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Screening and Genetic Analysis of Ethylene-Response Mutants in Etiolated Rice Seedlings

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[Abstract] Ethylene, the simplest gaseous plant phytohormone, is involved in the control of rice growth and development processes, but the mechanism of ethylene regulating these pathways remains unclear in rice. Recent studies have found that ethylene-signaling pathway is conserved but different between rice and *Arabidopsis*. The forward genetic analysis is an essential and efficient method to reveal fully the mechanism of ethylene signaling in rice plants. Here we provide a protocol of genetic analysis of rice ethylene-response mutant, including screening ethylene-response mutants, treatment with ethylene and a chemical reagent, and ethylene-responsiveness gene expression analysis.

Keywords: Rice, Ethylene response, Coleoptile, Root, Etiolated seedlings

[Background] The key molecular elements of ethylene-signaling pathway have been identified by molecular genetics and genomic approaches in *Arabidopsis* (Guo and Ecker, 2004). Ethylene plays important roles in rice growth, development, and environmental adaptation, including coleoptile and shoot elongation, aerenchyma development, and submergence response (Xu *et al.*, 2006; Fukao and Bailey-Serres, 2008; Ma *et al.*, 2010). Rice may have a distinct mechanism of ethylene-signaling pathway due to the different ethylene-regulated biological processes in rice and *Arabidopsis* (Yang *et al.*, 2015a). The forward genetic analysis is essential to reveal fully the mechanism of ethylene signaling pathway in rice. So, it is prerequisite to develop an efficient method to screen ethylene-response mutants in rice. Genetic screens that are based on the triple-response have been extensively conducted on *Arabidopsis* (Johnson and Ecker, 1998; Stepanova and Ecker, 2000). However, the genetic screens in rice have been hamper owing to the lack of ethylene-response phenotypes.

Ethylene promotes rice coleoptile elongation of dark-grown seedlings (Ku *et al.*, 1970). In our experimental conditions, root elongation can be inhibited by ethylene in etiolated rice seedlings. Over the past decade, based on the double-response of etiolated rice seedling, including ethylene-induced coleoptile growth promotion and root growth inhibition, several unique rice mutants have been identified. We named these ethylene-response mutants *maohuzi* (*mhz*) (Ma *et al.*, 2013). Loss-of-function of *MHZ7*/*OsEIN2* rice plants display insensitive to ethylene, including ethylene-insensitive coleoptile elongation and root growth. Conversely, *MHZ7*/*OsEIN2*-overexpression transgenic lines display ethylene hypersensitivity (Ma *et al.*, 2013). Characterization of *MHZ6*/*OsEIL1* revealed that MHZ6/OsEIL1 and its homolog gene OsEIL2 have functional diversification in rice ethylene response (Yang *et al.*, 2015b). Identifications of *MHZ4*/*ABA4* (Ma *et al.*, 2014) and *MHZ5*/*CRTISO* (Yin *et al.*, 2015)

revealed that ethylene inhibits root growth requiring ABA pathway in rice, which is different with that in *Arabidopsis*. The study of MHZ2/SOR1 provides a candidate mechanism that auxin acts downstream to modulate ethylene inhibition of root growth in etiolated rice seedlings (Chen *et al.*, 2018). The research of MHZ3 found that ethylene-induced MHZ3 stabilizes MHZ7/OsEIN2 and impeding protein ubiquitination to facilitate ethylene signaling pathway (Ma *et al.*, 2018). Here, we describe the method in detail for genetic analysis of ethylene response mutants in etiolated rice seedlings. This protocol can also be used in other monocotyledonous plants to detect ethylene response (Yang *et al.*, 2015a). Besides, the longer mesocotyl and coleoptile mutant *gaoyao1*(*gy1*) was also discovered by this method (Xiong *et al.*, 2017).

Materials and Reagents

1. Syringes with needle: 2-, 5-, and 60-ml capacity
2. Petri dish, 60 mm (Corning, PYREX®, catalog number: 3160-60)
3. Airtight plastic and cups:
   a. 5.5 L box (Lock & Lock, catalog number: HPL 836) matched with the 12-lattice sieve
   b. 520 ml (Lock & Lock, catalog number: HPL 931N) cup matched with the wire mash
      Note: The capacity of the cup is 520 ml when covered with its lid.
   c. 20 L box (FUDOGIKEN, catalog number: 112025) matched with the 100-lattice sieve
      Note: There is a hole drilled into to the side of each box and fitted with a silicon rubber stopper; Cups with lid and silicon rubber stoppers (Figure 1).
4. Plant seeds: Wild-type, mutants, varieties or transgenic lines
5. Ethylene gas
6. 1-MCP (1-Methylcyclopropene) (LuNuo Bio-Technology, Maxfresh)

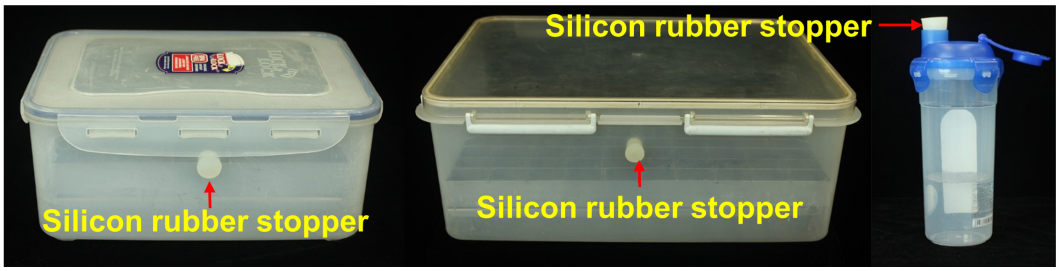

Figure 1. Airtight plastic boxes and cups. Each of the containers has a fitted silicon rubber stopper in order to inject ethylene gas.

Equipment

1. Beaker (BOMEX, 250 ml or 500 ml, depends on the number of seeds)
2. Dim green light source (ordinary commercial green light is OK)
3. Silicon rubber stopper (Laboran, catalog numbers: 9-860-03, 9-860-06, 9-860-09, 9-860-11 and

so on)

4. Stainless sieves (custom made) and wire mesh:
   a. 12-lattice sieve (240 mm length x 180 mm width x 33 mm height) for phenotype analysis
   b. 62 mm diameter wire mesh, and 100-lattice sieve (400 mm length x 300 mm width x 33 mm height) for mutant screening, positive seedlings selection and genetic mapping

   Note: Each sieve has four legs and each leg is 29 mm in height (Figure 2).
5. Graduated cylinders: 1,000-, 2,000-, and 4,000-ml capacity
6. Incubators for culturing rice seedlings set at 28 °C and 37 °C
7. Drying Oven
8. Watering can

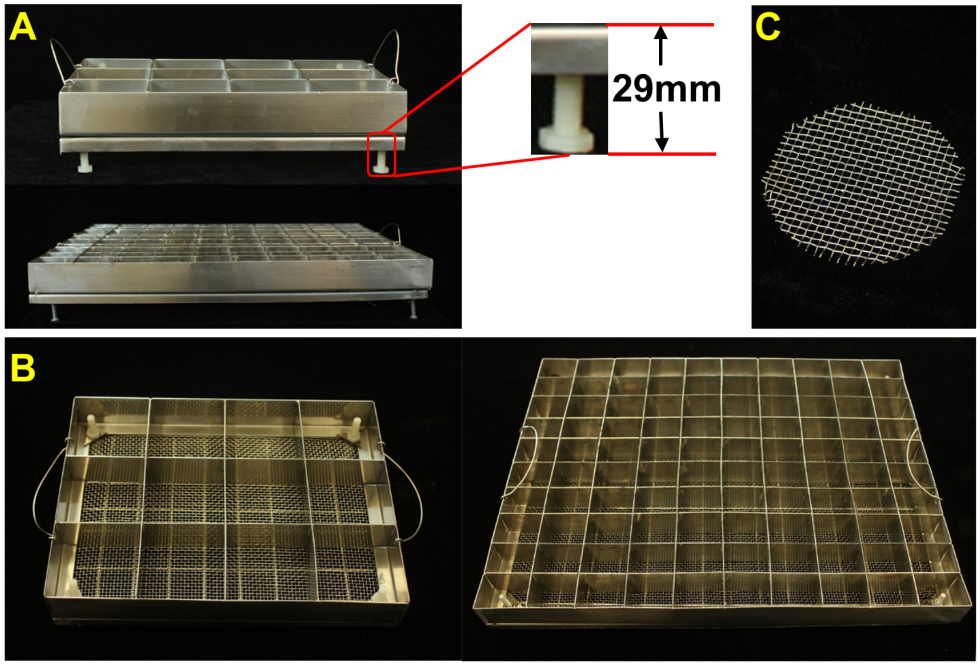

Figure 2. 12-lattice sieve, 100-lattice sieve, and wire mesh. A. Side view of the sieves. B. Top view of the sieves. C. 62 mm diameter wire mesh.

Procedure

A. Ethylene Treatment for etiolated rice seedlings
   1. Seed germination
      a. Place freshly harvested rice seeds (WT and/or mutant) in a drying oven at 40 °C for 5-10 days, then heat them at 50 °C for 48 h to break the dormancy of the seeds.

         Note: Old seeds that have been broken dormancy can be used directly.
      b. Select fully filled mature seeds with uniform size, 20-60 seeds are appropriate for each of lattice or 62 mm diameter wire mesh for genotype analysis.

         Note: Too many seeds will affect phenotypic analysis due to mechanical resistance.

c. Place the seeds into each lattice of the stainless sieve fitted to the 5.5-L or 20-L boxes. Add 2 L tap water into the 5.5-L box or 5.4 L into the 20-L box to soak the seed completely.
Place an appropriate amount of seeds into the beaker and add enough water to submerge the seeds when using the cups for phenotype analysis.
Note: Make sure all seeds submerged into the water. Seeds floating on the water surface are easily contaminated.

d. Place the boxes or beaker (without cover) in an incubator at 37 °C in the dark for 1.5-3 days until the white coleoptile length of most of the seeds reaches 1.5-3.0 mm. Change the tap water every day to avoid microbial infestations.

2. Ethylene Treatment

a. Withdraw the old water. Add 700 ml tap water into the 5.5-L box (Figure 3) or 1.9 L to the 20-L box, to make sure that the germinated seeds in each lattice are 15-16 mm above the water surface. Add 80 ml tap water with or without corresponding chemical reagent into the cups, so that the germinated seeds in each cup are 15-16 mm above the water surface. Then cover the boxes or cups with the airtight lids.
Note: This is the most important detail. Make sure the distance between germinated seeds and the water surface is 15-16 mm by adding the appropriate amount of water into the box or cup. So that maintain the moisture content of the atmosphere around the seeds and make sure the roots, especially the root tips, are completely exposed to ethylene.

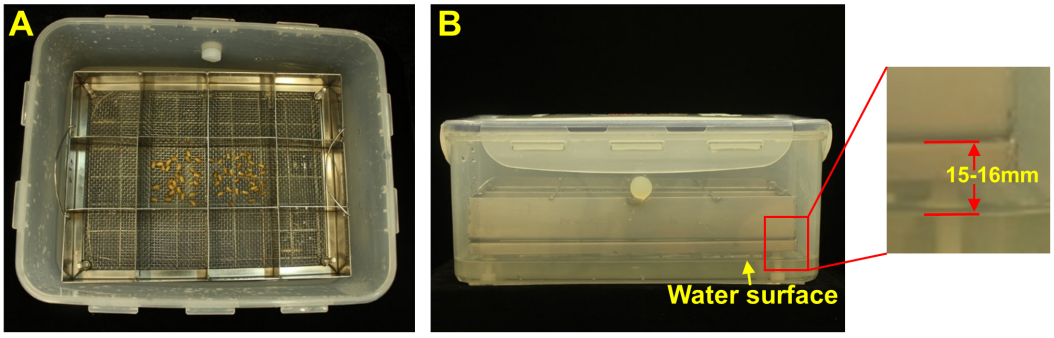

Figure 3. Phenotype analysis using 5.5-L box. A. Top view of the germinated seeds on the stainless sieve. B. Side view of the 5.5-L box with germinated seeds on the stainless sieve inside (In order to get a clear picture, these pictures were taken in daylight, but the processing needs to be done under dim green light source).

b. Inject the corresponding amount of ethylene gas into the boxes using an appropriate syringe. For the air control, open the box once every day to reduce the impact of endogenous ethylene.
Note: To reduce the effect of endogenous ethylene gas, mercuric perchlorate or activated carbon can be used.

c. Follow the steps below to obtain different concentrations of ethylene gas. First, remove 30

ml of air from a sealed 300-ml bottle with a 60-ml syringe, then inject 30 ml of ethylene into a 300-ml sealed bottle to obtain 10-fold diluted ethylene. Then inject 3 ml of 10-fold diluted ethylene into a sealed 300-ml bottle with a 5-ml syringe to get 1,000-fold diluted ethylene. Second, inject 3 ml of ethylene into a sealed 300-ml bottle with a 5-ml syringe to get 100-fold diluted ethylene, then inject 3 ml of 100-fold diluted ethylene into a sealed 300-ml bottle with a 5-ml syringe to get 10,000-fold diluted ethylene. According to the needs of the experimental design, inject the corresponding diluted ethylene into the corresponding volume of the sealed boxes or cups. For example, for ethylene treatment with 1 ppm (1 μl/L), inject 4.8 ml of 1,000-fold diluted ethylene into the 5.5-L box, 4.4 ml 1,000-fold diluted ethylene into the 520-ml cup with a 5-ml syringe or 1.9 ml 100-fold diluted ethylene into the 20-L box with a 2-ml syringe.

Note: Alternatively, a continuous-flow method may be used and it is more rigorous (Chen and Bleecker, 1995).

d. Culture the germinated seeds in an incubator at 28 °C in the dark for 2 days.

e. Withdraw the old water. Add 500 ml tap water into the 5.5-L box, 1,000 ml to the 20-L box or 65 ml tap water with or without corresponding chemical reagent into the 520-ml cups, so that the seeds are 19-20 mm above the water surface.

Note: The purpose is to maintain the entire roots are suspended in the air or ethylene by further reducing the content of water in the boxes or cups.

f. Then cover the boxes or cups with their lids, inject the appropriate amount of ethylene as in Step A2b, and culture the seedlings in the incubator at 28 °C in the dark for another 1-2 days.

Note: Culture the seedlings for another 1-2 days is suitable for mutant screening.

g. To keep the roots or shoots/coleoptiles of rice seedlings treated continuously with ethylene and other chemical reagents, such as ABA or inhibitor, the germinated seeds must be kept close to the water surface. Add 1.5 L water with or without chemical reagent into the boxes, 120 ml to the cups, so that the water surface reaches the germinated seeds. Then inject an appropriate amount of ethylene into the boxes or cups as in Step A2b. The other steps are the same as described above (refer to Ma *et al.*, 2014; Yin *et al.*, 2015).

Note: This improved method is very suitable for the use of expensive reagents or treating seedlings simultaneously with ethylene and other solution containing chemical reagents. Place 20-25 seeds on the wire mesh when using the cups to reduce the effects of endogenous ethylene.

3. Phenotype Analysis

Ethylene promotes coleoptile elongation but inhibits root growth of etiolated rice seedlings. Select mutant seedlings according to the morphology of adventitious roots, primary roots, and/or coleoptile (Figure 4). Measure the primary roots and/or coleoptiles length of rice seedlings at different concentrations of ethylene to quantitatively evaluate the unique ethylene-response.

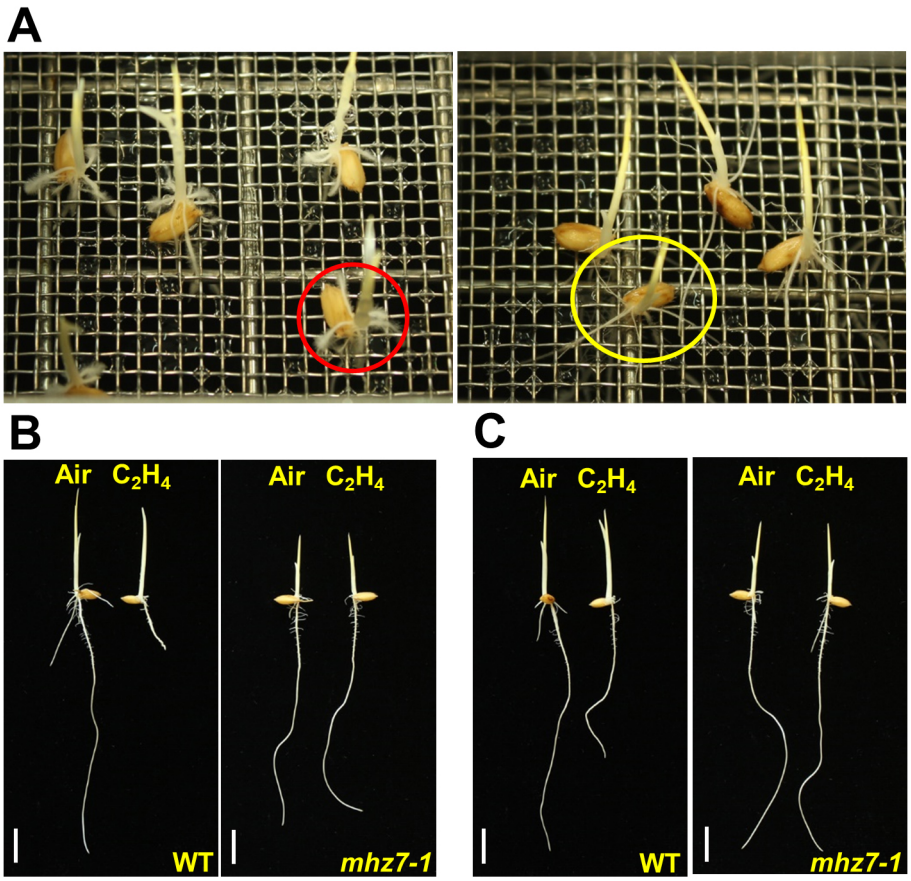

Figure 4. An example of a unique ethylene-response mutant phenotype of etiolated rice seedlings. A. Phenotypic comparison of wild-type and *mhz7-1*/*Osein2-1* in the sieve and the whole seedling. Ethylene inhibits the adventitious root growth of wild-type (red circle) but has no effect on the adventitious root growth in *mhz7-1*/*Osein2-1* (yellow cycle), which is an ethylene-insensitive mutant (Ma *et al.*, 2013). B and C. Phenotypic comparison of whole seedlings of wild-type and *mhz7-1*/*Osein2-1* that roots suspended in the air (B) or grown continuously in water (C), respectively. The root length of WT was inhibited 40-50% under the latter condition. *mhz7-1*/*Osein2-1* exhibits insensitivity in both roots and coleoptiles under two different conditions. Etiolated seedlings were treated with or without 10 ppm ethylene at 28 °C for 4 days (A) or 3 days (Figures 4B and 4C), respectively. Scale bars = 10 mm.

B. 1-MCP Treatment

1-MCP, the structure is similar with ethylene, is an extensively used competitive inhibitor. It can tightly bind to the ethylene receptors and hamper the ethylene perception and the ethylene-signaling pathway.

1. The protocol of seed germination is the same as described above.
2. After seed germination, withdraw the old water. Add 700 ml tap water into the 5.5-L boxes.
3. Place a small Petri dish with an appropriate amount of 1-MCP powder on the stainless sieve. Add a specific amount of water into the Petri dish to get 1-MCP with a final concentration of 5 ppm. For example, dissolving 1.555 mg 1-MCP powder (calculated according to the

manufacturer's instruction for releasing 5 ppm of 1-MCP) into 2 ml of water. Cover the box with its lid at once.

4. Culture the germinated seeds in an incubator at 28 °C for 3 or 4 days.
5. Investigate the phenotypes or the gene expression level as described above.

C. Ethylene Treatment for Investigating Gene Expression

1. Geminate seeds as described in Procedure A.
2. After seed germination, withdraw the old water, then add an appropriate amount of tap water to the boxes until the water surface reaches the sieve (Figure 5A).
3. Place the boxes without lids in an incubator and culture the germinated seeds at 28 °C for 2 or 2.5 days.
   Note: The boxes are open to avoid the effects of endogenous ethylene before treatment.
4. Withdraw the old water. To maintain the moisture inside of the boxes, add an appropriate amount of tap water that just cover the bottom of the boxes. Adjust the sieves to a higher position to make sure the roots are suspended fully and exposed to ethylene gas. Cover the boxes with their lids (Figures 5B and 5C).
   Note: Spray enough water to seeds and/or seedlings, side walls and lid of the boxes to maintain moisture inside. The purpose is to avoid drought damage.
5. Inject an appropriate amount of ethylene gas into the boxes using a syringe. Setting the corresponding air controls in parallel.
   Note: For ethylene-responsive gene expression analysis, each time point should have a control, especially for time-course treatment.
6. Culture the seedlings in the dark at 28 °C for an appropriate time according to the experimental design.
7. Harvest the ethylene-treated coleoptiles, shoots and/or roots. Then isolate the RNA for ethylene-responsiveness gene expression.
   Note: To avoid the effects of wound-induced ethylene, the tissues should be frozen in liquid nitrogen at once.

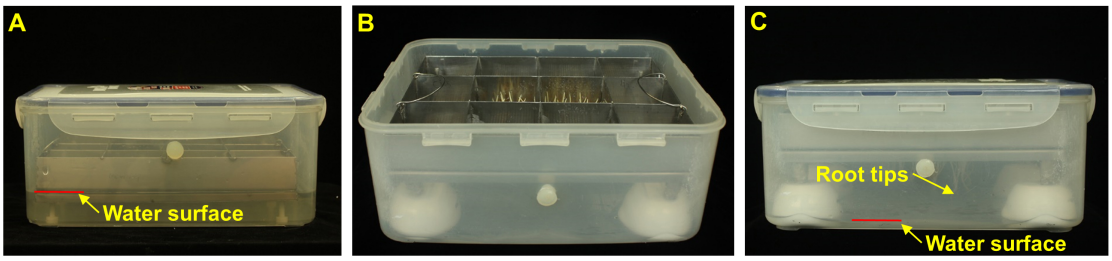

Figure 5. Gene expression analysis using 5.5-L box. A. Side view of the germinated seeds on the stainless sieve, the water surface was next to the germinated seeds for the 2 or 2.5 d seedlings culture. B. Top view of the 5.5-L box with 2 or 2.5 d seedlings on the stainless sieve inside; C. Side view of the 5.5-L box with ethylene treatment (In order to get a clear picture,

these pictures were taken in daylight, but the processing needs to be done under dim green light source).

Data analysis

To quantitatively evaluate the unique ethylene-response of each mutant, three biological replicates are needed. Measure the primary roots and/or coleoptiles length of rice seedlings at different concentrations of ethylene, such as 0, 0.1, 1, 10, 100 ppm. Relative coleoptile/root length can well reflect the ethylene response of WT and mutant. The relative coleoptile and root length are derived from ethylene-treated versus untreated in WT or mutant, respectively. Please refer to the Figure 3 in Plant Cell (Yin *et al.*, 2015).

Acknowledgments

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