Plant Biotechnology 23, 459–466 (2006)

Original Paper

A high-throughput evaluation system for *Arabidopsis* mutants for defense signaling

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Received June 13, 2006; accepted July 10, 2006 (Edited by T. Hashimoto)

Abstract This paper describes an efficient evaluation system of *Arabidopsis* mutants for defense responses. Young *A. thaliana* seedlings were analyzed for the induction of reactive oxygen species (ROS) as well as defense gene expression by chitin oligosaccharide elicitor in microtiter plates. Combined with the analysis of ROS in microtiter plates, the system enabled to evaluate thousands of mutant plants within a couple of weeks. Pharmacological studies could be applied and provided information about the molecular machinery involved in the defense responses. Experiments with the dissected organs showed that the roots were responsible for most ROS generation while the gene activation was observed in all the organs. An example of the successful application of this system for the screening of *Arabidopsis* muntats is also presented. Thus, the system provides a promising approach to screen, or evaluate, novel mutants for a confined defense signaling cascade downstream of a specific elicitor.

Key words: *Arabidopsis thaliana*, chitin elicitor, defense response, high-throughput evaluation, reactive oxygen species.

Multiple signal transduction cascades play pivotal role in the regulation of plant responses to various stimuli, including defense responses against pathogen attack, and the characterization of signaling cascades involved in these responses has been receiving global attention (Devoto and Turner 2005; Feys and Parker 2000; Stepanova and Alonso 2005). *Arabidopsis* mutants aberrant in signaling pathways are widely used in these studies due to greater feasibility in growing and handling the plants and the thorough understanding of its complete genome (Glazebrook et al. 2003; Ton et al. 2002).

High-throughput evaluation of mutants is a prerequisite for effective screening of novel mutants that would be useful for the dissection of a signaling cascade leading to a specific cellular response. In most cases so far, changes in the macroscopic phenotypes such as growth, shape, color variation, resistance to specific pathogens etc. have been used for the evaluation and screening of mutants because of the easy handling of a number of mutants. However, it is usually difficult to obtain the mutants confined to a specific biochemical process or signaling cascade selectively by these approaches. If an experimental system that enables the high-throughput evaluation of the mutants confined to a signaling cascade leading to a specific biochemical response is established, it would benefit the use of this model plant to dissect a signaling cascade more effectively.

Here we report a high-throughput evaluation system of *A. thaliana* mutants for the signaling cascade leading to a specific defense response, elicitor-induced reactive oxygen generation. To establish and examine the validity of the system, we used chitin oligosaccharide elicitor. Although chitin oligosaccharides have been shown to induce various defense responses in both monocots and dicots (Okada et al. 2002; Shibuya and Minami 2001) including *A. thaliana* (Zhang et al. 2002), the signal transduction cascade leading to the defense responses is largely unknown. As the ROS generation analyzed by this system reflects a confined cellular process starting from the activation of the elicitor receptor to reactive oxygen generation, it would be suitable for the screening of new mutants involving those for the signal transduction cascade .

We also show that the system can be effectively used

Abbreviations: DPI, diphenylene iodonium; LAR, localized acquired resistance; MS, Murashige and Skoog medium; SAR, systemic acquired resistance; ROS, reactive oxygen species.
This article can be found at http://www.jspcmb.jp/

for the analysis of gene expression profiles and for pharmacological studies.

Materials and methods

Elicitors

Chitosan oligosaccharides were kindly supplied by Yaizu Suisankagaku Industrial Co. Ltd (Shizuoka, Japan) and re-*N*-acetylated in our laboratory to obtain *N*-acetylchitooligosaccharides as described previously (Ito et al. 1997).

Plant growth

The seeds of *Arabidopsis* were surface-sterilized according to the method described previously (Clough and Bent 1998) and seeds were spread in petri plates containing MGRL nutrients (Naito et al. 1994) supplemented with 0.1% agarose and 1% sucrose. After sealing the plates with parafilm, they were placed at 4°C for 2 days to break dormancy and then incubated in a 16/18 h light/dark cycle at 22°C.

Preparation of seedlings for elicitation

Under sterile condition, seedlings at the age of 8 to 10 days were individually picked up using a sterile forceps and washed with filter-sterile (Millex®-GV, 0.22 μm, Millipore, Ireland) nutrient solution to remove the remnants of agarose over the plant surface. Without damaging the root system, seedlings were incubated in 96-well microtiter plate (Microtest™ U-bottom, BECTON DICKINSON, USA) containing appropriate volume of filter sterile nutrient solution containing 1% sucrose. Thus, in each well, a single seedling was placed and the microtiter plate was kept on a thermomixer Comfort (Eppendorf, USA) at 25°C and a mild shaking (300 rpm) was implied.

Elicitor treatments

Appropriate amounts of chitin or chitosan oligosaccharide elicitors (100 μg ml^{-1}) were added after 1 h of stabilization at 25°C. Various inhibitors were also added similarly (final concentrations: 5 μM k252a, 5 μM Staurosporine, and 20 μM DPI). The aliquots from control and treated seedlings were collected at every time points under sterile condition. The seedlings were recovered in this high-throughput approach and raised into full plants.

Chemiluminescence assay for H_2O_2

The reactive oxygen species released by *Arabidopsis* seedlings were quantified by the luminol chemiluminescence method as briefly described previously (Yamaguchi et al. 2003).

RT-PCR

Total RNA from roots, stems, leaves or entire *Arabidopsis* seedlings at indicated time points after chitin treatment was extracted according to the manufacturer's instructions (QIAGEN Science, Maryland, USA). Approximately 12 seedlings or dissected plant parts (roots, stems, and leaves) from 12 seedlings per treatment were combined together to get appropriate amounts of total RNA. RT-PCR was performed with 50 ng of *DNaseI*-treated total RNA using OneStep RT-PCR kit (QIAGEN Gmbh, Hilden, Germany) following the manufacturer's protocol. Amplication was carried out in the Applied Biosytems, GeneAmp® PCR System 9700 (PE Biosystems, California, USA) under the following conditions: reverse transcription for 30 min at 50°C: initial PCR activation step for 15 min at 95°C; followed by 3-step cycling of denaturizing at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min; final extension at 72°C for 10 min. The cycle numbers for each gene were: 25 for *actin1* and *PAL*; 26 for *pdf1.2* and *pr1*; 28 for *Nit4*, *rbohD* and *rbohF*; and 30 for *AsA1*, *PR-2* and *PR-5*. The gene-specific primer pairs were designed based on sequence data obtained from the NCBI website: *Actin1* (Accession number: M20016) forward primer (5′-ggcgatgaagctcaatccaaacg-3′) and reverse primer (5′-ggtcacgaccagcaagatcaagacg-3′); *AsA1* (Accession number: M92353) forward primer (5′-tgtgagtgccagaacaaagc-3′) and reverse primer (5′-tcgttctgctgccaagtatg-3′); *Nit4* (Accession number: U09961) forward primer (5′-ggtaagcaccgcaaactcat-3′) and reverse primer (5′-aaacatccaccctcaagtgc-3′); *PAL* (Accession number: X84728) forward primer (5′-attaacggggcacacaagag-3′) and reverse primer (5′-agttgagatcgcagccactt-3′); *PDF1.2* (Accession number: AY063779) forward primer (5′-tcacccttatcttcgctgct-3′) and reverse primer (5′-aaaccagaaatatgcatgtcataaag-3′); *PR-1* (Accession number: M90508) forward primer (5′-aggagcggtaggcgtaggtc-3′) and reverse primer (5′-acgtgttcgcagcgtagttg-3′); *PR-2* (Accession number: M90509) forward primer (5′-cgataccttgccaagtccat-3′) and reverse primer (5′-tgtaccggaatctgacacca-3′); *PR-5* (Accession number: M90510) forward primer (5′-cgtacaggctgcaactttga-3′) and reverse primer (5′-gcgttgaggtcagagacaca-3′); *rbohD* (Accession number: AF055357) forward primer (5′-ggatgggagacagcaggata-3′) and reverse primer (5′-tttggcgacacaaacacaat-3′); *rbohF* (Accession number: AB008111) forward primer (5′-aaggtgatgctcgttctgct-3′) and reverse primer (5′-agagggcttttggcttcttc-3′). The amplified products were separated on 2% agarose gels and visualized under UV light after staining with ethidium bromide.

Results

Establishment of high-throughput evaluation system

Although the reactive oxygen generation induced by pathogen attack, or elicitor treatment, has been considered to play a central role in plant defense, quantitative analysis of reactive oxygen species (ROS) has mostly been performed by using cultured cells and scarcely been reported for intact *Arabidopsis* plants (Joo et al. 2005). By using young *A. thaliana* seedlings of 8 to 10 days after *in vitro* culture, we could observe a typical

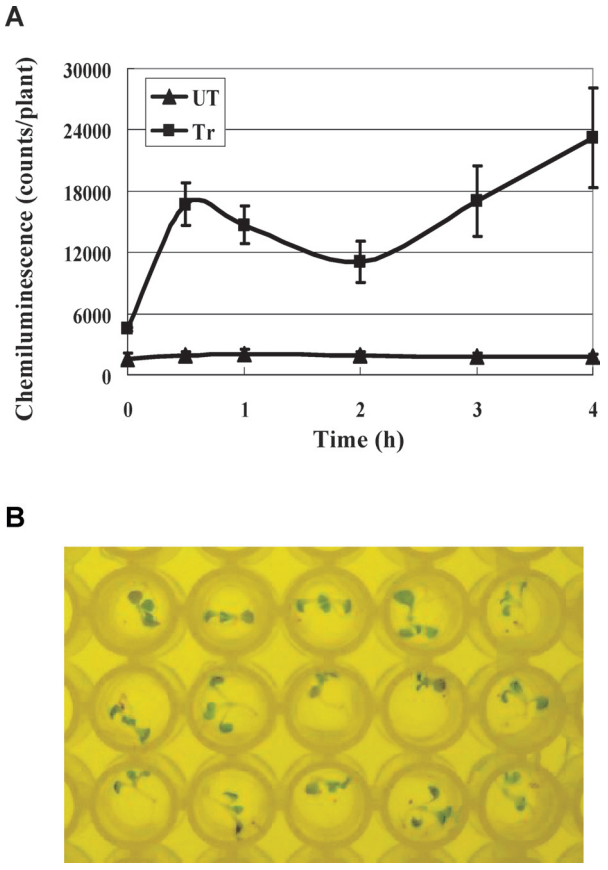

Figure 1. Effect of *N*-acetylchitooctaose on ROS generation in *Arabidopsis* seedlings. Seedlings were incubated in 96-well microtiter plate with or without *N*-acetylchitooctaose (100 μg ml^{-1}) and aliquots were collected at various time points for luminometric analysis of ROS generation (Figure 1A). Figure1B shows the picture of seedlings grown in the microtiter plate. Values are means of twelve replicates (n=12). Data presented correspond to one representative experiment. Tr, treated with elicitor; UT, control without elicitor. Error bars indicate standard deviation.

biphasic ROS generation induced by chitin oligosaccharide elicitor, which was reported for cultured rice cells previously (Yamaguchi et al. 2005) (Figure 1A). The use of 96-well microtiter plates for the elicitor treatment as well as ROS determination enabled a high-throughput analysis (Figure 1B).

Optimization of the system

The ability of the young *A. thaliana* seedlings to respond to the elicitor was significantly influenced by various factors including carbon source (glucose or sucrose), salt composition (MGRL, half-strength MS and Hoagland's nutrients), age of the seedlings, growth in antibiotics (carbenicillin and meropen) and others. The changes in plant growth and metabolisms by differences in media composition and culture conditions were shown for many plant species (Candela et al. 2001; Rosa et al. 2005). Figure 2 shows the effect of salt compositions on the reactivity of *A. thaliana* seedlings to the chitin oligosaccharide elicitor as an example. Thus, we optimized the experimental conditions to attain maximal responsiveness of the seedlings using microtiter plates. Briefly, the seeds were germinated and grown on a petri dish containing MGRL nutrients supplemented with 0.1% agarose and 1% sucrose. The seeds were first incubated 2 days at 4°C to break dormancy and then incubated for 6-8 days at 22°C before use.

Gene expression induced by the elicitor

The total RNA was extracted from the seedlings treated with chitin oligosaccharide elicitor and used for the analysis of expression of typical defense-related genes. As shown in Figure 3, the expression of *AsA1*, *Nit4*, *PAL*, *PDF1.2*, *PR-1*, *PR-2*, *PR-5*, *rbohD* and *rbohF* was significantly induced by the *N*-acetylchitooctaose treatment within 30 min.

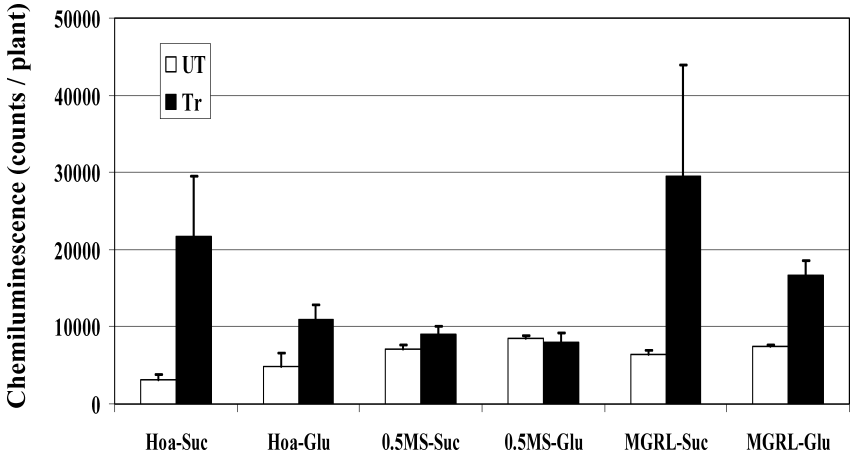

Figure 2. Effect of growth media on elicitor-induced ROS generation. Tested growth media were Hoagland (Hoa), half-strength MS (0.5MS) or MGRL, supplemented with 1% glucose or sucrose. The elicitor-induced ROS generation was analyzed after 30 min elicitor treatment as described for Figure 1. Values are means±standard deviations (indicated by vertical lines) of four replicates (n=4). Tr, treated with elicitor; UT, control without elicitor.

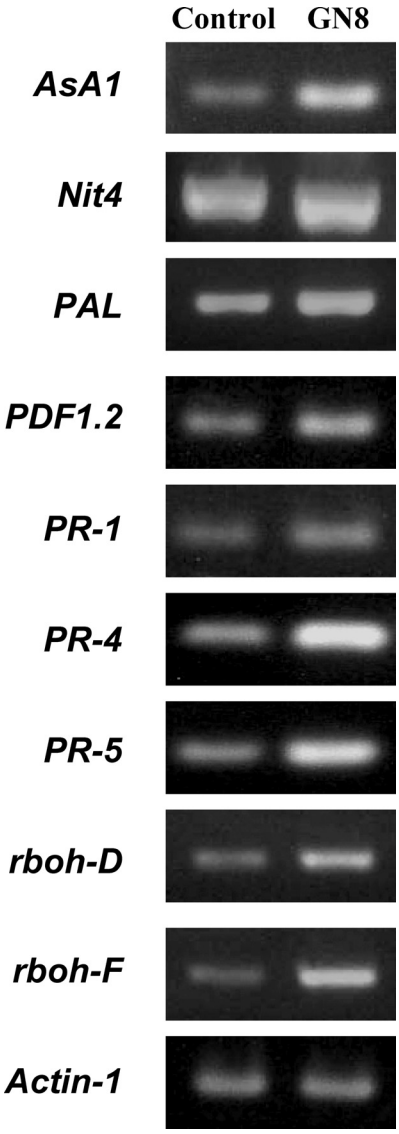

Figure 3. Gene expression induced by elicitor treatment. *Arabidopsis* seedlings were treated with *N*-acetylchitooctaose (100 μg ml^{-1}) for 30 min as similar to Figure 1. Total RNA was obtained from the whole seedlings of untreated (control) and 30-min *N*-acetylchitooctaose (100 μg ml^{-1}) treated (GN8) seedlings and used for RT-PCR analysis as described in materials and methods. The expression of *actin1* was used as an internal control.

Recovery of the tested seedlings

After tested for the ROS generation under aseptic conditions, the seedlings could be recovered and grown to get the next generation seeds, thus enabling the selection of candidate mutants and their use for further genetic studies.

Response of A. thaliana seedlings to chitin/chitosan oligosaccharides

The young *A. thaliana* seedlings exposed to chitin oligosaccharides clearly exhibited a size and dose dependent sensitivity (Figure 4A, B). The maximum induction in ROS generation was observed in chitin-

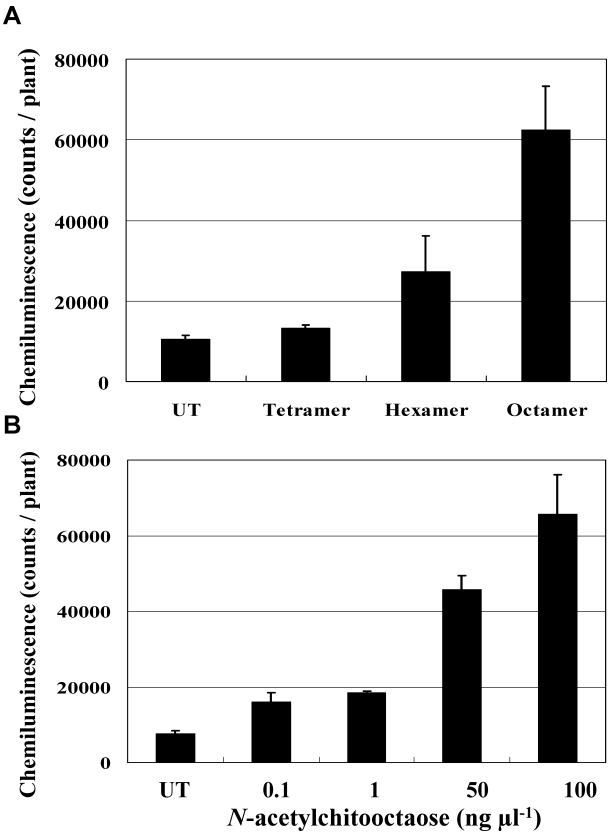

Figure 4. Effects of the sizes of *N*-acetylchitooligosaccharides (A) and concentrations of *N*-acetylchitooctaose (B) on ROS generation. *Arabidopsis* seedlings were treated with each *N*-acethylchitooligosaccharide (100 μg ml^{-1}) for 30 min. *N*-Acetylchitooctaose was used for the concentration dependency studies. Values are means±standard deviations (indicated by vertical lines) of twelve replicates (n=12).

octamer at the concentration of 0.1 μg/μl. Unlike in pea (Kendra and Hadwiger 1984), soybean (Kohle et al. 1985), and parsley (Conrath et al. 1990), the chitosan (de-acetylated chitin) oligosaccharides were not effective in eliciting ROS accumulation in *Arabidopsis* seedlings (Figure 5). Consistent with this data, cell suspension cultures of rice (Yamada et al. 1993) and tomato (Baureithel et al. 1994) exhibited no or very weak elicitation by deacetylated chitooligosachharides. The structural preference for chitin oligosaccharides by *A. thaliana* seedlings seems to be very similar to that of suspension-cultured rice cells (Yamada et al. 1993).

Pharmacological studies

Figure 6 shows the effects of various inhibitors on the induction of ROS generation by chitin-elicitor. The two potent inhibitors for protein kinases namely k252a and staurosporine significantly reduced the chitin-induced ROS in *Arabidopsis* seedlings as previously shown for ROS generation in cultured rice cells (Yamaguchi et al. 2003) and *A. thaliana* cells (Desikan et al. 1996) and also for the gene expression in the cultured rice cells (He

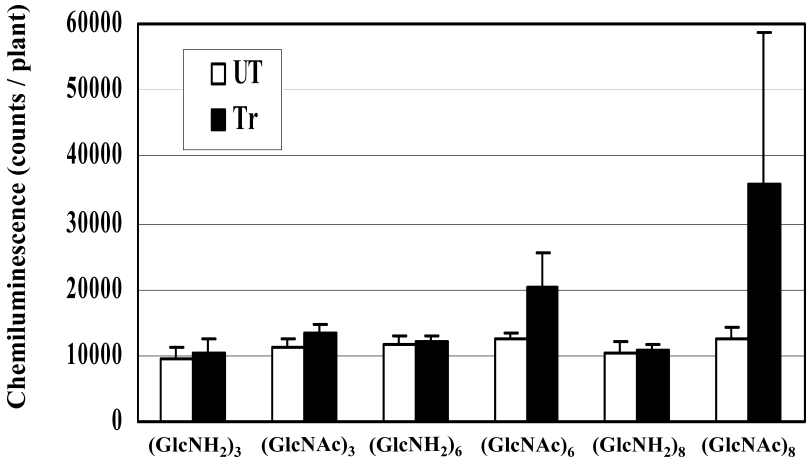

Figure 5. Effects of chitin and chitosan oligosaccharides on ROS generation. *Arabidopsis* seedlings were treated with chitin, $(GlcNAc)_n$, or chitosan, $(GlcNH_2)_n$, oligosaccharides (100 μg ml^{-1}) of different sizes for 30 min. Values are means ± standard deviations (indicated by vertical lines) of twelve replicates (n = 12).

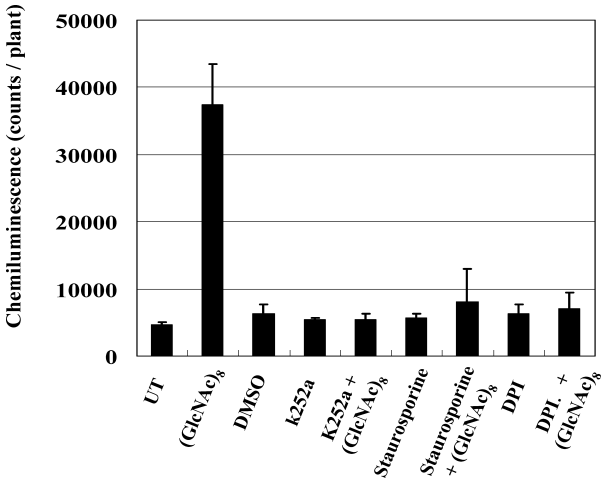

Figure 6. Effects of various inhibitors on ROS generation. *Arabidopsis* seedlings were treated with *N*-acetylchitooligosaccharides (100 μg ml^{-1}) containing one of the following inhibitors for 30 min. Inhibitor concentrations were: 5 μM k252a, 5 μM Staurosporine, and 20 μM DPI. Values are means ± standard deviations (indicated by vertical lines) of twelve replicates (n = 12).

et al. 1998) and *Arabidopsis* seedlings (Zhang et al. 2002). Complete suppression of the ROS generation by DPI indicated the involvement of NADPH oxidase in this response as similar to several other plant systems (Grant and Loake 2000).

Characterization of chitin-responses in dissected organs

Elicitor responses in different organs of *Arabidopsis* seedlings were analyzed by using dissected roots, stem and leaves that were separately treated with the elicitor. An extensive ROS production was induced in the dissected roots by chitin elicitor treatment, while no significant response was observed in the dissected stem and leaves (Figure 7A). These results clearly showed that the roots are the major source of ROS generation induced in the seedlings treated with chitin oligosaccharide elicitor.

Gene expression profiles induced by chitin elicitor treatment in the dissected roots, stem and leaves were also analyzed (Figure 7B). Contrary to the ROS accumulation, each of the excised plant organs showed the increase of the transcript levels of *PR-1* and *rbohD*, a gene reported to be responsible for most ROS generation in *Arabidopsis* triggered by gene-for-gene resistance (Torres et al. 2002). These results indicated that the elicitor could be perceived by individual parts of the *Arabidopsis* seedlings but the responses were different depending on each organ.

Screening of mutants for defense responses

We have been applying the evaluation method for the screening of novel mutants for defense responses. Figure 8 shows an example of the elicitor response of mutants thus obtained. One of the mutants, number 5, showed almost no response to the chitin oligosaccharide elicitor compared to the parent line of *A. thaliana*. Although the genetic as well as biochemical characterization of such mutants are still in progress, the result shows the potential of this screening method to find out novel mutants for chitin signaling leading to defense response.

Discussion

Validity and usefulness of the system

The results reported here showed that the system enables a high-throughput screening of the mutants for a confined signaling cascade from an elicitor receptor to reactive oxygen generation, though such mutants could also involve those for the ROS generation system. For such application, mutant seedlings evaluated for the elicitor-induced ROS generation should be recovered for the production of next generation for further genetic

studies and it can be easily achieved by this system. Although we used chitin oligosaccharides as an elicitor in this paper, the method can be applied to any elicitor that induces ROS generation in *A. thaliana*.

The system can also be applicable for high-throughput analysis of signaling cascade leading to the defense responses by using already established mutants. Successful application of pharmacological studies as shown in Figure 6 further supports the usefulness of the system for the dissection of signaling cascade by a combined approach with both genetic as well as pharmacological methods. Pharmacological studies will also help to characterize the mutants obtained by the screening, for example, by examining whether a specific mutant is located upstream of protein phosphorylation or not. We already applied this system to screen over 30,000 *A. thaliana* T-DNA insertional mutant plants for the survey of defense signaling mutants. Finding of the candidates for such mutants as shown in Figure 8 indicates the usefulness of this approach. So far as we tried, less than 0.5% of the tested plants showed some changes in the elicitor-induced ROS generation compared to the wild type *A. thaliana* and recovered for the second screening.

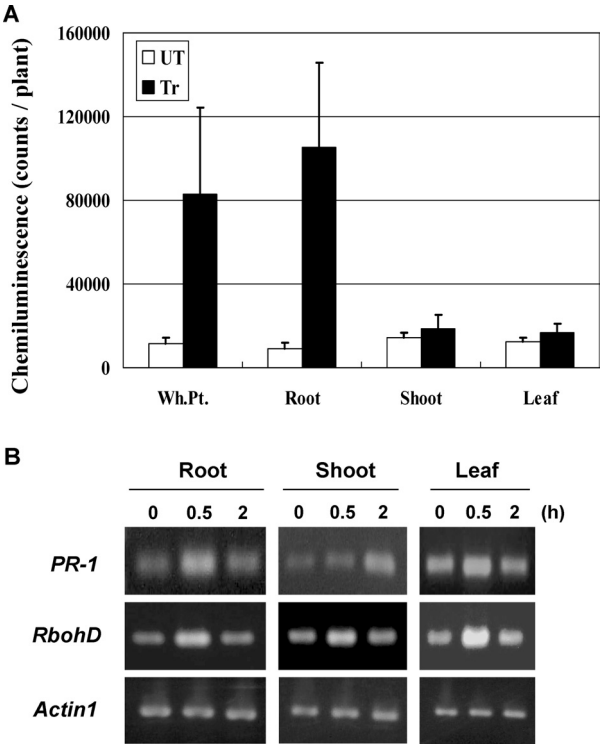

Figure 7. Defense responses induced in excised roots, stems and leaves by *N*-acetylchitooctaose. Excised organs were incubated in 96-well microtiter plate with *N*-acetylchitooctaose ($100\,\mu g\,ml^{-1}$). The ROS generation was analyzed after 30 min treatment (A). Total RNA was extracted after 30-min and 2 h elicitor treatments respectively, and used for RT-PCR analyses (B). The expression of *actin1* was used as an internal control.

Response of A. thaliana seedlings to chitin oligosaccharide elicitor

The recognition specificity of chitin oligosaccharides by *A. thaliana* observed in this study seems to be similar to that of cultured rice cells (Nishizawa et al. 1999; Yamada et al. 1993; Yamaguchi et al. 2003) in its preference to the larger sized oligosaccharides such as octamer, as also reported by Zhang et al (Zhang et al. 2002). Regulatory machinery for the elicitor-induced ROS generation seems also similar to the one observed for the rice cells in the involvement of protein phosphorylation upstream of the ROS generation (Yamaguchi et al. 2003) and also the involvement of DPI-sensitive enzymes, most possibly NADPH oxidase (Desikan et al. 1996; Torres et al. 2002). For the biphasic ROS generation induced by elicitor treatment or pathogen attack and the contribution of distinct NADPH oxidase genes has been suggested for *A. thaliana* (Torres et al. 2002; Torres et al. 1998),

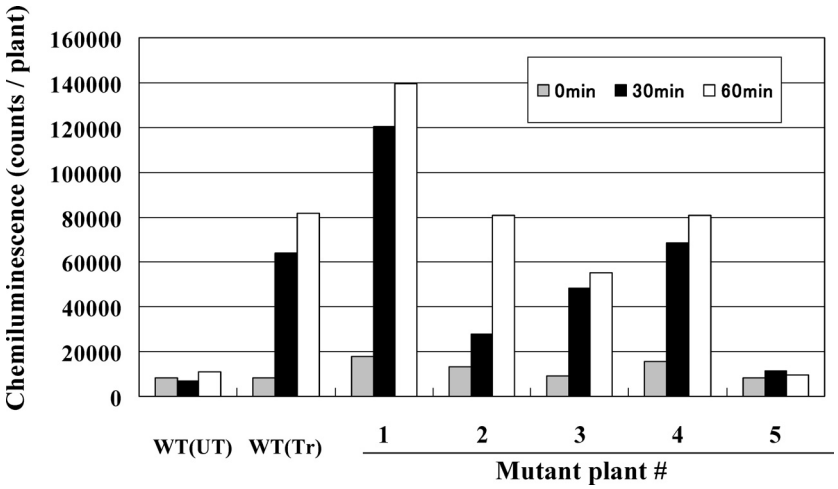

Figure 8. Screening of mutants for ROS response induced by *N*-acetylchitooligosaccharides. The numbers indicate each mutant plant. Note that #5 mutant showed almost no response to *N*-acetylchitooligosaccharide elicitor compared to the ROS induced by the elicitor in the wild type plant (WT-Tr).

Nicotiana benthamiana (Yoshioka et al. 2003) and potato (Yoshioka et al. 2001). The similarity observed in the specificity of the elicitor recognition in rice and *A. thaliana* indicates the conservation of chitin recognition system both in monocots and dicots (Okada et al. 2002). Overall similarity of the regulatory system indicates the possible compatibility of the knowledge obtained in either system.

Defense responses induced by the elicitor is LAR rather than SAR

Concerning to the question whether the elicitor was perceived and responded by each organ, or the responses in leaves or stem are the result of systemic signaling from the roots, the fact that the dissected organs responded independently to the elicitor (Figure 7) clearly showed the former mechanism. Thus, the differences in the responses to the chitin oligosaccharide elicitor in the roots, shoots and leaves seem to reflect the differences in the cellular responses induced by the same elicitor in each organ. In other word, the defense responses observed in this system is more related to local acquired resistance (LAR) rather than systemic acquired resistance (SAR).

Further, in some plant species such as tomato, the elevated level of hydrogen peroxide is required for the induction of defense genes (Orozco-Cardenas et al. 2001). In our present study, both *PR-1*, a well studied defense gene, and *rbohD* encoding a ROS generating NADPH oxidase were induced by chitin elicitor regardless of the ROS levels in excised *Arabidopsis* stem and leaves, indicating that at least for some genes ROS is not required as second messenger for gene expression.

The system can be efficiently used for gene expression studies

To demonstrate the efficiency of the method, we examined the expression profiles of typical genes involved in various defense and metabolic pathways. Chitin oligosaccharide elicitor induced several genes such as pathogenesis related proteins (*PR-1*, *PR-2*, *PR-5*) involved in SAR (Rogers and Ausubel 1997), *AsA1*, a gene encoding the alpha subunit of anthranilate synthase in *Arabidopsis* (Niyogi and Fink 1992), *PDF1.2*, a gene jointly activated by JA/ET signaling pathway (Penninckx et al. 1998), *Nit4*, a gene involved in auxin biosynthetic pathway (Bartel and Fink 1994) and two homologues of the human respiratory burst oxidase ($gp91^{phox}$) such as *rbohD* and *rbohF* (Torres et al. 1998).

Contrary to the results of Ramonell et al. (Ramonell et al. 2002), we were able to detect the induction of PAL in intact *Arabidopsis* seedlings by chitin-octamer using both RT-PCR (Figure 3) and Northern blot analyses (data not shown) indicating the efficient elicitation of gene activation through this high-throughput method.

In conclusion, this study demonstrates that *Arabidopsis* mutants for a confined defense response can be effectively screened through the high-throughput and non-invasive detection of ROS generation. The system enables to recover the seedlings for further molecular genetic studies. It can also be applicable for the dissection of signaling cascade by the use of established mutants as well as pharmacological approach.

Acknowledgements

We thank Yaizu Suisan Kagaku Industrial Co. Ltd (Tokyo, Japan) for the supply of chitosan oligosaccharides. This work was supported in part by the Program for Promotion of Basic Research Activities for Innovative Bioscience (Tokyo, Japan) and also a grant from the Ministry of Education, Culture, Sports, Science and Technology. A.P. acknowledges Japan Society for the Promotion of Science for the post doctoral grant.

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