A genetic screen of chitosan-insensitive mutant in *Arabidopsis thaliana*

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ABSTRACT

Forward genetic approach is a powerful tool to elucidate plant response to elicitors. Chitosan, the biodegradable polymer deacetylated form of chitin, plays a role as an elicitor in many plants. To discover the chitosan response mechanism, we achieved a screening for the EMS-mutagenized *Arabidopsis* population with chitosan-insensitive characters that has not been previously reported. At the seedling stage, high dosage of chitosan reduced plant growth both shoot and root systems. Mutant phenotype is characterized by the larger plant size and/or longer root length compared with wild type plant on 80 mg/l chitosan supplemented plates. Mutants also showed leaf defect phenotypes such as leaf shape and leaf coloration. A number of the putative mutants were sterile, with no flowers or showed early senescence before completion of their life cycles. At least 5 chitosan-insensitive mutant lines showed the larger shoots or longer root length compared to wild type in chitosan treated condition. The genetic inheritance analysis with L*er* background crosses showed heterosis phenotype, interfering the growth-related trait of chitosan-insensitive characters. To avoid the heterosis interference, the mutants were crossed with *Col* background. To identify the mutant population with a single recessive mutated gene, F_2 progeny of each cross was evaluated. Only three mutant lines, 106A, 129A and 161A showed the potential to contain the single recessive mutated genes involving in chitosan response. However, the 129A line did not showed the consistent chitosan-insensitive phenotype after the repeated phenotyping of the M_3 population. Therefore, only 106A and 161A will be used for further chitosan-responsive gene characterization as the next step.

Keywords: *Arabidopsis thaliana*; chitosan; mutant; genetic screen

INTRODUCTION

Chitosan is a deacetylated derivative of chitin. It is becoming a popular natural substance in agricultural utilization particularly for plant growth enhancement and plant disease control (Abdelbasset *et al*., 2010). Although, chitosan has been widely used as a plant growth stimulant in many crops, the mechanisms of chitosan response are still unknown. Transcription profiling of chitosan-responsive genes were previously reported in *Brassica napus* (Yin *et al*., 2006) and *Arabidopsis thaliana* (Povero *et al*.,

2013). Nevertheless, they focused on the plant disease responses, which were the majority of chitosan research in plants due to the elicitor property of chitosan. It was previously shown to induce a set of genes commonly recognized as pathogen-associated molecular patterns (PAMPs), responding by membrane depolarization, reactive oxygen species activation, plant hormone biosynthesis, callose formation, secondary metabolite biosynthesis, and the expression of biotic/abiotic stress responsive genes (Vidhyasekaran, 2014). In addition, chitosan also promotes plant growth in several plants including monocots and dicots, for examples, potato (Asghari-Zakaria *et al*., 2009) and maize (Guan *et al*., 2009). It was shown to increase the number of protocom-like body production, shoot induction and plantlet regeneration of Dendrobium 'Eiskul' *in vitro* (Pornpienpakdee *et al*., 2010). It can enhance rice seedling growth under drought condition and increase drought-resistant ability of indica rice (Pongprayoon *et al*., 2013). However, chitosan can have the negative impact on plant growth, if the inappropriate concentration and types were applied (Pornpienpakdee *et al*., 2010).

To annotate gene function, a forward genetics approach is a powerful tool to identify the responsive genes and isolate the large-scale of mutants who carry a particular phenotype of concern in mutagenized population. A success of genetic screening consisted of two components, which are the well-defined genetic information organism such as *A. thaliana* as well as a simple and steady protocol (Page and Grossniklaus, 2002). *Arabidopsis* has a small genome, short life cycle, self-fertility, and large number of progeny (Koornneef and Scheres, 2001). These characters promote *Arabidopsis* to be a flowering plant model system and a powerful resource of forward and reverse genetics. With development of forward genetics approaches, the simple way to generate mutagenized population is the use of mutagenic agents e.g. ethyl methanesulfonate (EMS). EMS mutagenesis becomes a standard procedure in *Arabidopsis*. EMS induces nucleotide modification from the original G/C to A/T resulting in transition point mutation by randomly generated across the genome. It can produce large-scale of mutagenized plants with an easy procedure (Kim *et al*., 2006).

In this work, we are intrigued in searching for the responsive genes involved in chitosan response particularly growth and development. The advantage of the clear root growth response of *A. thaliana* provided phenotype for chitosan responsive gene mutation screening. The high concentration of chitosan treatment on *Arabidopsis* seedlings resulted in root growth inhibition. We accomplished a large-scale screening for EMS-mutagenized mutant of *A. thaliana* lacking chitosan responses in root growth. The mutant analysis will provide the gene(s) that play a role in chitosan responses in plants.

MATERIALS AND METHODS

EMS-mutagenized *Arabidopsis* seeds preparation

Arabidopsis thaliana ecotype Columbia-0 (Col-0) seeds were mutagenized by treating with ethyl methanesulfonate (EMS) according to laboratory's protocol. In brief, seeds were rinsed with 4 ml distilled water with 0.1% Tween 20 in a scintillation vial. The vial was agitated at 180 rpm for 15 min, and the solution discarded. Seeds were washed four times with 4 ml distilled water at 180 rpm for 5 min. After washing, seeds were treated with 25 mM EMS and agitated at 180 rpm for 17 h. Treated seeds were washed five times with distilled water at 180 rpm for 5 min and stored at 4°C until sowing. Mutagenized seeds (M_1) were planted on soil (Sunshine Professional Peat-Lite mix 4, SunGro

Horticulture, Vancouver, BC) in a growth room at 22°C ± 2°C for 2 months to obtain M_2 seeds. After that, seeds of 5-10 plants per pool were collected to subject for chitosan resistance screening.

Seed sterilization

Seeds were placed in a 1.5ml micro-centrifuge tube and put in a desiccator. The desiccator contained a beaker filled with 100 ml Clorox® (5.25% Sodium Hypochloride) and 3 ml concentrated HCl. The tubes were opened in desiccator for 3 hours for fuming. All process was done in a fume hood. After that, the tubes were closed until used or added sterile water for stratification at 4°C.

Screening of chitosan-insensitive mutagenized *Arabidopsis*

Workflow of mutant screening is showed in Fig 1. The pools of mutagenized seed were selected on ½ MS medium (pH 5.8) with oligomeric chitosan with 80% degree of deacetylation (O80) at concentration of 0 or 80 mg/l for 10 days. The pools of M_2 seed were collected from 5 - 10 M_1 plants per seed pool, and spread on chitosan selective medium (½ MS medium with 80 mg/l chitosan). As a consequence of chitosan effect on plant growth, obtained mutants should grow nearly as same as its growing in normal conditions or grow better than wild type plant in chitosan condition, for example, having bigger cotyledons or leaves, having more lateral roots or having longer roots. Putative mutants that met the criteria for chitosan-insensitive phenotype were transferred to soil and grown for another 2 months to produce offspring.

The selected plants were grown individually on soil for 2 months, and the M_3 seeds from each plant were separately collected for the second round of screening. To determine chitosan-insensitive mutant plant, the mutagenized *Arabidopsis* M_3 seeds were surface-sterilized and grown on ½ MS medium with 0 or 80 mg/l of chitosan for 10 days. The positive candidate M_3 plants were crossed to background line (Col-0) to check their genetic characteristics i.e. phenotypes and mode of inheritance. The F_1 seeds were planted on soil to generate F_2 progeny. The pools of F_2 progeny from each putative line were selected on the selective medium with the same condition as described above.

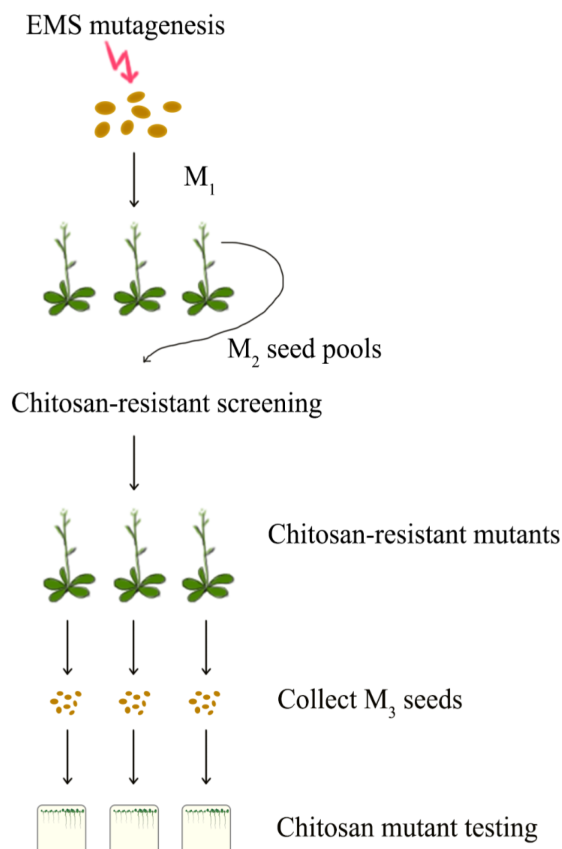

Figure 1 Workflow of chitosan-insensitive mutant screening. *Arabidopsis* seeds were mutagenized with EMS. M_1 generation were grown on soil and self-fertilized. M_2 seed pools were collected from 5-10 M_1 individuals and were used for chitosan-insensitive screening. Selected M_2 plants were self-fertilized, and the M_3 seeds were individually collected and used for chitosan mutant testing.

RESULTS AND DISCUSSION

The effects of 80 mg/l of chitosan on plant growth

At the concentration of 80 mg/l of chitosan, plants showed dwarf phenotype both shoot and root systems such as small plant size and short root length (Fig. 2). These characters were clear and easily distinguished by eyes. This condition was suitable for screening of mutant plants that were resistant to chitosan, so it could be used for further mutant selection.

EMS mutagenesis of *Arabidopsis* and isolation of putative chitosan-insensitive mutants

At least 120,000 M_2 plants were screened on the chitosan selective medium. Several phenotypes of mutagenized seedlings were broadly detected. Most of them showed dwarf phenotype that could be observed by eyes such as small leaves, short hypocotyls, small cotyledons, and short roots. Together with early lethal-effect mutation mutagenized, plants showed a high number of no germination and albino plants that indicated the efficiency of the EMS mutagenesis (Berná *et al.*, 1999). About 350 putative mutant plants from large-scale screening of M_2 generation showed the chitosan-insensitive phenotype by comparing the plant size or root length to wild type plant grown on the same plate. The putative M_2 mutants were transferred from plate to soil in an individual pot, then grown for another 2 months. Mutants were allowed to self-fertilize to obtain M_3 seeds.

In pot-grown plants, the putative M_2 mutants showed different leaf shapes including normal and mutant in leaf formation. Leaf shape of mutants can be categorized into eight phenotypic classes defined by Berná *et al.* (1999) as shown in Table 1. Incidentally, about 40 putative M_2 mutants died prior completing their life cycles, had an early senescence, had no flower or were sterile. We could not go through further step for these lines.

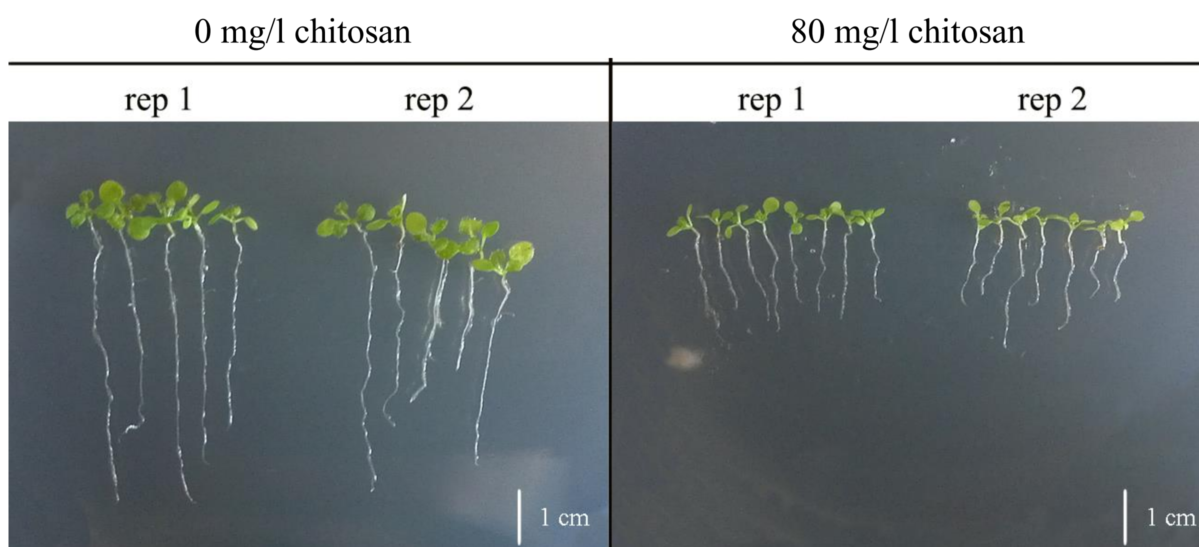

Figure 2 Phenotypic response of *Arabidopsis* to chitosan at the concentration of 80 mg/l.

Table 1 Phenotypic classes of chitosan-insensitive EMS-mutagenized *Arabidopsis* leaves according to definition by Berná *et al.* (1999)

Phenotypic class	Leaf phenotype of each class
Denticulata (Den)	Pointed lamina with dentate margins
Elongata (Elo)	Narrow and elongated lamina and long petiole
Rotunda (Ron)	Broad and rounded lamina
Scabra (Sca)	Rounded and protruded lamina
Ultracurvata (Ucu)	Lamina spirally rolled downward
Dentata (Dea)	Serrated margins
Incurvata (Icu)	Involute margins
Transcurvata (Tcu)	Margin obliquely revolute

M_3 seeds from each putative M_2 plant were separately collected for the chitosan mutant testing. The M_3 and wild type seeds were surface-sterilized and grown on ½ MS medium with or without 80 mg/l of chitosan for 10 days. In order to see the chitosan effects on root growth, plants were grown vertically. Some putative mutants showed retarded growth when exposed to chitosan but less than that observed in wild type plants. The effects varied in different lines. For example, some of putative mutants showed higher growth than wild type in normal conditions, but showed lower or equal growth in chitosan condition. Chitosan response of plant phenotype can be separated into 3 categories as shown in Table 2. Category 1, mutant plants showed higher growth than wild type in normal conditions and showed less retarded growth in chitosan condition. Category 2, mutant plants showed normal growth similar to wild type in normal conditions and showed less retarded growth in chitosan condition. Category 3, mutant plants showed normal growth similar to wild type in normal conditions and showed more retarded growth in chitosan condition. In the last category, it might be because the homozygous mutation occurs in the lethal genes in *Arabidopsis* genome in M_3 generation. More than 50 seedling-lethal mutants were identified in *Arabidopsis* (Budziszewski *et al.*, 2001)

In M_3 generation, other leaf defect phenotypes were observed. Coloration of mutant leaves varied from normal green, darkened green, pale green or purple leaves. These characters might be affected by pigmentation defects in mutants that have been reported in different classification of mutant screening (Jurgens *et al.*, 1991). Pale-green-leaf M_3 plants previously showed chitosan-insensitive phenotype and normal green leaves in their M_2 generation. A week after germination, lots of putative mutant lines showing pale green leaves could not show the insensitive phenotype. The slower growth rate was found in these mutants when compared with wild type plant under chitosan condition (Fig. 3a, b). Additionally, leaves of a few putative mutants turned darken or purple (Fig.3c, d). They also showed less chitosan-insensitive phenotype than in their M_2 generation. The darker color has been observed previously in seedling-lethal mutant screening. They suggested that these phenotypes might be the consequence of anthocyanin accumulation in

Table 2 Chitosan response phenotype of wild type and mutants in the second screening.

Category	Normal condition		Chitosan condition	
	Wild type	Mutant	Wild type	Mutant
1	+	++	--	-
2	+	+	--	-
3	+	+	-	--

+ represents normal growth

++ represents higher growth

- represents less retarded growth than wild type

-- represents more retarded growth than wild type

cotyledons (Kubasek *et al.*, 1992). Chitosan can also increase the flavonoid content including anthocyanin via its elicitor activity (Ferri and Rassoni, 2011). This phenotype showed the positive chitosan response, thus it was not surprising for loss of the resistance ability of mutants.

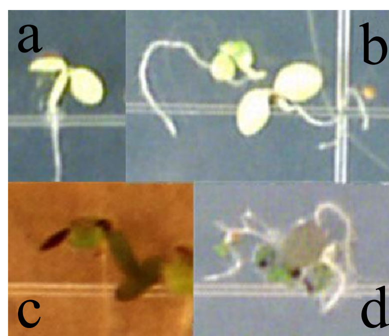

Figure 3 Phenotype of mutant *Arabidopsis* showing pale green leaf (a, b) or darkened leaf (c, d).

After the M_2 mutant screening, 60 putative mutants showed the larger size than wild type plants on chitosan plates. However, when the growth phenotype on chitosan plates was confirmed in M_3 plants, various phenotypes were found. Some mutant lines showed low seed germination percentage, some were sterile or had poor fertilization ability. Therefore, these lines were discarded as these would cause the difficulties in the back-crossing step to evaluate mode of heritability. Only the mutant lines showing the chitosan insensitive phenotype on chitosan plates were carried over to the evaluation step.

After the confirmation in M_3 generation, 5 putative lines, which were 21I, 86B, 106A, 129A, and 161A (Fig. 4a-e) were obtained. Mutant line 21I (Fig. 4a) and 86B (Fig. 4b) showed clearly chitosan-insensitive phenotypes, which were larger plant size and longer roots. Mutant line 129A (Fig. 4c) and 106A (Fig. 4d) showed slightly longer roots. Mutant line 161A exhibited larger shoots with slightly longer roots. Although its roots were shorter than other positive mutant lines, they are longer than that of wild type (Fig. 4e). The other weak chitosan-insensitive phenotype mutant lines were shown using line 39A (Fig. 4f) as a representative.

Test cross with Landsbergerecta (L*er*) ecotype

M_3 individuals from these remaining 5 lines were crossed with the background line to determine the inheritance pattern in F_1 and F_2 progeny. Firstly, the testcross of M_3 individuals was performed with commonly used background line, Landsberg*erecta* (L*er*). However, heterosis was found in a hybrid offspring of wild type (Col x L*er*). Hybrid wild type

plants showed higher growth and development than their parents (Col or L*er*) such as an increase in plant size and root length or more lateral roots (Fig. 5). These characters dramatically confound the chitosan-insensitive phenotype that we used for screening. Heterosis is a phenomenon of epistasis in genetics. Heterosis resulted in the higher vigor of F_1 hybrid over their parents. In *Arabidopsis*, heterosis is prevised to be low; however, it can be observed in various *Arabidopsis* hybrid. Col and L*er* ecotypes share closely related genetic backgrounds thus heterosis is occurred in Col x L*er* hybrid. The study of heterosis using recombinant inbred lines (RILs) from Col and L*er* revealed the different heterozygous regions between in RIL x Col and in RIL x L*er* within the same RIL. The homozygous regions in Col crosses change to heterozygous in L*er* crosses leading to higher vigor. The high vigor of F_1 hybrid is dependent on total heterozygosity and individual chromosome heterozygosity in L*er* crosses but not necessary in Col crosses (Syed and Chen, 2005). To solve the problem, we changed to perform the testcross within Col to avoid the confounding effect of hybridization to L*er*. It was very important with genes that have a relatively subtle effect. We did the testcross for all 5 lines before knowing that the

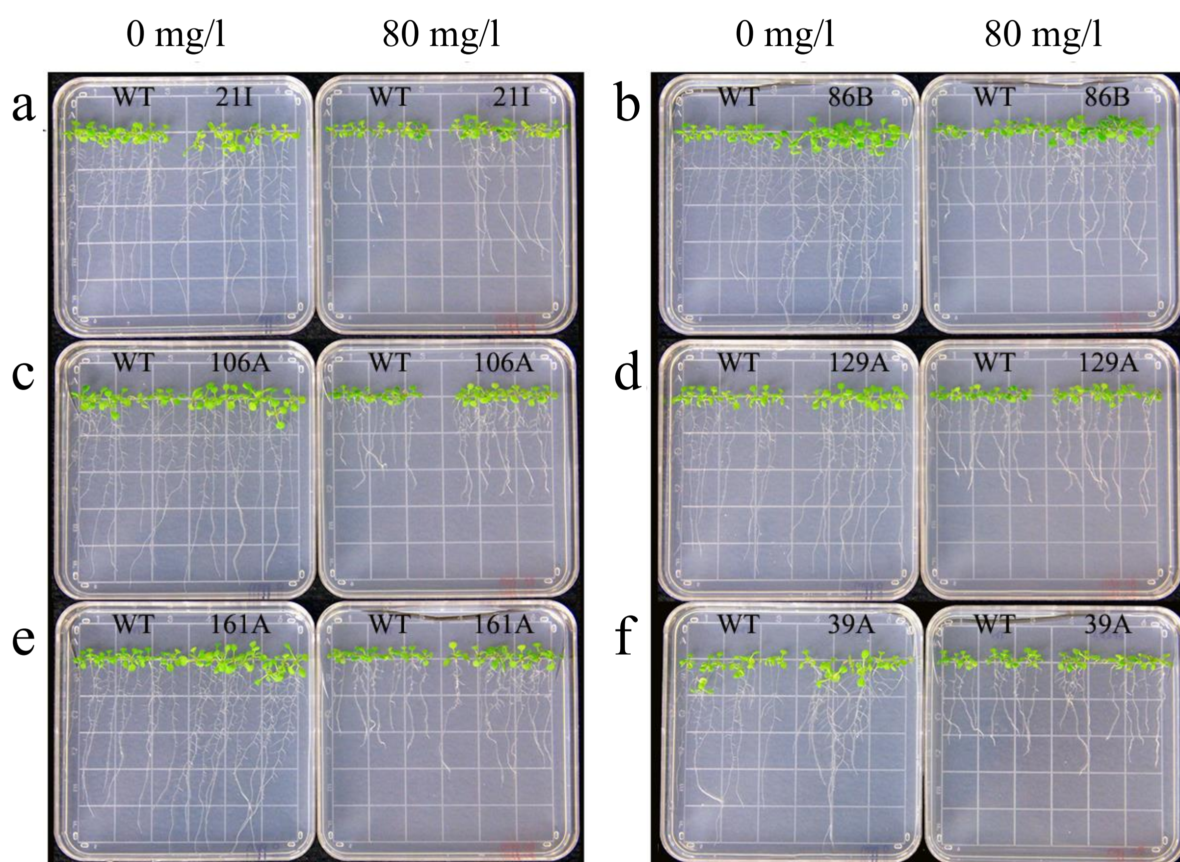

Figure 4 Putative chitosan-insensitive mutants from chitosan mutant testing. Left-handed side on each plate was wild type plants and right-handed side one was mutant plants. a: mutant line 21I; b: mutant line 86B; c: mutant line 106A; d: mutant line 129A; e: mutant line 161A; f: mutant line 39A. (left plate: 0 mg/l of chitosan; right plate: 80 mg/l of chitosan)

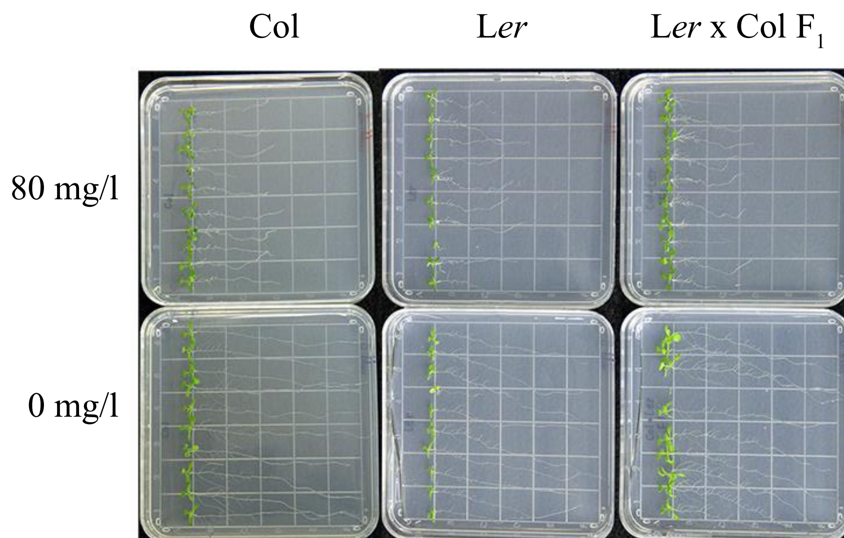

Figure 5 Phenotype of wild type *Arabidopsis* Col, L*er* and hybrid of L*er* x Col.

heterosis effect of Col x L*er* that confound the insensitive phenotype. We investigated the pattern of inheritance of F_2 population of mutants in L*er* cross before solving the problem. This might roughly screen for genetic inheritance of candidate mutants while we produced the new progeny of Col cross.

When the putative mutant was crossed with L*er,* all F_1 progeny had the short root phenotype (Table 3) similar to what was found in wild type, suggesting that these mutated genes were recessive genes. The segregation ratio of the short root: long root phenotype was collected as shown in Table 3. As F_2 of L*er* x 21I and L*er* x 86B show the segregation ratio of 10:1 and 24:1, respectively, these suggested that multiple mutations in the genome contributed to chitosan response. On the contrary, F_2 of the others showed the segregation ratio of short: long root phenotype as 3:1, suggesting the mutated chitosan responsive genes in these lines were recessive genes. *P* value of *Chi*-square test with the null hypothesis of 3:1 segregation for short root : long root plants were also shown in Table 3. The distribution of F_2 progeny with various root length was shown in Fig. 6. Moreover, not only the longer root phenotype was found in these 3 mutant lines, the larger shoot was also detected under 80 mg/l chitosan treatment.

Confirmation of M_3 mutant phenotype and the segregation of F_2 in Col background

The M_3 mutant phenotype of 129A, 106A and 161A were confirmed two more rounds to investigate the stability of the phenotype. It was found the 129A line showed the inconsistent phenotype, while 106A and 161A lines performed consistently long root on chitosan supplemented plates. In order to eliminate the heterosis effects of L*er* background, 106A and 161A lines were crossed with *A. thaliana* ecotype Col. Approximately 300 seeds of each F_2 progeny were grown to investigate the segregation of phenotypes. It was displayed that the mutated phenotypes were regulated by a single recessive gene by showing short: long root phenotype of 3:1 in F_2 progeny on the chitosan supplemented plates. Therefore, these two lines could be used for further characterization.

Table 3 F_1 phenotype and segregation ratio of the F_2 root phenotype on the medium supplemented with 80 mg/l chitosan

Cross	F_1 phenotype	Segregation ratio of (short root: long root) F_2	*P* value*
L*er* x 21I	All have short roots	10:1	0.0002
L*er* x 86B	All have short roots	24:1	0.0000
L*er* x 106A	All have short roots	3:1	0.8173
L*er* x 129A	All have short roots	3:1	1.0
L*er* x 161A	All have short roots	3:1	1.0

**P* value when the null hypothesis was based on the segregation ratio of short root: long root = 3:1

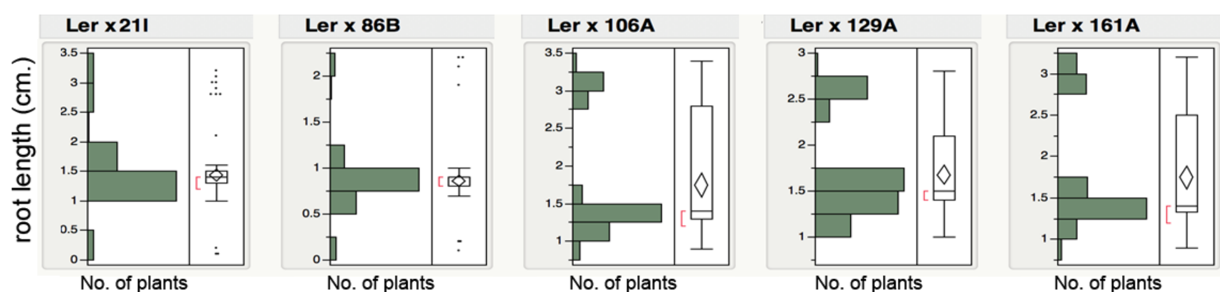

Figure 6 Distribution of root length of the F_2 progeny of wild type x mutant line.

To the best of our knowledge, we reported the first isolation of chitosan-insensitive *Arabidopsis* mutants. It is possible that chitosan plays multiple actions depending on its properties. This makes it difficult to study chitosan response in plants. For further investigation, the putative mutants will be sequenced and mapped to identify the mutated genes that could play crucial roles in chitosan response.

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