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IDENTIFICATION OF RICE (*ORYZA SATIVA* L.) MUTANT WITH INCREASED SENSITIVITY TO THE PHYTOTOXIC METALLOID GERMANIUM

T.H. TAI[1*], S.C. MAGEE[1], K.C. SHIM[2], H.J. KIM[3], and A.V. LIEBSCHER[1]

[1]Crops Pathology and Genetics Research Unit, USDA-ARS, Davis, CA, USA
[2]Department of Crop Science, Chungnam National University, Republic of Korea
[3]Department of Plant Sciences, University of California, Davis, USA
*Corresponding author's email: thomas.tai@usda.gov
Email addresses of co-authors: sarah.magee@driscolls.com, kcshim@cnu.ac.kr, mmmss00@gmail.com, anna94gutierrez@gmail.com

SUMMARY

Germanium (Ge) is a metalloid with chemical properties similar to silicon (Si). In Si accumulators like rice (*Oryza sativa* L.), micromolar concentrations of Ge can result in necrotic lesions and chlorosis. Ge phytotoxicity has been employed to great effect to identify rice mutants defective in silicon (Si) uptake, leading to the cloning of the first Si transporters in plants. A population of M_4 mutants derived from chemical mutagenesis of the temperate japonica Kitaake underwent Ge screening to identify mutants with more rapid development of Ge-induced lesions than the wild type. From 30 mutant lines identified in the preliminary screening, the line designated KDS-557B emerged as hypersensitive, exhibiting a more rapid development of necrotic lesions even at 5 μM GeO_2, a 10-fold lower concentration than the normal used for screening. An analysis of mapping populations from crosses of KDS-557B with Kitaake ($\chi2 = 2.63$, df = 1, P = 0.105, not significant at $P \leq 0.01$) and a tropical japonica variety, Sabine ($\chi2 = 0.04$, df = 1, P = 0.841, not significant at $P \leq 0.01$) supported a single gene recessive mutation model. Further characterization of KDS-557B will contribute to understanding the interaction of metalloid elements and plants.

Keywords: Rice (*O. sativa* L.), induced mutations, germanium, toxicity, hypersensitivity

Key findings: Identification of a rice (*O. sativa* L.) mutant exhibiting hypersensitivity to the phytotoxic metalloid germanium was successful. This mutant, which exhibits a single gene recessive mode of inheritance, and its further characterization will contribute to advancing our understanding of the interaction of crop plants with metalloid elements in the environment.

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INTRODUCTION

The metalloid element germanium (Ge), closely related to silicon (Si), exhibits very similar chemical properties that largely govern its interactions in soil-plant systems (Wiche *et al.,* 2018). However, Ge is more chemically reactive than Si and can also be toxic to plants at even moderate levels (Takahashi *et al.,* 1976; Puerner *et al.,* 1990; Halperin *et al.,* 1995), unlike Si, which, in general, is beneficial to plant growth and development (Ma and Yamaji, 2006; Coskun *et al.,* 2019). In Si accumulators, such as rice (*Oryza sativa* L.), Ge can cause toxic symptoms, i.e., the formation of necrotic lesions even at low concentrations (Matsumoto *et al.,* 1975; Takahashi *et al.,* 1976). This attribute has succeeded in its employment to screen for rice mutants defective in the uptake of Si (Ma *et al.,* 2001), leading to the identification of the first Si transporters in plants (Ma *et al.,* 2006, 2007).

Takahashi *et al.* (1976) were the first to examine the uptake of Ge and Si across several plant species and determine that rice roots were unable to distinguish between the two elements in hydroponic culture (Ma *et al.,* 2001a). Species with high Si concentrations typically have correspondingly high Ge and, in particular, grasses, such as rice, have tenfold greater Si and Ge content than herbaceous plants (Ma *et al.,* 2001b; Wiche *et al.,* 2018). Differences in Ge uptake result from active and passive uptake mechanisms, as well as the availability of Ge (Wiche *et al.,* 2018). Pioneering work on the identification of Si transporters in rice using the attributes of Ge (Ma *et al.,* 2001a) to screen for Si/Ge uptake defective mutants (Ma *et al.,* 2002, 2006, 2007) has resulted in the identification of several genes in rice that encode transporters (Ma and Yamaji 2006; Yamaji *et al.,* 2015). This work has been extended to other grass species (Chiba *et al.,* 2009; Mitani *et al.,* 2009; Montpetit *et al.,* 2012) and some Si-accumulating dicots (Desmukh *et al.,* 2013; Sun *et al.,* 2017), although most dicot species lack an active uptake mechanism for Si/Ge (Ma and Yamaji, 2006).

While significant progress has occurred regarding the active uptake of Ge through its association with Si, relatively few reports have come out on the mechanism of Ge toxicity in plants. This is likely due to low concentrations in the Earth’s crust (1.3–1.6 µg g^{-1}; Wiche *et al.,* 2018) that make it unlikely for plants to encounter levels high enough to elicit toxic effects (Halperin *et al.,* 1995). However, in Ge-rich soils, such as those in mining areas, concentrations of Ge are in the range that could have toxic effects on Si accumulators like rice (Matsumoto *et al.,* 1975) and barley (Halperin *et al.,* 1995). Understanding the mechanism of uptake, as well as tolerance to the toxic effects of Ge, is important to the practicality of phytoaccumulation or phytomining as an economical means of concentrating this essential industrial metalloid (Halperin *et al.,* 1995; Wiche and Heilmeier, 2016).

Few genetic studies have emerged regarding germanium toxicity in plants. Hayes *et al.* (2013) reported the mapping of two quantitative trait loci (QTL) associated with Ge toxicity in barley. More recently, Talukdar *et al.* (2015) employed genome-wide association mapping and recombinant inbred mapping to characterize natural variations in a rice diversity panel and identify several QTL associated with sensitivity to Ge. Some of these loci contain homologs of the *Lsi1* and *Lsi2* genes, but neither of those genes nor *Lsi6* (another Si transporter) were evident.

This study reports the identification of a novel mutant exhibiting a “hypersensitivity” to Ge from a sodium azide-mutagenized population of the Japanese temperate japonica rice variety, Kitaake. The more rapid development of Ge-induced lesions in this mutant, designated KDS-557B, was also visible at a 10-fold lower concentration than the normal used for the initial screen that detected it. Genetic analysis of KDS-557B supports a single gene recessive mutation mode of inheritance for the Ge hypersensitive phenotype. Identifying the underlying gene will contribute to our understanding of plant-metalloid interactions in the environment.

MATERIALS AND METHODS

Plant materials and germanium screening

Preliminary screening of a sodium azide-mutagenized population (M_4 and M_5 lines; n = 879) developed from the temperate japonica rice (*O. sativa* L.) cultivar, Kitaake (Monson-Miller *et al.,* 2012), with 50 µM Ge (GeO_2; germanium [IV] oxide; Ma *et al.,* 2001a), revealed about 30 non-segregating lines that showed greater sensitivity to Ge toxicity by producing more rapid or stronger symptoms than wild-type Kitaake. KDS-557B exhibited the strongest response to Ge (detailed in Results) from these lines and thus became a selection for further characterization using the remaining seed.

Ge dose response of KDS-557B and wild-type cultivars

The KDS-557B mutant identification as hypersensitive to Ge succeeded at a concentration of 50 µM Ge based on the timing of the appearance of Ge toxicity symptoms (i.e., necrotic lesions on leaf sheaths and blades) relative to wild-type Kitaake. The conduct of a dose-response experiment determined the Ge sensitivity of KDS-557B and three wild type varieties (Kitaake, Nipponbare, and Sabine), which have served to generate various mutant populations in the laboratory (Till *et al.,* 2007; Monson-Miller *et al.,* 2012; Tai, 2015). Placing single 3-leaf stage seedlings of each genotype proceeded in a magenta box containing 350 ml of 0.5X Kimura B with Ge at five different concentrations (0, 5, 15, 50, and 150 µM). Each treatment had four replications (a total of four seedlings, one seedling per replicate), with the seedlings visually evaluated every 24 hours for the appearance of Ge-induced necrotic lesions.

Genetic crosses and phenotyping of F_2 populations

Genetic crosses were initially performed between KDS-557B and the U.S. tropical japonica variety, Sabine, used the KDS-557B as the female parent. Additionally, reciprocal crosses between KDS-557B and the wild-type progenitor variety Kitaake were made. F_1 plants were selfed to produce F_2 seeds for genetic analysis of the KDS-557B mutant phenotype.

Phenotyping of the KDS-557B/Sabine F_2 population, as initially conducted, used a solution culture, as described above, except using 1X instead of 0.5X Kimura B. A few F_2 (n = 45) underwent the Ge treatment along with the parents KDS-557B and Sabine. Seeds entailed placement in inlet hose washers (1.905-cm diameter) with stainless steel-cone filters that were placed in 24-cell culture plates with deionized water and underwent germination in a 28 °C incubator for a few days. Afterward, their transfer to a large plastic tray in the greenhouse continued with 12 L of 1X Kimura B for 10 days, at which time the plants were at the 2–3 leaf stage, and changing the solution to 1X Kimura with 50 µM Ge. Seedlings entailed visual evaluation for the appearance of necrotic lesions every 24 hours for four days. Additional phenotyping proceeded with a second population of KDS-557B/Sabine F_2 plants (n = 132) using a modified Ge-treatment protocol. Briefly, F_2 seedlings and controls (KDS-557B, Sabine, and Kitaake) underwent germination and growing in UC Davis rice soil mix (80% sand, 20% sphagnum peat moss with oyster shell calcium at 2.5 lbs. per cubic yard and dolomite lime at 7.5 lbs. per cubic yard) in about 7.6-cm square pots (Kord Products, Toronto, Canada) for 2–3 weeks. For Ge treatment, plants/pot arrangement ensued in five 4 × 7 carrier trays and one column of seven pots in a plastic-lined basin, with 30 L of 10 µM Ge in water added to reach the level of the soil surface in the pots. Mixing an additional 25 L of 25 µM Ge occurred six days later, maintaining water level at the soil surface. Plants received qualitative evaluation for Ge sensitivity in comparison with the controls (five replicates per genotype) every few days, with the final evaluation performed three weeks after the initiation of Ge treatment.

F_1 seeds from reciprocal crosses between KDS-557B and Kitaake reached germination in petri dishes before transplanting after seven days to UC Davis rice soil mix in

8.89-cm square Kord pots and growing in the greenhouse. Approximately three weeks later, putting the F_1 plants (n = 6) for each cross ensued in two plastic trays along with one each of KDS-557B and wild-type Kitaake. Filling each tray with 2.5 L of 50 µM Ge in water followed, with the plants qualitatively evaluated for Ge sensitivity in comparison with the controls. After completing the assessment, removing plants from their pots continued with rinsing the soil from the roots before re-potting the plants in clean soil in about 14-cm square pots to produce self (F_2) seeds. Based on the phenotyping for Ge sensitivity of the F_1, germinating seeds from one KDS-557B/Kitaake F_1 succeeded (n = 193) in water in a 30 °C incubator. Meanwhile, the transfer of F_2 and parental (KDS-557B and Kitaake) seedlings to the greenhouse enabled their growth for about 10 days before being transferred to plug flats and placed in two trays containing six liters of 0.5X Kimura B per tray. After about one week, the 3-leaf seedlings received treatments with 50 µM Ge in 0.25X Kimura B and evaluation daily for the appearance of lesions. Segregation ratios for all F_2 populations sustained Pearson's $\chi 2$ test for goodness-of-fit to the single gene recessive mode of inheritance.

RESULTS

Initial evaluation of Ge response occurred on rice (*O. sativa* L.) accessions that were parents of various mapping and mutant populations generated in our research program. Among these accessions were Kitaake and a few Kitaake M_4 generation mutants (n = 25) derived by sodium azide seed mutagenesis, referred to as the 'KDS' mutant population (Monson-Miller *et al.,* 2012). Three-to-four leaf-stage rice seedlings treated with 50 µM Ge exhibited symptoms of Ge toxicity in the form of necrotic lesions on leaf sheaths and blades in three to four days. Similar responses were evident for Kitaake and the KDS mutants, except for the mutant line KDS-557B, which showed symptoms 1–2 days earlier than wild-type Kitaake (~2–3 days after initiation of Ge exposure) and appeared to be qualitatively different in the severity of the lesions over time (Figure 1). Based on this pilot screening, a larger-scale screen of 855 mutant lines (M_5 generation) took place. Preliminary screening revealed about 30 mutant lines that appeared to show greater sensitivity to Ge than wild-type Kitaake by producing more rapid or stronger symptoms (i.e., formation of lesions and eventual death), but none produced as pronounced a response as KDS-557B, which attained selection for further investigation.

Examining the Ge response of KDS-557B more closely led to treating seedlings of the mutant and three wild-type cultivars (Kitaake, Nipponbare, and Sabine) with five different concentrations of Ge (0, 5, 15, 50, and 150 µM) and visually assessing daily for the appearance of Ge-induced lesions (n = 4 for each genotype and treatment) (Figure 2). Under the assay conditions used, necrotic lesions emerged on the KDS-557B seedlings on day 3 in all treatments, except the control (0 µM). Lesions appeared on Kitaake and Nipponbare on day 3 for the 50 and 150 µM treatments, although these seedlings exhibited fewer than 15 lesions compared with too many lesions to count on the KDS-557B seedlings. At lower concentrations of 5 and 15 µM, lesions occurred on Kitaake by day 5 and day 4, respectively, with lesions observed on Nipponbare a day after Kitaake in each treatment. Of the wild-type cultivars observed, Sabine seemed the most tolerant, with no lesions appearing with the 5 µM Ge treatment over the 10 days of observing the seedlings, with lesions observed on day 6, day 4, and day 3 at 15, 50, and 150 µM, respectively. Meanwhile, lesions at the 150 µM Ge treatment were initially noticeable at the same time as the other wild types and KDS-557B (i.e., day 3); the number was < 5 lesions and only appeared on three of the four seedlings in contrast to Kitaake and Nipponbare, where all seedlings exhibited about double the number of lesions.

In examining the mode of inheritance of the KDS-557B Ge hypersensitive phenotype, initial genetic crosses took place with the tropical japonica cultivar Sabine. KDS-557B, when crossed, served as the female parent, with the resulting F_1 plants determined to be

Figure 1. Symptoms of Ge toxicity in rice seedlings. Three-to-four leaf-stage rice seedlings of wild-type Kitaake (left) and KDS-557B mutant (right) grown in hydroponic culture with 50 µM GeO_2. Seedlings shown are four days after initiation of the Ge treatment.

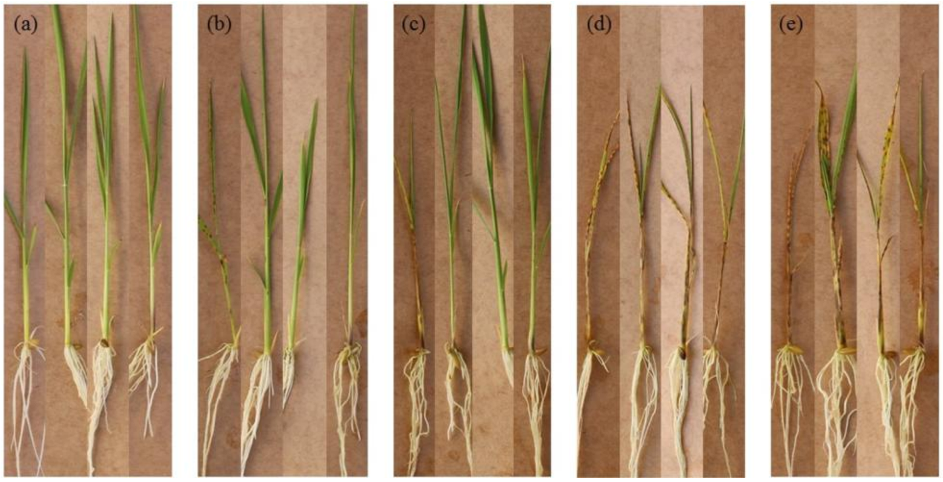

Figure 2. Response of KDS-557B and wild-type cultivars Kitaake, Nipponbare, and Sabine to different concentrations of Ge six days after initiation of treatment. (a) KDS-557B, Kitaake, Nipponbare, and Sabine (left to right) in 0 µM Ge, (b) 5 µM Ge, (c) 15 µM Ge, (d) 50 µM Ge, and (e) 150 µM Ge.

true hybrids based on differences in plant type, as well as inferior fertility. Initially, a small set (n = 45) of F_2 seedlings underwent phenotyping in solution culture with 50 µM Ge, and 14 of the F_2 appeared to develop lesions more rapidly (i.e., 2 days post initiation of Ge treatment). The observed segregation ratio was consistent with a single gene recessive mutation conferring the Ge hypersensitivity in KDS-557B (χ2 = 0.896, df = 1, P = 0.344, not significant at $P \leq 0.01$). During the phenotyping of the F_2, it was notable that many of the F_2 had developed lesions on day 3, suggesting that phenotyping a larger mapping population under these conditions could be difficult.

Based on the response of KDS-557B and wild-type cultivars to different concentrations of Ge and indications that plants grown in soil amended with Ge could support phenotyping (Ma *et al.,* 2007; T. Tai, unpublished observations, and this study), the conduct of a second phenotyping experiment used a larger KDS-557B/Sabine F_2 population (n = 132) grown in soil and then treated with lower concentrations of Ge (10 µM, followed by 25 µM Ge). A total of 32 F_2 received scores as having strong symptoms (i.e., size, number, and distribution of necrotic lesions) similar to the KDS-557B controls. From the remaining 100 F_2, reduced Ge toxicity symptoms (i.e., fewer lesions, mostly on leaf sheaths) were visible in several F_2 (≥ 11), but most plants seemed to be non-Ge hypersensitive as they had no obvious Ge-induced lesions. Considering the Ge hypersensitive phenotype, the segregation ratio was in agreement with a single gene recessive mutation model ($\chi2$ = 0.04, df = 1, P = 0.841, not significant at $P \leq 0.01$). F_2 individuals exhibiting reduced Ge toxicity symptoms (i.e., less severe than KDS-557B parent) in this KDS-557B/Sabine population were consistent with the differential response of KDS-557B, Kitaake, and Sabine to varying concentrations of Ge. In other words, these F_{2s} may be an indication of other loci affecting Ge toxicity in rice based on reports by Talukdar *et al.* (2015).

As the generation of the KDS-557B/Sabine F_2 populations occurred before the observation of the differential response of Kitaake and Sabine to Ge, reciprocal crosses were made between KDS-557B and Kitaake to develop additional mapping populations and begin developing a mutant line free of background mutations that may confound more detailed characterization of the effect of the KDS-557B mutation. In confirming true F_1 individuals, six putative F_1 seedlings (2–3 leaves) of the KDS-557B/Kitaake cross succeeded in their transplanting into soil and growing for about two weeks before transferring the pots to trays containing 50 µM Ge in water. In addition to the KDS-557B control, lesions were noteworthy on one of the six putative F_1 plants three days after initiation of the treatment, with no lesions on the Kitaake control. This result indicated the Ge-hypersensitivity mutations are recessive, and five plants were true F_1 progeny, with one plant being an accidental selfed progeny, KDS-557B, as it was as Ge-sensitive as the KDS-557B parent. The wild-type response of the F_1 was consistent with the mutant phenotype being recessive. Phenotyping of F_2 (n = 193) of one of the KDS-557B/Kitaake populations proceeded in solution culture with 50 µM Ge, scoring 135 as wild type, and 58 exhibited a KDS-557B mutant response, a segregation ratio consistent with previous results ($\chi2$ = 2.63, df = 1, P = 0.105, not significant at $P \leq 0.01$).

DISCUSSION

Germanium (Ge) is a metalloid element sharing similar chemical properties with boron (B), Si, and arsenic (As) (Hayes *et al.,* 2013; Wiche *et al.,* 2018). An important raw material for the manufacture of electronics, semiconductors, and other high-tech devices, Ge exists in the Earth's crust but at relatively low concentrations (Halperin *et al.,* 1995; Wiche *et al.,* 2018) and rarely in the form of deposits amenable to mining operations (Wiche and Heilmeier, 2016). As a result, an economical approach to concentrating Ge would greatly help to meet the rapidly growing demand for this valuable trace metal. One such approach is phytomining, which compares favorably to other bioaccumulators (Halperin *et al.,* 1995). Among plant species, grasses have some of the highest concentrations of Ge owing to the active Si transport systems, which also accommodate Ge (Ma and Yamaji, 2006). Given that Ge concentrations in the natural environment are 100-fold less than those inducing symptoms of toxicity in grasses, such as barley (Halperin *et al.,* 1995) and rice (Matusmoto *et al.,* 1975; Takahashi *et al.,* 1976; Ma *et al.,* 2001a), more research is vital to determine if Si/Ge accumulators could be effectively applicable for phytomining of Ge.

This study employed a forward genetics approach to identify putative rice (*O. sativa* L.) mutants with altered sensitivity to Ge using a concentration (50 µM GeO_2), previously reported for the identification of rice mutants defective in Si uptake (Ma *et al.,* 2001a, 2006, 2007). As previous studies focused on identifying mutants with enhanced Ge tolerance to find Si uptake mutants, we hypothesized that mutants with greater sensitivity to Ge could be identified. As a result, the identification of several putative mutants succeeded, the most striking of which was KDS-557B, a Ge hypersensitive mutant exhibiting a more rapid and stronger response than the wild type.

A comparison of the qualitative response of KDS-557B and three wild-type cultivars (Kitaake, Nipponbare, and Sabine) revealed the timing of the appearance of toxicity symptoms in the KDS-557B mutant was the same over the range of 5 to 150 µM GeO_2. Meanwhile, the wild types exhibited delayed symptoms at lower concentrations. Interestingly, the evaluation of the wild types at different treatment concentrations also showed natural variation concerning Ge sensitivity, suggesting that the tropical japonica cultivar Sabine is more tolerant than the temperate japonicas, Kitaake and Nipponbare. Such natural variation is not surprising, as reported by Talukdar *et al.* (2015).

Genetic analysis of the KDS-557B disclosed the Ge hypersensitive phenotype appears to be controlled by a single gene recessive mutation. The analysis of F_2 from the KDS-557B/Sabine cross revealed phenotypes differing from the parents, consistent with an earlier observation that there appears to be natural variation in Ge sensitivity between Sabine and Kitaake. The KDS-557B/Kitaake F_2 mapping population should be amenable to mutation mapping and discovery approaches (Abe *et al.,* 2012). A continued backcrossing of individuals exhibiting the Ge hypersensitive phenotype will eliminate spurious background mutations, resulting in "clean" lines for more detailed phenotypic studies of Ge uptake, accumulation, and sensitivity resulting from the KDS-557B mutation.

Determination of the Ge and Si content of KDS-557B is underway to provide preliminary evidence for altered uptake or post-uptake metabolism of Ge as the basis for the hypersensitivity observed. Ultimately, identification of the gene underlying the KDS-557B phenotype will be essential in substantiating which mechanism is responsible.

CONCLUSIONS

A forward genetics approach employed by this study identified rice (*O. sativa* L.) mutants with increased sensitivity to the metalloid element Ge. The mutant KDS-557B exhibited a significantly faster and stronger phytotoxic response than the wild type and other mutants identified in this screen. Genetic analysis indicates the hypersensitivity of KDS-557B has the control of a single gene recessive mutation. Further characterization of this mutant will contribute to our understanding of Ge phytotoxicity and the interaction of metalloids and plants.

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