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Research article

Induction of rice mutations by high hydrostatic pressure

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ABSTRACT

High hydrostatic pressure (HHP) is an extreme thermo-physical factor that affects the synthesis of DNA, RNA and proteins and induces mutagenesis in microorganisms. Our previous studies showed that exposure to 25–100 MPa HHP for 12 h retarded the germination and affected the viability of rice (*Oryza sativa* L.) seeds, increased the tolerance of rice plants to cold stress and altered gene expression patterns in germinating rice seeds. However, the mutagenic effect of HHP on rice remains unknown. In this study, exposure to 25, 50, 75 or 100 MPa for 12 h HHP could efficiently induce variation in rice plants. Furthermore, presoaking time and HHP strength during HHP treatment affected the efficiency of mutation. In addition, the Comet assay revealed that exposure to 25–100 MPa HHP for 12 h induced DNA strand breakage in germinating seeds and may have been the source of mutations. Our results suggest that HHP is a promising physical mutagen in rice breeding.

1. Introduction

High hydrostatic pressure (HHP) is an extreme thermo-physical factor that affects multiple cellular processes such as protein synthesis, cell survival and the morphology of microorganisms [1–3]. In some mesophilic microorganisms, cell growth was completely inhibited at about 50 MPa of continuous HHP [4]. In living *Escherichia coli* cells, the synthesis of DNA, RNA and protein was inhibited by 50, 58 and 77 MPa of continuous HHP [5,6]. Furthermore, the gene expression patterns of HeLa S3, pheochromocytoma, human chondrosarcoma and astrocyte cells were affected by exposure to 41.4 MPa for 15 min, 41.4 and 68.9 MPa for 20 min, 5 and 30 MPa for 3, 6 and 12 h, and 0.08 MPa HHP for 6, 24, and 48 HHP, respectively [7–10]. In *Saccharomyces cerevisiae*, many genes involved in defense response, membrane metabolism and protein degradation were up-regulated after exposure to 200 MPa HHP for 30 min [11,12]. HHP greater than 100 MPa for 10 min induced cytoplasmic petite, tetraploid or homozygous diploid forms of mutations to *S. cerevisiae* cells [13] while 96 MPa HHP treatment for 4 h induced the petite mutation in *S. cerevisiae* cells [14,15]. Furthermore, a series of piezo-tolerant and heat-resistant *E. coli* strains were isolated after treatment with 220–700 MPa HHP for 15 min [16]. More recently, our group investigated the effect of 25–100 MPa HHP for 4–12 h on rice (*Oryza sativa* L. cv. 'Yuefengzhan'). After adding pressure, rice seed germination was delayed and seedling growth was retarded [17]. Exposure to 25, 50, 75 and 100 MPa HHP for 12 h also decreased the viability of rice seeds [18]. Some rice plants that survived showed greater resistance (the plants showed higher chlorophyll content and photosynthetic efficiency than the control) to cold stress (5 °C) after seeds were exposed to 25–100 MPa HHP for 2–12 h [19]. Transcriptional analysis revealed that exposure to 75 MPa HHP for 12 h altered the expression patterns of genes involved in metabolism, defense response, signal transduction and transcriptional regulation in germinating rice seeds [18]. In addition, HHP induced the heritable alteration of DNA methylation patterns in miniature inverted repeat transposable elements, *mPing* and *Pong*, and activated their mobilization in rice plants [20,21]. These studies clearly indicate that HHP affects growth and development processes in rice. Despite this, the mutagenic effect of HHP on rice remains unknown.

Abbreviations: DMN, dimethylnitrosamine; HHP, high hydrostatic pressure; SDW, sterile distilled water; LMA, low-melting agarose; NMA, normal-melting agarose; ENU, ethylnitrosourea; UV, ultraviolet.

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In the present study, our key objective was to ascertain the mutagenic effect of HHP on rice seeds and subsequent plant growth and development by testing pre-soaking period and HHP strength. We were also interested in discovering whether such mutations could be stably inherited to the M_2 generation. Finally, using the Comet assay, we wanted to observe whether there was any damage to DNA.

2. Results

2.1. HHP induces inheritable mutagenesis in rice

After separate batches of rice seeds were presoaked in sterile distilled water (SDW) for 12 h and then treated with 25, 50, 75 or 100 MPa HHP for 12 h in a pressure valve (Fig. 1A, B), the seeds from different pressure treatments were mixed and planted. Seeds exposed to normal atmospheric pressure conditions were used as the control. Seeds were harvested on July, 2007 and planted to generate M_2. Finally, the phenotypes of the plants were characterized throughout their lifespan. A large subset of mutants was discovered, including super tillering, internode elongation, dwarf, early flowering, long awns, long leaf, big spike, purple leaf sheath, brown husk, among others (Fig. 2A–K). These mutants remained stable in the offspring. Our findings suggest that exposure to HHP could induce inheritable mutagenesis in rice.

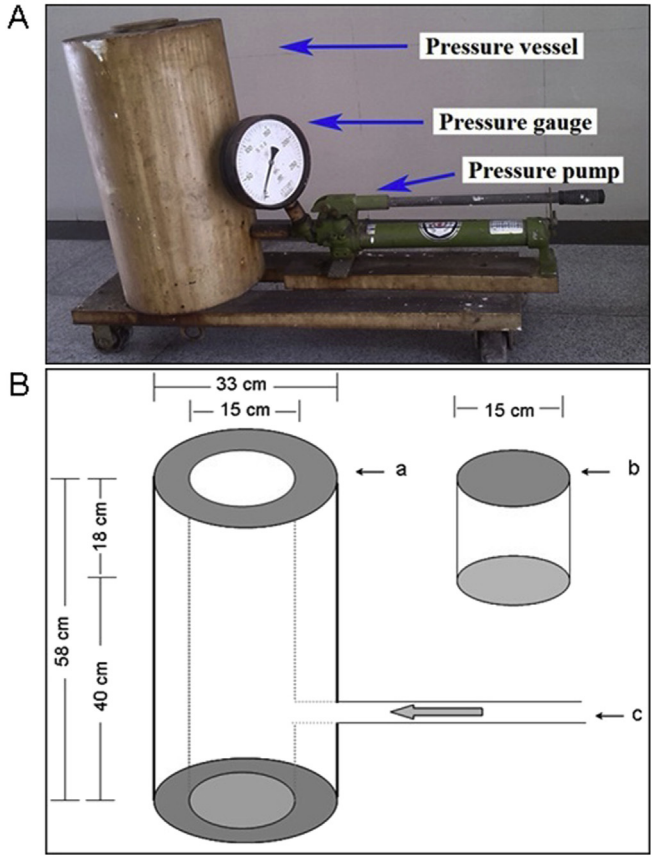

Fig. 1. High hydrostatic pressure (HHP) equipment used for inducing rice mutants. (A) The HHP equipment. The pressure vessel, gauge and pump are indicated. (B) Schematic structure of the pressure vessel. a is the pressure reactor, b indicates the cap, and c is the passage. Procedures of HHP treatment: 200 g of rice seeds are packaged and placed into the reactor, which is then filled with SDW (about 7×10^{-3} L). After closing the cap, the pressure pump pushes water into the reactor through the passage, and the pressure is indicated by the pressure gauge. A batch of $1–3 \times 10^{-3}$ L seeds can usually be treated at once.

2.2. Presoaking time affects mutation efficiency of HHP in rice

Presoaking is important for rice seed germination. Rice seeds show different physiological states following various periods of soaking. The effect of presoaking time on induction efficiency was studied. Since 1000 seeds were planted per treatment, the mutation level was calculated by the number of mutants divided by 1000. Only dwarf mutants (0.4% mutation level) were obtained after soaking for 4 h (Table 1). In the group soaked for 20 h, four types of mutants, including long stem, thin stem, long awns and a brown husk (0.9% mutation level) were scored (Table 1). Six mutant types (1.3% mutation level) were obtained after soaking for 36 h and the characteristics of these mutants included dwarfing, reduced tillers, high position of the tiller, long awns, brown husk, short-rounded grains and late maturation (Table 1). This data set demonstrates that a longer presoaking period could increase the induction efficiency of HHP in rice.

2.3. Strength of HHP pressure influences mutation induction efficiency in rice

As strength is a key factor in HHP treatment, the effect of HHP intensity on the mutant induction efficiency was studied. Only dwarf mutants (0.4% mutation level) were observed after exposure to 25 MPa HHP for 12 h. When seeds were treated with 50 MPa HHP for 12 h, four mutant types (0.9% mutation level) could be identified (Table 2). In the 75 MPa-treated group, eight mutant types (1.3% mutation level) were indentified, the phenotypes of mutants including dwarf, long stem, few tillers, high position of tillers, incomplete panicle exertion, late maturation, long awns and a brown husk (Table 2). Thus, the level and type of mutation increase as HHP pressure increases.

2.4. HHP exposure induces damage to DNA

After observing that HHP can induce mutations in rice, the integrity of DNA in rice seeds following exposure to HHP was verified. The Comet assay (alkaline single cell gel electrophoresis) is a highly sensitive method for detecting DNA damage in single cells [22]. Damaged cells show “tail” shapes on a microscope after electrophoresis, and the tail length is measured to evaluate the level of DNA damage. In the present investigation, the Comet assay was performed to detect the integrity of DNA and to evaluate DNA repair capacity in germinating rice seed embryos after exposure to HHP. DNA repair capacity was determined by examining how long the majority of HHP-induced DNA breaks could be repaired under normal pressure conditions.

Significant pressure-dependent DNA breakage was observed with increasing pressure (Fig. 3A, B). After exposure to HHP intensity of 0.1 (control), 25, 50, 75 and 100 MPa for 12 h, the average medium tail length (value $\pm$ SE) of single cells in seed embryos increased significantly from 8.91 $\pm$ 0.19 pix in the control to 30.28 $\pm$ 0.85 pix in the 100 MPa treatment ($P < 0.001$) (Fig. 3A). The level of DNA damage in HHP-treated groups of all pressures was significantly higher than that of the control group ($P < 0.05$). This indicates that exposure to HHP induced DNA damage in germinating rice seeds.

To evaluate the DNA damage repair capacity in germinating rice seeds after exposure to HHP, the kinetics of damaged DNA repair caused by 100 MPa HHP was studied. Immediately after exposure to HHP, damage to DNA was tested (Fig. 3C). After 8 h of incubation, the average tail length decreased significantly from 30.28 $\pm$ 0.85 to 14.85 $\pm$ 0.38 pix. However, after incubation for 16 h–40 h, the damage to DNA was not significantly ($P < 0.05$) different (with respect to tail length) than the control (6.90 $\pm$ 0.78 pix) (Fig. 3C),