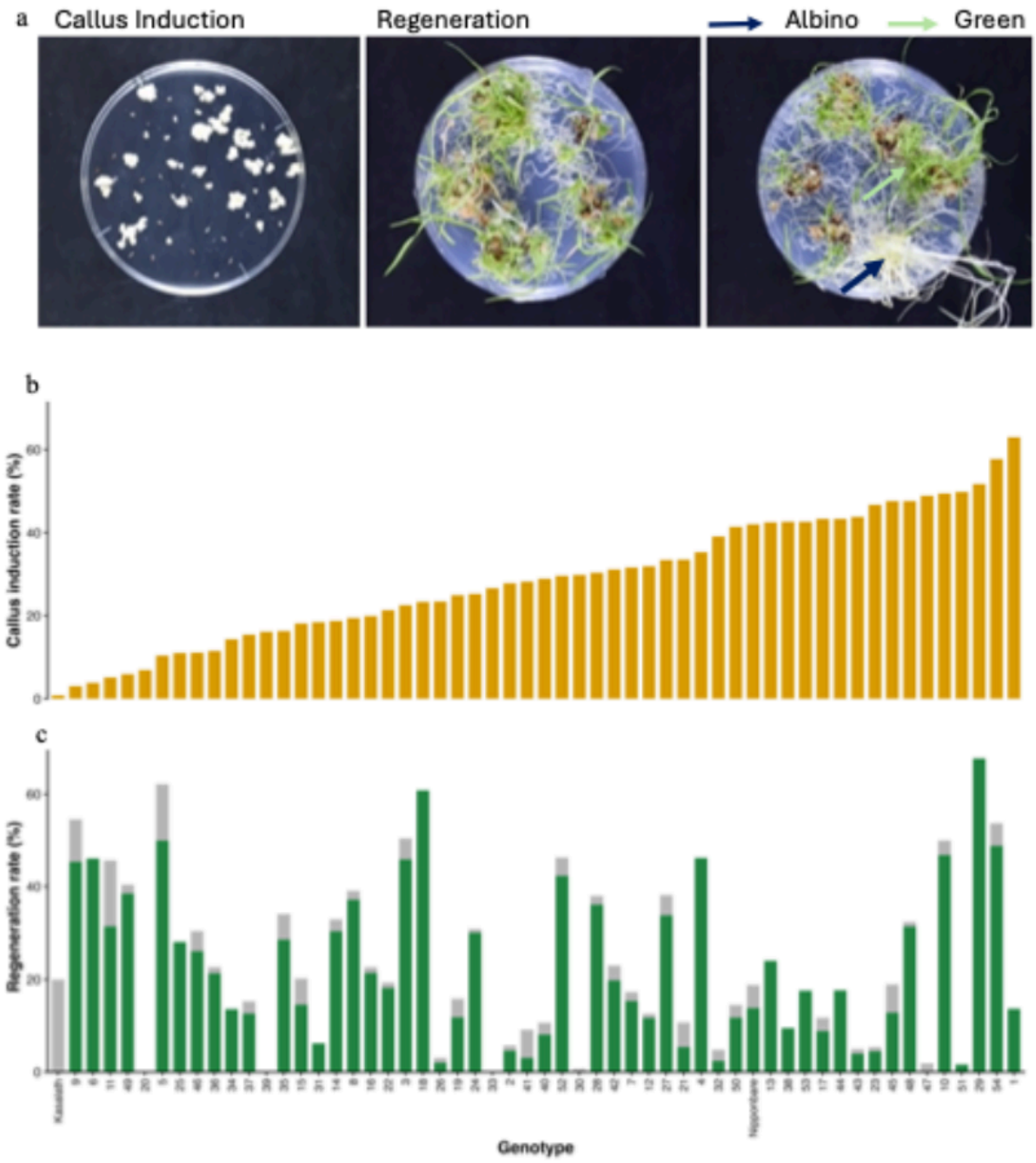

Figure 2

Variation in anther culture traits among chromosome segment substitution lines (CSSLs). (a) Representative images of callus induction and plant regeneration, including regenerated green and albino plants. Red and blue arrows indicate green and albino plants, respectively. (b) Callus induction rates of the CSSLs and parental genotypes. Genotypes are arranged in ascending order of callus induction rate. (c) Regeneration rates and proportions of green and albino plants for the corresponding genotypes. Green and gray bars represent green and albino plants, respectively

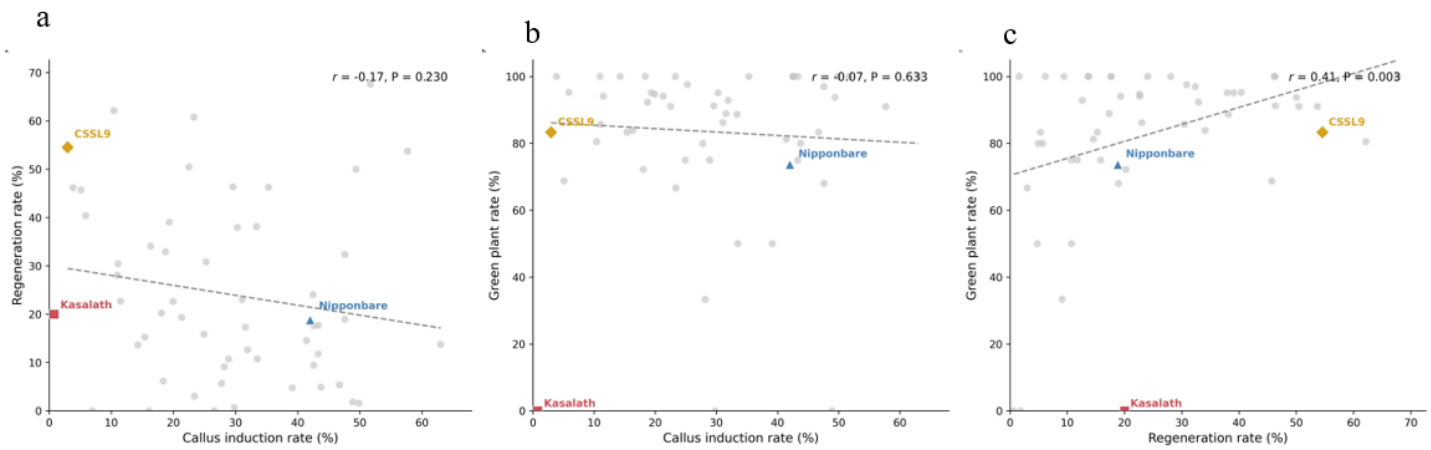

Figure 3

Relationships among anther culture traits in the CSSL population. Scatter plots show the relationships between (a) callus induction and regeneration rates, (b) callus induction and green plant rates, and (c) regeneration and green plant rates. Dashed lines represent linear regression fits. Pearson's correlation coefficients (*r*) and corresponding *P* values are indicated in each panel. Gray circles represent other CSSLs; CSSL9, Nipponbare, and Kasalath are indicated by the symbols and colours shown in the legend

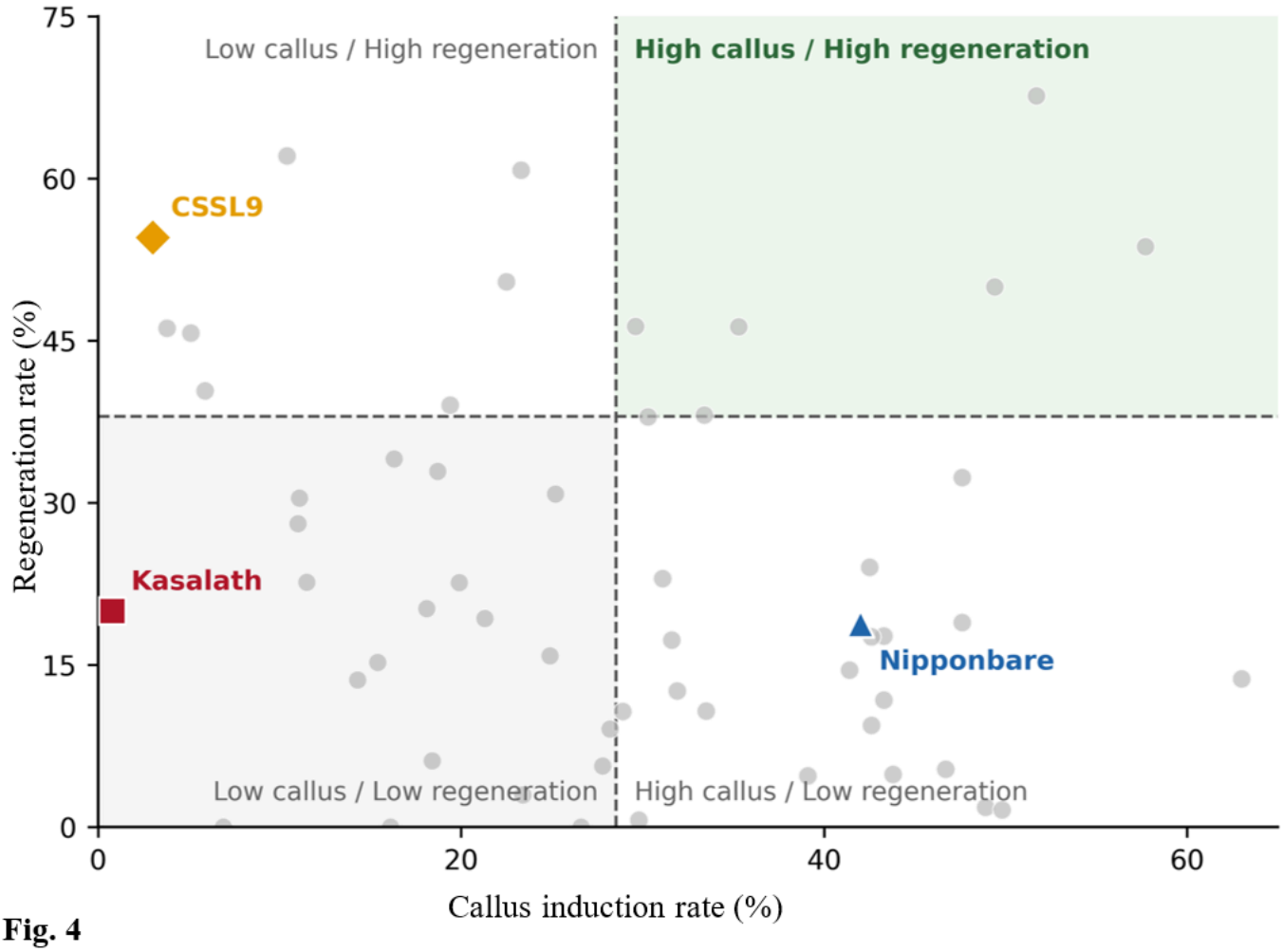

Fig. 4

Figure 4

Classification of CSSLs according to callus induction and regeneration rates. Scatter plot showing callus induction rate versus regeneration rate for the CSSLs and parental genotypes Nipponbare and Kasalath. Vertical and horizontal dashed lines indicate the thresholds used to divide the population according to callus induction and regeneration rates, respectively. The shaded upper-right region indicates genotypes with high callus induction and high regeneration. CSSL9, Nipponbare, and Kasalath are highlighted using the symbols shown in the legend

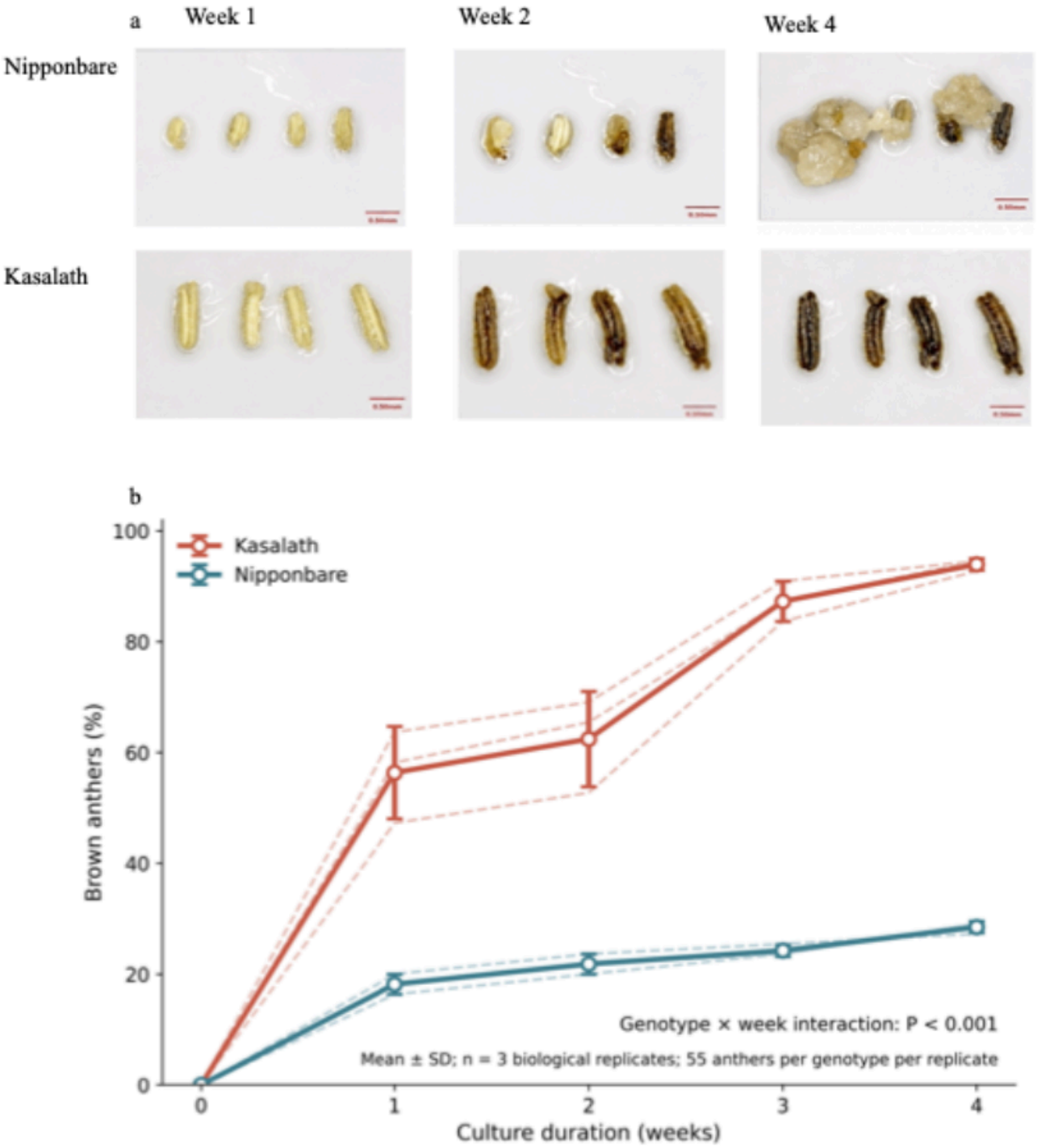

Figure 5

Time-course analysis of anther browning in Nipponbare and Kasalath during anther culture. (a) Representative images of cultured Nipponbare and Kasalath anthers at Weeks 1, 2, and 4 after culture initiation. Scale bars = 0.50 mm. (b) Percentage of browned anthers in Nipponbare and Kasalath from Week 0 to Week 4 of culture. Points represent mean ± SD from three biological replicates, with 55

anthers evaluated per genotype in each replicate. Browning progression differed significantly between genotypes (genotype × culture-week interaction, $P < 0.001$)

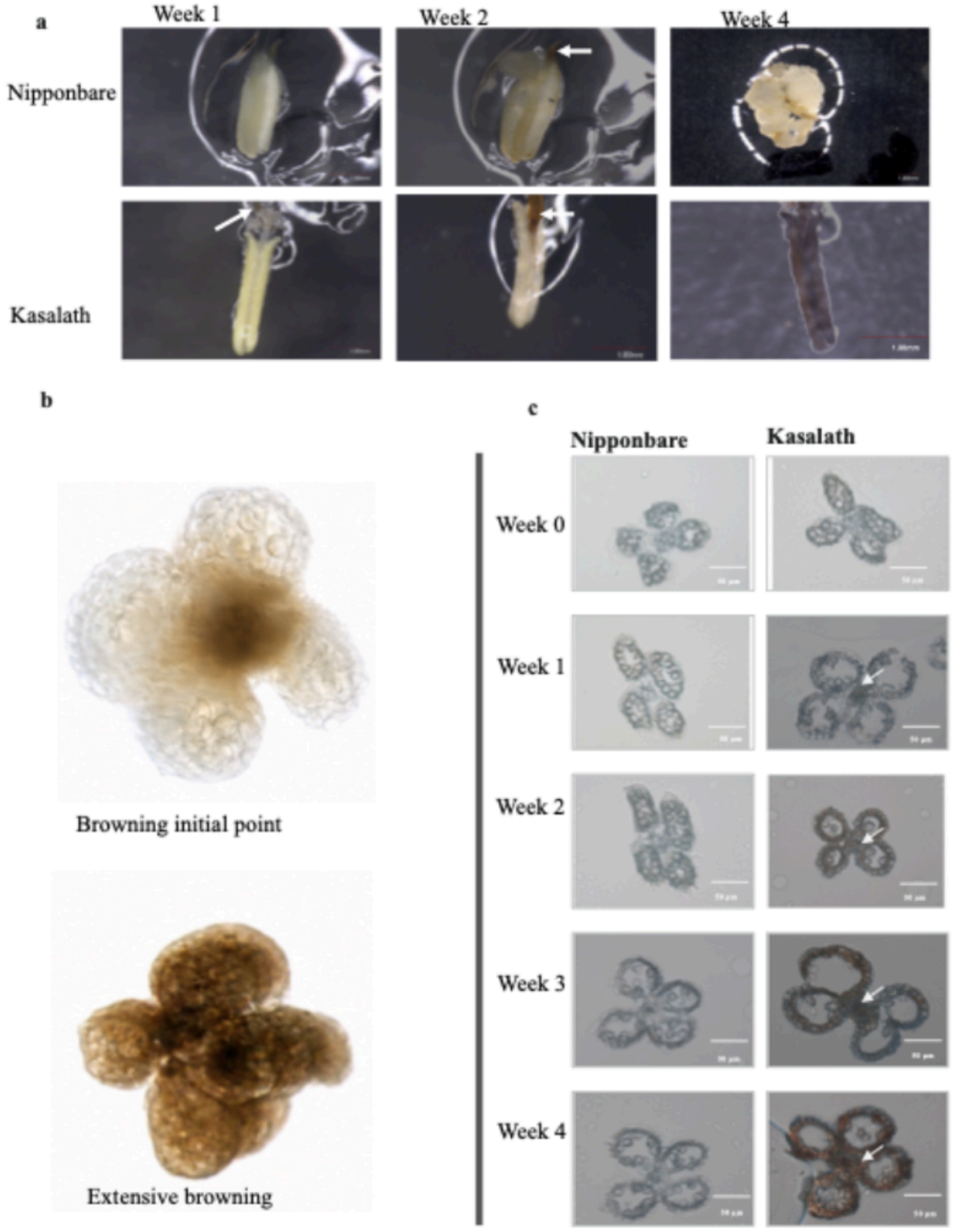

Figure 6

Macroscopic and cryostat observations of anther browning during culture. (a) Representative images of cultured Nipponbare and Kasalath anthers at Weeks 1–3. Red arrows indicate visible browning. (b)

Representative cut sections of Kasalath anthers showing localized and extensive browning. (c) Transverse cryostat sections of Nipponbare and Kasalath anthers from Weeks 0–4 of culture. White arrows indicate sites of visible browning in Kasalath sections. Scale bars = 50 μm

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- NachilimaetalSupplementaryTables.xlsx.xlsx
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