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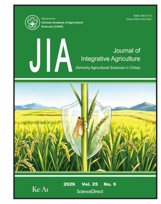

LETTER

Comprehensive phenotypic, physiological, and genetic analyses reveal stronger seedling-stage drought tolerance in *xian*/*indica* than *geng*/*japonica* rice

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Highlights

- *Xian/Indica* (XI) exhibits stronger seedling-stage drought tolerance (DT) than *geng/japonica* (GJ), as shown by delayed leaf rolling, higher survival rates, and improved physiological performance under drought stress.
- A total of 37 genes display haplotype-dependent effects on drought survival, revealing the genetic basis of DT divergence and informing subspecies-targeted breeding.

Rice (*Oryza sativa* L.) is one of the most important cereal crops, supporting more than half of the global population (Huang *et al*. 2024). Ensuring stable rice yield is essential for safeguarding global food security, particularly amid increasing climate variability (Jiao *et al.* 2024). Drought is one of the most serious abiotic stresses limiting rice production worldwide (Fang and Xiong 2015). Enhancing drought tolerance (DT) in rice through genetic improvement has thus become a major focus in modern breeding programs.

Xian/Indica (XI) and *geng/japonica* (GJ) are two geographically and genetically divergent subspecies of cultivated rice. XI cultivars are typically grown in tropical and subtropical regions at lower latitudes, and GJ cultivars are mainly cultivated in temperate regions at higher latitudes (Campbell *et al.* 2020). Consequently, GJ cultivars exhibit greater cold tolerance, while XI cultivars display better adaptability to warmer climatic conditions (Mackill and Lei 1997). Several genes have been identified as key targets of selection during domestication, contributing to these adaptive differences. For example, *COLD1*^{jap} and *bZIP73*^{jap} have been selected to enhance cold tolerance in GJ rice, while *SLG1*^{indica} contributes to heat adaptation in XI rice (Ma *et al.* 2015; Liu *et al.* 2018; Xu *et al.* 2020). Given that high temperature and drought often occur simultaneously, XI rice may also possess stronger DT than GJ rice. However, comprehensive comparative studies investigating DT divergence between the two subspecies remain limited.

To compare DT between XI and GJ rice, 292 accessions (144 XI and 148 GJ cultivars) (Appendix A) at the four-leaf stage were subjected to a 7-day drought treatment followed by rewatering (Appendix B). Phenotypic analysis revealed that leaf rolling occurred significantly later in XI (4–5 d post-stress) than in GJ (2.5–3.5 d post-stress) (Fig. 1-A and B). Consistently, the average survival rate of XI accessions after rewatering was significantly higher than that of GJ accessions (Fig. 1-C and D). Correlation analysis further confirmed a positive correlation between leaf rolling time and survival rate (Fig. 1-E). These results indicated that XI accessions possess superior DT than GJ accessions at the seedling stage. To elucidate the physiological basis of DT divergence, four extremely segregated XI and GJ groups were subjected to drought treatment at the four-leaf stage. Under drought stress, plant morphological performance ranked as XI-DT>GJ-DT>XI-DS>GJ-DS (Fig. 1-F). We further assessed physiological responses by measuring relative water content (RWC), chlorophyll relative content (SPAD), malondialdehyde (MDA) content, antioxidant activity, and osmotic adjustment (Appendix B). As shown in Appendix C, RWC was similar among groups at 0 h but declined under drought, ranking as XI-DT>GJ-DT>XI-DS>GJ-DS. This indicates superior water retention in XI-DT under stress. SPAD values were similar among groups during the initial 24 h. From 48 h, SPAD values remained stable or slightly increased in DT groups, but significantly declined in DS groups. At 72 h, XI-DT accessions

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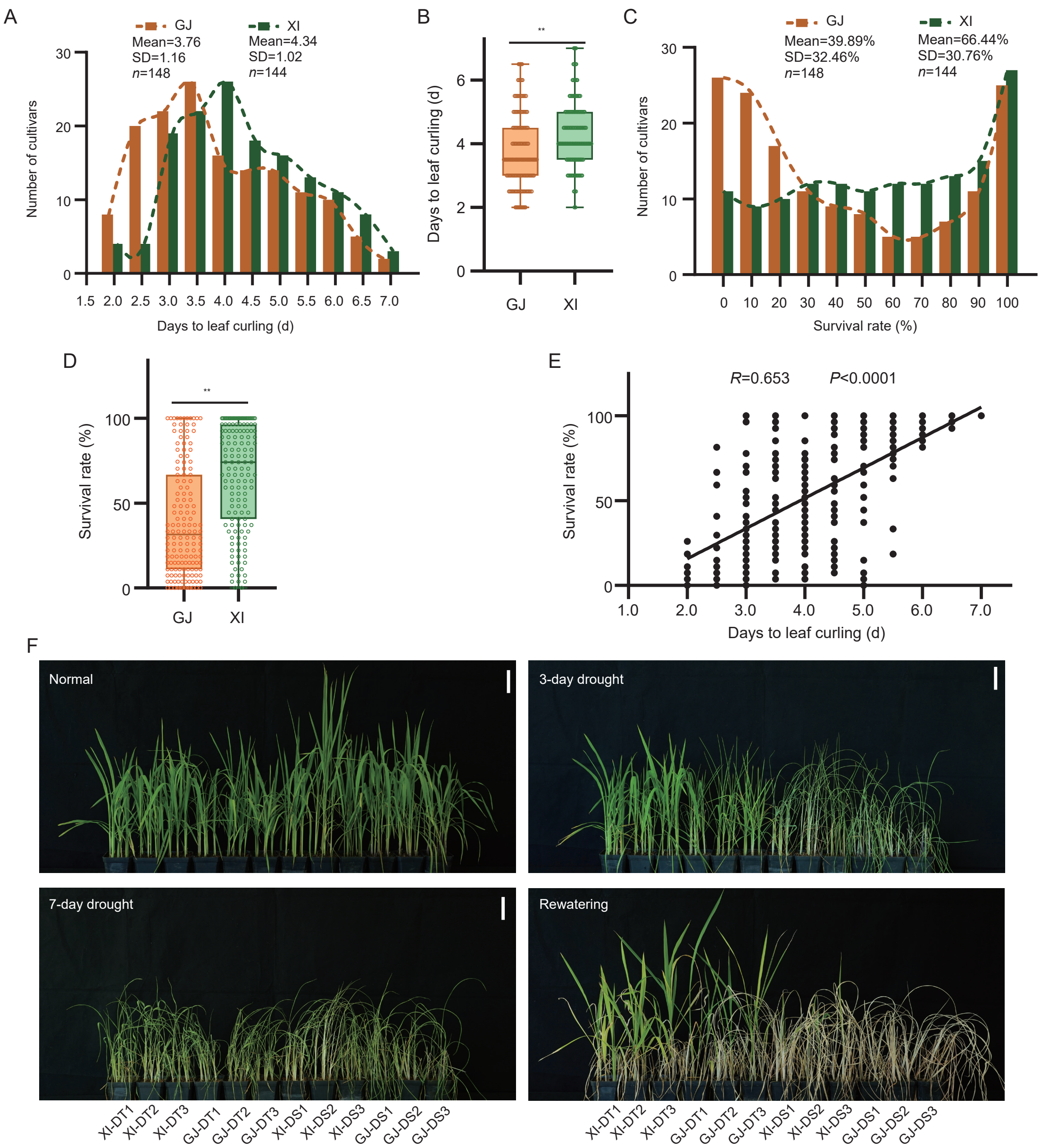

Fig. 1 Differences in drought tolerance (DT) between *xian/indica* (XI) and *geng/japonica* (GJ) rice. A, frequency distribution of days to leaf-rolling in XI and GJ groups. B, comparison of leaf rolling days between XI and GJ rice. C, frequency distribution of survival rates in the two groups. D, comparison of survival rates between XI and GJ rice. E, correlation between survival rate and days to leaf rolling. F, morphology of extremely segregated XI and GJ rice groups under drought stress (DS). ** indicates $P<0.01$ (Student's *t*-test). Scale bar=5 cm.

showed the highest SPAD values, indicating better chlorophyll retention and maintained photosynthetic capacity under drought. Membrane damage was evaluated by MDA content, which progressively increased in all groups, following the order: GJ-DS>XI-DS>GJ-DT>XI-DT. Consistently, antioxidant enzyme activities (superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT)) increased in all groups up to 48 h, continuing to increase in DT groups but declining in DS groups by 72 h post-stress, with activity levels ranked XI-DT>GJ-DT>XI-DS>GJ-DS. At 72 h post-stress, the highest soluble sugar (SS), soluble protein (SP), and proline (Pro) content were observed in XI-DT, followed by GJ-DT, XI-DS, and GJ-DS. These results indicated that XI-DT may maintain superior water balance, membrane integrity, antioxidant capacity, and osmotic adjustment capacity under drought stress.

To explore the genetic basis of DT divergence between XI and GJ subspecies, we performed population differentiation and haplotype analyses of 253 major DT-related genes (Appendix B). Among them, 74 genes showed significant differentiation (F_{st}>0.90) between the two subspecies (Appendix D). Of these 74 genes, 37 were further identified whose haplotypes displayed significant differences in survival rate. This suggests divergent selection on drought-resistance loci during subspecies evolution. Favorable haplotypes were subsequently defined and showed contrasting distribution patterns between XI and GJ accessions in the 3K Rice Genome Project (3KRG) (Fig. 2-A). Notably, the favorable haplotypes of nine genes — including *DSM1*, *OsEBP89*, *OsRbohB*, *OsDIRP1*, *Os9BGlu33*, *OsFTIP6*, *OsMYB6*, *OsEF1A*, and *OsANN9* — have not been widely selected in XI rice (Fig. 2-A), highlighting their considerable potential for future application in XI rice DT breeding. Moreover, haplotypes of eight genes (*DSM1*, *OsEBP89*, *GUDK*, *Os9BGlu33*, *Os6BGlu24*, *OsMYB6*, *OsOFP6*, and *OsNR1.2*) showed significant differences in both leaf rolling (Fig. 2-B) and post-drought survival rate (Fig. 2-C).

Almost 37 DT-related genes exhibited generally higher leaf expression in XI than GJ, with 16 significantly upregulated in XI based on four accessions per subspecies (Appendix E), suggesting a contribution of expression divergence to the enhanced DT of XI. For the other genes, differences in DT may arise from structural variation or other regulatory mechanisms. Many of these genes are involved in regulating the various DT physiological traits mentioned above. For example, *OsbZIP23* enhances DT in rice by regulating ABA signaling and activating dehydration-responsive genes, thereby improving water retention, increasing SS and Pro, and reducing membrane peroxidation (Zong *et al.* 2020). Similarly, plants overexpressing *OsMYB6* exhibit enhanced DT, as evidenced by higher Pro accumulation and antioxidant enzyme activities, along with lower MDA levels compared with the wild type (Tang *et al.* 2019). Moreover, our previous studies identified several key genes, including *OsNCED2* (Huang *et al.* 2024), *OsDRAP1* (Huang *et al.* 2018), and *OsbHLH59* (Ning *et al.* 2025), which confer DT by modulating physiological responses related to water balance and redox homeostasis. Collectively, these findings provide a coherent molecular and evolutionary explanation for the pronounced physiological and phenotypic divergence in DT between XI and GJ rice. This first systematic analysis of DT mechanisms in the two subspecies offers valuable insights for subspecies-targeted breeding of drought-tolerant rice.

Understanding the physiological and genetic mechanisms underlying DT in rice is essential for developing climate-

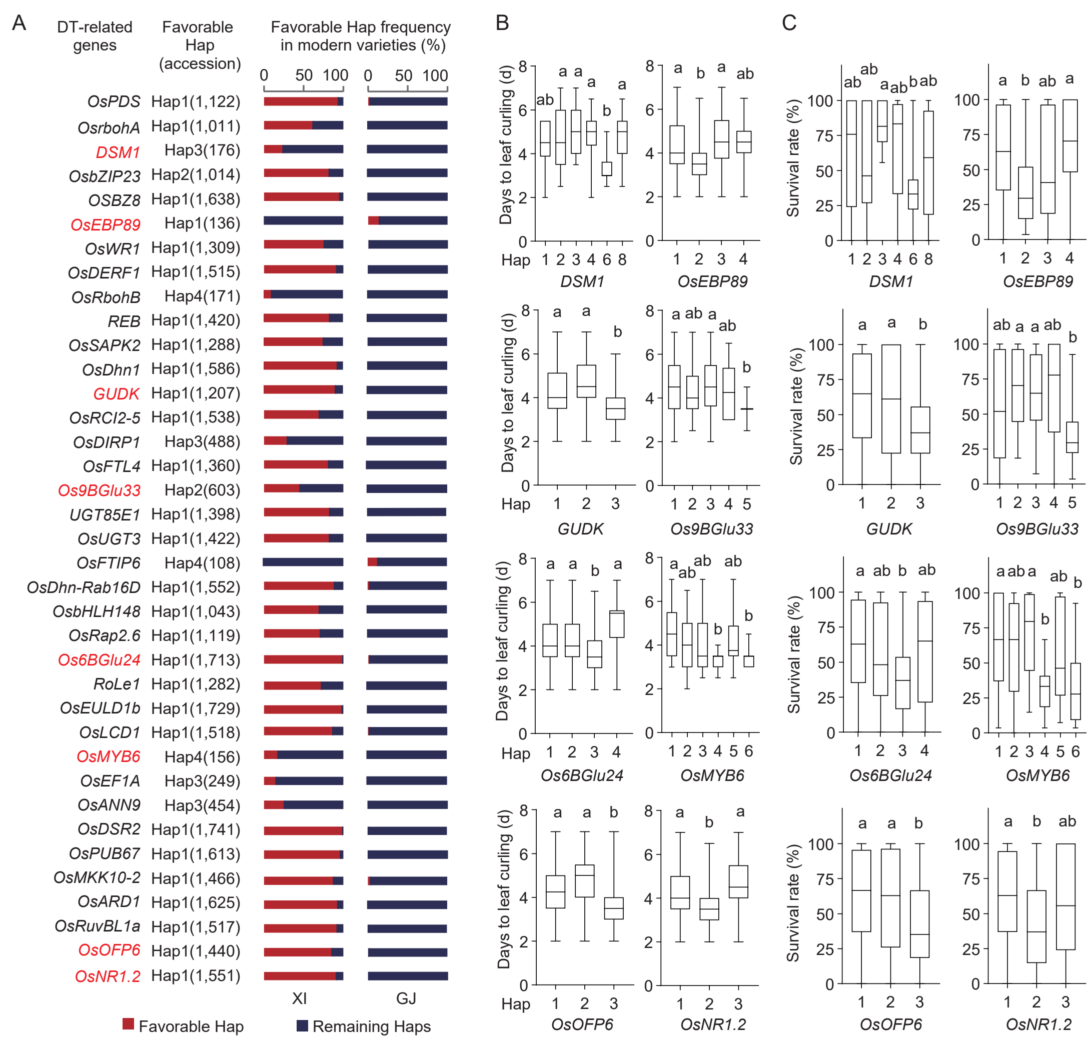

Fig. 2 Haplotype (Hap) analysis of drought tolerance (DT)-related genes. A, frequencies of the favorable Haps of 37 key DT-related genes affecting survival rate in *xian*/*indica* (XI) and *geng*/*japonica* (GJ) rice. B and C, haplotypes of eight DT-related genes and their effects on days to leaf rolling (B) and survival rate (C). The favorable Hap of each gene is defined as the Hap with the highest survival rate. Accessions indicate the number of rice accessions carrying the favorable haplotype in 3KRG. Eight DT-related genes are highlighted in red. Horizontal line inside the box indicates the median; upper and lower lines indicate the maximum and minimum values, respectively. Different letters on the boxplots indicate statistically significant differences at $P<0.05$ based on Tukey's multiple comparisons test.

resilient cultivars, especially in the face of increasingly frequent co-occurring abiotic stresses such as drought and heat. The superior drought and heat tolerance observed in XI rice may be partly attributed to its long-term adaptation to tropical and subtropical environments, where drought and heat stress often co-occur, as water deficits are frequently accompanied by low soil and air humidity and elevated temperatures (Lipiec *et al.* 2013). Such environmental pressures likely exerted strong natural and artificial selection for XI genotypes capable of maintaining water balance, stabilizing cellular homeostasis, and sustaining photosynthetic capacity under combined stress conditions.

Co-occurring stressors are one of the main reasons why stress-resistant crops remain a scientific promise rather than a farming reality (Kong *et al.* 2025). Therefore, targeting pleiotropic genes to develop crops with multiple stress tolerances through multi-gene engineering represents a promising strategy. Several differentiated genes have been identified as responsive to both drought and heat stress, including *SNAC3* (Fang *et al.* 2015), *OsRab7* (El-Esawi and Alayafi 2019), and *OsWRKY11* (Wu *et al.* 2009). From a breeding perspective, identifying shared genetic components that confer tolerance to both drought and heat stress offers a valuable strategic advantage. Introgression of key loci from XI into elite GJ backgrounds could accelerate the development of climate-resilient cultivars. Given the increasing frequency of combined drought and heat events under climate change, integrating such genetic and molecular insights into rice breeding programs is crucial for sustaining stable productivity.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, the authors used ChatGPT 4.0 and ChatGPT 5.3 to assist in improving the clarity and readability of the language. All content generated was carefully reviewed and revised by the authors as necessary, and the authors take full responsibility for the final content of the article.

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