Direct RT-PCR amplification of mature mRNAs in cytoplasm micropipetted from barley coleoptile epidermal cell – A model system for analyzing gene expression in host cells attacked by powdery mildew

Nobuyuki Moriura*, Yoshinori Matsuda*, Kazuhisa Fujita*, Mizuki Wada*, Koji Kakutani** and Hideyoshi Toyoda*

*Laboratory of Plant Pathology and Biotechnology, Faculty of Agriculture, Kinki University, 3327-204 Nakamachi, Nara 631-8505, Japan.
**Pharmaceutical Research and Technology Institute, Kinki University, Higashi-Osaka, Japan

Synopsis

Single-cell RT-PCR was used to detect gene expression *in situ* in single selected cells of detached barley coleoptile epidermis inoculated with *Blumeria graminis* f. sp. *hordei*. The cytoplasm was removed with a micropipette using a light microscope and directly used for RT-PCR, followed by nested PCR. Three intron-containing genes, vacuolar ATPase B-subunit gene, cytosolic triosephosphate isomerase gene, and glycolitic glyceraldehyde-3-phosphate dehydrogenase gene were constantly expressed in this tissue and were therefore used as indicators, because shorter-size PCR-products produced by splicing are easy to detect. By simultaneously amplifying both target and indicator genes, the transcription of some stimuli-induced genes such as chitinase 2 and acidic β-1,3-glucanase genes could be precisely detected in powdery-mildew-invaded and noninvaded, neighboring cells of the coleoptile epidermis. In addition, micropipetting only the cytoplasm without the nucleus prevented contamination with genomic DNA, which leads to miss-amplification of corresponding genomic DNA sequences of the intron-less genes during RT-PCR and subsequent nested PCR. Thus, this technique can be successfully used with coleoptile epidermal cells on which the powdery mildew fungus has attempted to invade and/or form a haustorium or with epidermal cells in the vicinity of infected cells.

Barley coleoptile epidermis—powdery mildew fungus is a model system suitable for analyzing cytological and molecular events in host-parasite interactions because cytological responses of host cells attacked by the pathogen can be microscopically tracked (Bushnell *et al.*, 1967). In addition, micropipette techniques can be used to detect gene expression in infected cells using a light microscope (Toyoda, 1993). A micropipette-manipulation technique has been refined in our laboratory to introduce foreign materials, such as foreign genes for transient gene expression and direct transformation (Toyoda *et al.*, 1990), labeled probes for *in situ* hybridization detection (Matsuda *et al.*, 1992; 1994), primers for in situ RT-PCR amplification of transcribed mRNAs (Matsuda *et al.*, 1997) and antifungal enzymes for plant disease prevention (Toyoda *et al.*, 1991). into target cells in plant and fungal systems. In the present study, we further refined this technique to remove the

cytoplasm from single cells through a micropipette and to subsequently amplify mature mRNAs in single cell RT-PCR. This paper describes the successful removal of intracellular contents with or without the nucleus from targeted single cells of barley coleoptile epidermis and the effective amplification of mature mRNAs involved in sucked cellular contents during RT-PCR followed by nested PCR (N-PCR).

First, we selected seven barley genes as PCR-targets from the cDNA database in the DNA databank of Japan (DDBJ): i) vacuolar ATPase B-subunit (ATB) gene (accession number in DDBJ, L11862), ii) cytosolic triosephosphate isomerase (TPI) gene (U83414), iii) glycolitic glyceraldehyde-3-phosphate dehydrogenase (GAP) gene (X60343), iv) chitinase 2 (CHI) gene (AJ276226), v) acidic β-1,3-glucanase (GLC) gene (M91814), vi) tonoplast ABC transporter (ABC) gene (AB063580) and vii) BARE-1 retrotransposon protease (BRP) gene (Z17327). For trapping transcripts of these genes, two sets of the primers were artificially constructed on the basis of their nucleotide sequences on the inside regions of the cDNAs. The primers were at-1 and at-2 for ATB, tp-1 and tp-2 for TPI, ga-1 and ga-2 for GAP, ch-1 and ch-2 for CHI, gl-1 and gl-2 for GLC, ab-1 and ab-2 for ABC, and br-1 and br-2 for the BRP gene and used for RT-PCR and N-PCR, respectively. The nucleotide sequences of the primers were given in the legend of Fig. 2.

Prior to single-cell RT-PCR of mRNAs in coleoptile epidermal cells, genomic DNA was extracted from the first leaves of 10-day-old seedlings of barley (*Hordeum vulgare* L., cv. Gose-shikoku) by standard protocols (Murray and Thompson, 1980) and amplified with the N-PCR primers to determine whether the genes contained intron sequences in the amplified regions. These genes were amplified under the same conditions—pre-heating at 95 C for 2 min, and 30 cycles for denaturing at 95 C for 50 sec, annealing at 60 C for 40 sec and extension at 72 C for 90 sec. The PCR products obtained were electrophoresed with 2% agarose gels, and the lengths of the amplified products were compared with those of the original cDNAs to determine the existence of the introns within the genes. The DNA fragments were extracted from the electrophoresis gels and inserted in a pGEM-T easy vector for sequencing (Promega, WI, USA). The nucleotide sequence of the amplified region was determined using Big Dye Terminator Cycle Seqencing Ready Reaction Kit from Applied Biosystems (Tokyo, Japan) on an ABI Prism 310 Genetic Analyzer (Perkinson Elmer Applied Biosystems, Tokyo, Japan). As a result, three, two, three, two, two and four intron sequences were detected in the genome-PCR products of ATB, TPI, GAP, CHI, GLC and ABC genes using primers at-2, tp-2, ga-2, ch-2, gl-2 and ab-2, respectively. There was no intron in the BRP gene with primer br-2. The nucleotide sequences determined were registered with the DDBJ; the accession number assigned was AB120302 for ATB, AB120303 for TPI, AB120301 for GAP, AB120304 for CHI, AB120305 for GLC and AB120306 for ABC introns. Thus, the present PCR primers for the genes were designed so that the introns and exons were put between sense- and antisense-primers; therefore the nucleotide length to be amplified by genome PCR and RT-PCR (followed by N-PCR) was 819 and 403 base pairs (bp) for ATB, 646 and 199 bp for TPI, 634 and 300 bp for GAP, 833 and 435 bp for CHI, 605 and 352 bp for GLC, 966 and 515 bp for ABC genes. On the other hand, the product of BRP gene by genome PCR was a 305 bp fragment.

The inner epidermis was detached from coleoptile tissues of 10-day-old seedlings of barley (cv. Gose-shikoku), placed onto an agar plate on a glass slide and inoculated with conidia of barley powdery mildew (*Blumeria graminis* f. sp. *hordei*, race I) according to the method described previously (Toyoda *et al.*, 1987). The agar slide with inoculated coleoptile epidermis was placed in a moistened Petri dish and incubated at 20 C for 24 hr under continuous illumination at 4,000 lux with fluorescent lights. This coleoptile epidermis was used for micropipetting the intracellular

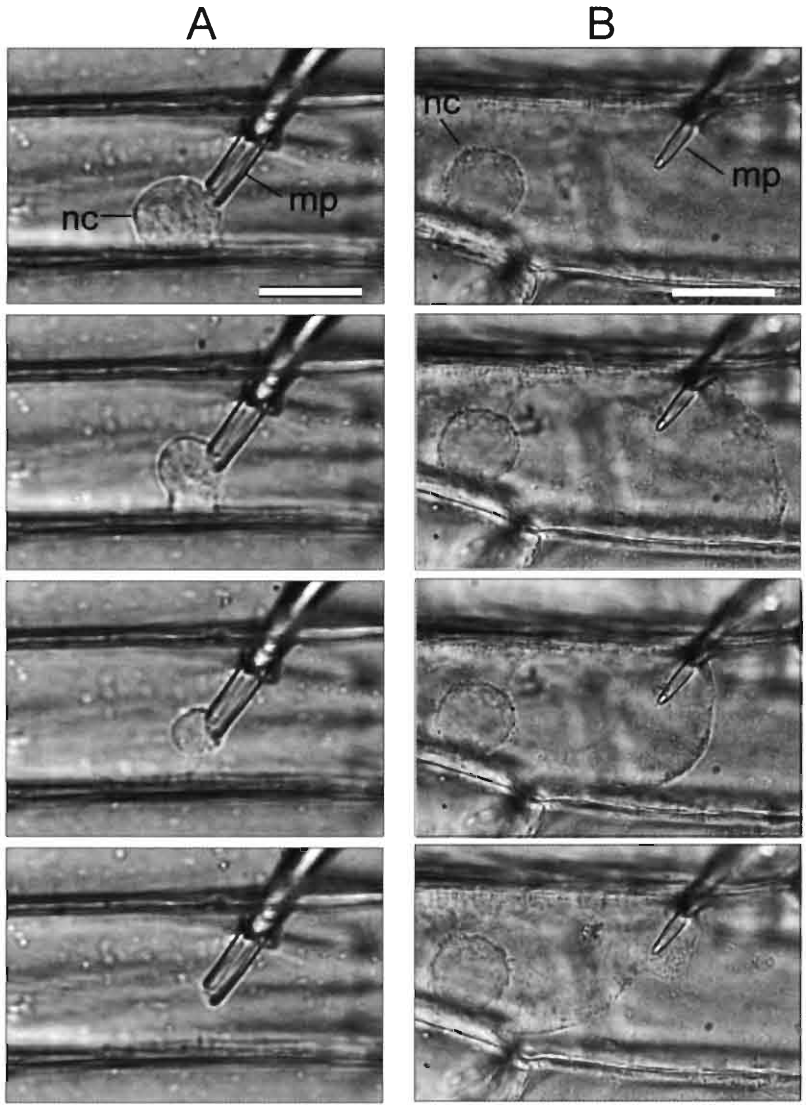

Fig 1. Removal of intracellular contents from targeted single cells of barley coleoptile epidermis with a glass micropipette. A: Suctioning of nucleus (nc)-containing cellular contents by the micropipette (mp) (tip diameter, 2 μm). B: Suctioning cytosolic contents (without nucleus) with the pipette (tip diameter, 5 μm). The pipette with larger tip-diameter was used to effectively suction nuclei. Photographs were consecutively taken at the same focal point. Bar represents 20 μm.

contents of single cells.

The intracellular contents were removed with an autoclaved glass micropipette (tip diameter, 2 and 5 μm) held by a micromanipulator on an Olympus Injectoscope (Toyoda *et al.*, 1988). The micropipette was filled with silicon oil and inserted into a haustorium-harboring cell of the coleoptile epidermis or non-infected cell laterally adjacent to the infected cell. The inner pressure of the micropipette was gently decreased to suction the cellular contents. Successful suctioning was microscopically confirmed by the influx of the contents into the micropipette and the movement of the interface between cellular contents and silicon oil in the micropipette. In Fig. 1A, the nucleus has been successfully removed from a single cell in a non-inoculated coleoptile epidermis. The cellular contents (0.3 to 1.0 nl) were directly ejected into 15 μl of the RT-PCR solution (RT-PCR High-Plus-Kit, Toyobo Co., Osaka, Japan) in

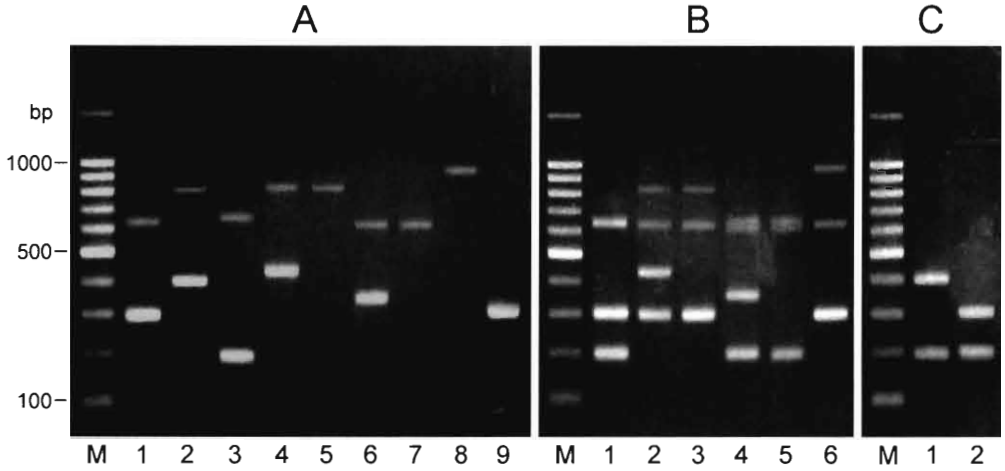

Fig 2. Electrophoresis of DNA fragments amplified through the suc-PCR (RT-PCR and subsequent N-PCR) of intracellular contents suctioned from single cells of barley coleoptile epidermis with the micropipette. A: Amplification of transcripts of seven genes GAP (lane 1), ATB (2), TPI (3), CHI (4 and 5), GLC (6 and 7), ABC (8) and BRP (9) from the nucleus-containing samples with the primers ga-1/ga-2, at-1/at-2, tp-1/tp-2, ch-1/ch-2, gl-1/gl-2, ab-1/ab-2, and br-1/br-2 in the RT-PCR/N-PCR process, respectively. B: Dual amplification of two genes from nucleus-containing samples by suc-PCR with the mixed primers; GAP and TPI (lane 1), CHI and GAP (2 and 3), GLC and TPI (4 and 5) and ABC and GAP (6) with ga-1 and tp-1/ga-2 and tp-2, ch-1 and ga-1/ch-2 and ga-2, gl-1 and tp-1/gl-2 and tp-2, ab-1 and ga-1/ab-2 and ga-2 in the RT-PCR/N-PCR process, respectively. C: Dual amplification of two genes from nucleus-free samples by suc-PCR with mixed primers; ATB and TPI (lane 1) and BRP and TPI (2) with at-1 and tp-1/at-2 and tp-2, br-1 and tp-1/br-2 and tp-2 in the RT-PCR/N-PCR process, respectively. The molecular weight marker (M) corresponds to the 100 bp DNA ladder.

at-1: 5'-TTTAACGGCTCTGGAAAACC-3'/5'-GCTGTTGTTAGCGCAATACG-3' at-2: 5'-TAATGGCCCTCCGATATTGC-3'/5'-TTCGATCGTGGGATCATTTG-3'

tp-1: 5'-AATGGAACCGTCGAGCAG-3'/5'-AAGAATGACCCAGGGAACG-3' tp-2: 5'-AGAGGCGATCGTCCAAACTC-3'/5'-GTTGGCAAGCATCTCAGCAC-3'

ga-1: 5'-GCTGTTGGTAAGGTTCTTCCTG-3'/5'-CTGGGTCTTGGCCATGTG-3' ga-2: 5'-CGGTATGTCTTTCCGGGTTC-3'/5'-CGGATCAGGTCGACAACAC-3'

ch-1: 5'-CAAGCTCTCGTTGAGCTATACATT-3'/5'-TTGCTCACAAGGTCCTTCC-3' ch-2: 5'-TGTCAGCTGAAGGTGAGCTAGT-3'/5'-GGCGAGATCGTAGTTGGACT-3'

gl-1: 5'-GTGTTGCTTCCGTGCTTG-3'/5'-GCTGGGAGGATGGTCTTCT-3' gl-2: 5'-TTGTTTTCCTGGCAGCATTC-3'/5'- GCGATGTAGCGGAAGGAGA-3'

ab-1: 5'-TCAGGGAACAAATGTCCAAA-3'/5'-TGAACAAGGTGTTCGCTCTC-3' ab-2: 5'-TGAAAATGATGGGGAAGTTGAG-3'/5'-CTTCGTCCAGGAGGAGCAC-3'

br-1: 5'-GGTTCCAAGGTTGATGCAAT-3'/5'-GCGCTTTACACCAATATGACC-3' br-2: 5'-CGTCGGCACAATTTCACTTC-3'/5'-AGCGGCAGTGCCATAAAAAC-3'

a 200 μl-Eppendorf tube. The solution contained the RT-PCR primers at 6 pmol. After defined cycles of RT-PCR (reverse transcription at 50℃ for 30 min, pre-heating at 95℃ for 2 min, and then 40 cycles for denaturing at 95℃ for 50 sec, annealing at 58℃ for 40 sec and extension at 72℃ for 60 sec, for all genes), the reaction mixture was mixed with 25 volumes of the N-PCR solution (Taq PCR Master Mix Kit, Qiagen Co., Tokyo, Japan) containing the N-PCR primers at 20 pmol. The amplification for gene transcripts was carried out by N-PCR (pre-heating at 95℃ for 2 min, 40 cycles for denaturing at 95℃ for 50 sec, annealing at 60℃ for 40 sec and extension at 72℃ for 60 sec). This successive amplification of the suctioned samples by RT-PCR and N-PCR was designated as suc-PCR. The final products were electrophoresed with 2% agarose gels, and all bands were separately extracted from the gels to determine their nucleotide sequences.

Table 1. Detection of gene transcripts using suc-PCR of cellular contents micropipetted from single cells of non-inoculated barley coleoptile epidermis

Primers used for suc-PCR		Gene transcripts	Number of cells with positive amplification
PR-PCR	N-PCR	amplified	of the transcripts/total pipetted cells
ga-1	ga-2	GAP	25/25
at-1	at-2	ATB	25/25
tp-1	tp-2	TPI	25/25
ch-1	ch-2	CHI	15/25
gl-1	gl-2	GLC	8/25
ab-1	ab-2	ABC	0/25
br-1	br-2	BRP	25/25

Fig. 2A shows the electrophoretic products obtained by the suc-PCR for the samples of nucleus-containing cellular contents micropipetted from non-infected epidermal cells, and Table 1 shows the frequency of successful detection of mature mRNA-derived transcripts in suctioned samples. The transcripts of three genes (GAP, ATB and TPI) were always amplified in all samples, while the transcripts of CHI and GLC genes were in 30 to 60% of the samples. As shown in Fig. 2A (lanes 1 to 4, 6), suc-PCR generated not only intense bands for the products originating from the mature mRNAs at the expected migration positions, but also faint bands for the products originating from genomic DNA. Both bands were extracted from the gels, and their nucleotide sequences were determined to confirm that the longer fragments contained the introns and the shorter no intron. These results indicated that intron-spliced mRNAs were successfully amplified during the present suc-PCR of single-cell samples, and the faint bands were amplified from contaminated genomic DNA sequences during RT-PCR or subsequent N-PCR.

As mentioned, the major intense electrophoretic bands were not detected in some cases of suc-PCR for CHI and GLC genes (lanes 5 and 7 in Fig. 2A), although the faint bands were detectable. To clarify whether this unsuccessful detection of major bands was due to technical errors, we conducted dual amplification of transcripts for two genes; target (CHI and GLC) and indicator genes. In this experiment, constantly detectable genes (GAP, ATB and TPI) were used as indicators for successful suc-PCR. Specifically, the suctioned sample was transferred to the first solution containing two sets of the RT-PCR primers (ch-1/ga-1 or gl-1/tp-1), and then the reaction mixture was mixed with the second solution containing two sets of the N-PCR primers (ch-2/ga-2 or gl-2/tp-2). The combination of primer sets for the indicator genes (ga-1/tp-1 in the first and ga-2/tp-2 in the second step) was also tested as a positive control. Fig. 2B shows the electrophoretic products amplified by dual suc-PCR for nucleus-containing samples obtained from non-infected epidermal cells. With the primer combination for the two indicator genes (GAP and TPI), two major intense bands (and two close minor faint bands) were consistently detected in all sucked samples (lane 1 in Fig. 2B, Table 2), indicating that mature mRNA-derived transcripts of two genes were successfully amplified even when the primers for two genes were together. On the other hand, suc-PCR with the mixed primers to detect either the GAP/CHI or TPI/GLC genes yielded two types of electrophoretic patterns; one produced two major bands and two faint bands (lanes 2 and 4), and the other, one major (corresponding to the indicator gene) and two faint bands (lanes 3 and 5 in Fig. 2B). In the latter electrophoresis, the major bands of the indicator genes were clear, comparable to

those of the previous electrophoretic data. The frequency for these two types was also comparable to those obtained with the primers singly used in the suc-PCR amplification (Table 2). These results strongly suggest that the lack of major bands resulted from no or negligibly low transcription of these genes in sucked cells, but not artificial errors in the suc-PCR process. A similar result was obtained when the sample was amplified through scu-PCR using ab-1 and ab-2 (lane 8 in Fig. 2A) or ga-1/ab-1 and ga-2/ab-2 primers (lane 6 in Fig. 2B) in the first and second step, respectively. Thus, the ABC gene was considered as not being expressed in epidermal cells of detached coleoptile tissues.

Only one intense band was amplified by suc-PCR with primers br-1 and br-2 for the BRP gene (lane 9 in Fig. 2A). In this case, however, it was difficult to deny contamination of the products derived from miss-amplification of the corresponding genomic DNA sequence because the gene has no intron in its amplified region; therefore, the contaminated product would be the same size as the mRNA-derived RT-PCR product. To solve this problem, we attempted to remove the cytosol without taking the nucleus into the micropipette. This approach was expected to prevent contamination with genomic DNA. In Fig. 1B, the cytosol is successfully removed from the cell without the nucleus. This cytosol was used for suc-PCR with the mixed primer sets. First, the sample was subjected to the suc-PCR together with the primers (tp-1/at-1 and tp-2/at-2) for the indicator genes, and the amplified products were electrophoresed (lane 1 in Fig. 2C). Two major bands from spliced mRNAs of TPI and ATB genes were detected without any faint bands. These clear bands were comparable to those bands amplified via suc-PCR of nucleus-containing samples. In Fig. 2C (lane 2), electrophoresis of the

Table 2. Detection of gene transcripts using simultaneous suc-PCR of cellular contents micropipetted from single cells of barley coleoptile epidermis non-inoculated or inoculated with conidia of powdery mildew of barley (*B. graminis* f. sp. *hordei*, race I), using mixed primers for target and indicator genes

Gene as		Primers used for suc-PCR	Coleoptile epidermis used for pipetting	Number of single cells pipetted	Number of cells with positive amplification of gene transcripts		
Target (T)	Indicator (I)	RT-PCR N-PCR			T+I	I	T
GAP	TPI	ga-1/tp-1 ga-2/tp-2	Non-inoculated	30	30	0	0
			Inoculated				
			Epidermal cells with haustorium	30	30	0	0
			Non-infected, adjacent epidermal cells	30	30	0	0
CHI	GAP	ch-1/ga-1 ch-2/ga-2	Non-inoculated	30	18	12	0
			Inoculated				
			Epidermal cells with haustorium	30	6	24	0
			Non-infected, adjacent epidermal cells	30	3	27	0
GLC	TPI	gl-1/tp-1 gl-2/tp-2	Non-inoculated	30	9	21	0
			Inoculated				
			Epidermal cells with haustorium	30	15	15	0
			Non-infected, adjacent epidermal cells	30	0	30	0
ABC	ATB	ab-1/at-1 ab-2/at-2	Non-inoculated	30	0	30	0
			Inoculated				
			Epidermal cells with haustorium	30	0	30	0
			Non-infected, adjacent epidermal cells	30	0	30	0
BRP	TPI	br-1/tp-1 br-2/tp-2	Non-inoculated	30	30	0	0
			Inoculated				
			Epidermal cells with haustorium	30	30	0	0
			Non-infected, adjacent epidermal cells	30	30	0	0

products with the combination of primers for the BRP and indicator (TPI) genes, two major bands migrated; one was the intron-spliced product of the TPI without the faint band. The same result was consistently reproduced in all targeted cells of the coleoptile epidermis (Tables 1 and 2). Thus, we could successfully remove by only the cytosol by micropipetting, leaving the nucleus within the cell to avoid or minimize contamination with genomic DNA in suc-PCR. The technique was especially useful for detecting non-intron genes expressed in epidermal cells of the coleoptile.

In the following experiment, invaded epidermal cells and non-infected epidermal cells adjacent to the invaded cells were selected as targets. Table 2 shows the efficiency of positive transcription of the genes for the target cells. The transcription of four genes GAP, ATB, TPI and BRP was constant in both pathogen-invaded cells and their neighbors, while the expression of the ABC gene was not detected in either pathogen-invaded (haustorium-harboring) cells, those adjacent or non-invaded. The expression of CHI and GLC genes was affected after inoculation with the pathogen. The rate of CHI gene transcription decreased conspicuously in both pathogen-invaded and non-invaded cells, and GLC expression was promoted only in the invaded cells and suppressed in the adjacent cells.

The coleoptile epidermis of barley was a useful material for applying single-cell suc-PCR to detect gene expression in microscopically pinpointed specific host cells responding to the pathogen. In this approach, the use of indicator genes was essential to evaluate positive amplification of transcripts during suc-PCR. For this purpose, we selected some intron-containing genes in barley after a computer search, then tested whether these genes could be expressed constitutively in detached coleoptile epidermis of barley. Cytosolic triosephosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase catalyze vital steps in glycolysis, and H+-ATPase on the cytoplasmic side of the vacuolar membrane generates the proton gradient for the secondary transport of ions and metabolites (Berkelman *et al.*, 1994). These enzymes are present in all eukaryotes, and in the present study, the constant expression of their genes was proven in detached barley coleoptile epidermis, irrespective of infection by the pathogen. Thus, ATB, GAP and TPI genes were concluded to be acceptable as indicator genes for single-cell suc-PCR.

With confirmation of successful amplification of the indicator gene transcripts, the transcription of stimuli-activated genes can be evaluated in response to powdery mildew infection. The BARE-1 retrotransposon was transcribed in some callus cultures and leaf-derived protoplasts of barley (Suoniemi *et al.*, 1996). The transcripts of BARE-1 retrotransposon in these tissues were initiated at different sites on the long terminal repeats (LTR), indicating the involvement of different mechanisms in the transcription regulation in different tissues of barley. Although the LTR for the entire BARE-1 retrotransposon was not analyzed in the detached coleoptile epidermis, the BRP gene involved in the retrotransposon was always transcribed in this tissue. No transcription of this gene was detected in intact barley coleoptile epidermis (data not shown), suggesting that transcription of BARE-1 was induced as a result of the detaching stimulus. It was evident that this transcription was not affected by pathogen infection.

The basic β-1,3-glucanase is preferentially produced as one of the pathogenesis-related (PR) proteins in barley cultivars resistant to powdery mildew, and the early-stage secretion of β-1,3-glucanase in the intercellular space of inoculated leaves was closely correlated to resistance conditioned by the major genes (Jutidamrongphan *et al.*, 1991). An acidic homologue to the basic β-1,3-glucanase gene was expressed constitutively in healthy roots and induced in leaves in response to biotic and abiotic stresses (Malehorn *et al.*, 1993). In addition, the endochitinases were produced in cell suspension cultures (Kragh *et al.*, 1991), aleurone cells (Swegle *et al.*, 1989; Jacobsen *et al.*, 1990) and leaves of barley inducing resistance to

the powdery mildew (Kogel *et al.*, 1994). The present study revealed that both acidic β-1,3-glucanase and chitinase genes were inconsistently activated in pathogen-invaded cells of coleoptile epidermis inoculated with powdery mildew as well as those not inoculated. Interestingly, the expression of these two genes was completely suppressed in non-infected epidermal cells directly adjacent to pathogen-infected epidermal cells, suggesting that an intercellular signal(s) to suppress transcription for the PR-protein genes was dispatched from infected cells to non-infected neighbors in a compatible combination of the host and its powdery mildew pathogen. Thus, this microsuctioning method provided a useful cytological and molecular tool for pinpoint analysis of gene expression in cell-to-cell interactions at the site of powdery mildew infection in a susceptible host.

ACKNOWLEDGEMENTS

This work was supported in part by a Grant-in-Aid (No. 12660050) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We would like to acknowledge Dr. Beth E. Hazen for critically reading the manuscript.

REFERENCES

Berkelman, T., Houtchens, K. A. and DuPont F. M. (1994) Two cDNA clones encoding isoforms of the B subunit of the vacuolar ATPase from barley roots. Plant Physiol. 104, 287-288.

Bushnell, W. R., Dueck, J. and Rowell, J. B. (1967) Living haustoria and hyphae of Erysiphe *graminis* f. sp. *hordei* with intact and partly dissected host cells of *Hordeum vulgare*. Can. J. Bot. 45, 1719-1732.

Jacobsen, S., Mikkelsen, J. D. and Hejgaard, J. (1990) Characterization of two antifungal endochitinases from barley grain. Physiol. Plant. 79, 554-562.

Jutidamrongphan, W., Andersen, J. B., Mackinnon, G., Manners, J. M., Simpson, R. S. and Scott, K. J. (1991) Induction of β-1,3-glucanase in barley in response to infection by fungal pathogens. Mol. Plant-Microbe Interact. 4, 234-238.

Kogel, K-H., Beckhove, U., Dreschers, J., M nch, S. and Rommé, Y. (1994) Acquired resistance in barley. The resistance mechanism induced by 2,6-dichloroisonicotinic acid is a phenocopy of a genetically based mechanism governing race-specific powdery mildew resistance. Plant Physiol. 106, 1269-1277.

Kragh, K. M., Jacobsen, S., Mikkelsen, J. D. and Nielsen, K. A. (1991) Purification and characterization of three chitinases and one β-1,3-glucanase accumulating in the medium of cell suspension cultures of barley (*Hordeum vulgare* L.). Plant Sci. 76, 65-77.

Malehorn, D. E., Scott, K. J. and Shah, D. M. (1993) Structure and expression of a barley acidic β-glucanase gene. Plant Mol. Biol. 22, 347-360.

Matsuda, Y., Toyoda. H., Nogi, Y., Tamai, T. and Ouchi, S. (1992) Foreign gene expression in barley coleoptile epidermis: An improved system for gene transfer and in situ detection of gene expression by dual microinjection. Plant Tissue Cult. Lett. 9, 154-163.

Matsuda, Y., Toyoda, H., Morita, M., Ikeda, S. Tamai, T., Nishiguchi, T. and Ouchi, S. (1994). A novel method for *in situ* hybridization in fungal cells based on pricking micro-injection of photobiotin labelled probes. J. Phytopathol. 141, 133-142.

Matsuda, Y., Toyoda, H., Kurita, A. and Ouchi, S. (1997) *In situ* PCR technique based on pricking microinjection for cDNA cloning in single cells of barley coleoptile and powdery mildew pathogen. Plant Cell Rept. 16, 612-618.

Murray, M. G. and Thompson, W. F. (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8, 4321-4325.

Suoniemi, A., Narvanto, A. and Schulman, A. H. (1996) The BARE-1 retrotransposon is transcribed in barley from an LTR promoter active in transient assays. Plant Mol. Biol. 31, 295-306.

Swegle, M., Huang, J-K., Lee, G. and Muthukrishnan, S. (1989) Identification of an endochitinase cDNA

clone from barley aleurone cells. Plant Mol. Biol. 12, 403-412.

Toyoda, H., Matsuda, Y., Shoji, R. and Ouchi, S. (1987) A microinjection technique for conidia of *Erysiphe graminis* f. sp. *hordei*. Phytopathology 77, 815-818.

Toyoda, H., Matsuda, Y., Utsumi, R. and Ouchi, S. (1988) Intranuclear microinjection for transformation of tomato callus cells. Plant Cell Rept. 7, 293-296.

Toyoda, H., Yamaga, T., Matsuda, Y. and Ouchi, S. (1990) Transient expression of the ß-glucuronidase gene introduced into barley coleoptile cells by microinjection. Plant Cell Rept. 9, 299-302.

Toyoda, H., Matsuda, Y., Yamaga, T., Ikeda, S., Morita, M., Tamai, T. and Ouchi, S. (1991) Suppression of the powdery mildew pathogen by chitinase microinjected into barley coleoptile epidermal cells. Plant Cell Rept. 10, 217-220.

Toyoda, H. (1993) Transformation in *Hordeum vulgare* L. (Barley). In Biotechnology in Agriculture and Forestry Volume 22 (Bajaj, Y. P. S., ed). Berlin: Springer-Verlag, pp. 220-233.

オオムギ子葉鞘表皮細胞から顕微抽出した細胞質に存在するmRNAのDirect RT-PCR法による増幅 - 植物病原菌感染宿主細胞における遺伝子発現解析のモデルシステム

森浦展行*・松田克礼*・藤田和久*・和多泉季*・角谷晃司**・豊田秀吉*

*近畿大学農学部、**近畿大学薬学総合研究所

要　約

オオムギうどんこ病菌（*Blumeria graminis* f. sp. *hordai*）は、宿主植物であるオオムギに接触すると、速やかに宿主認識を行い、数分後には第一次発芽管を伸長する。その後、植物組織へ付着する器官として新たに付着器を分化し、表皮細胞への侵入を試みるとともにその細胞内に栄養吸収器官である吸器を形成する。一方、本病原菌の感染を受けた表皮細胞は、その感染過程の進行に対応し、異なる反応を示すと考えられている。しかしながら、これらの宿主細胞の反応が感染を受けている細胞に限定されること、また、その反応が数分単位で進行することから、病原菌の感染過程で誘導される遺伝子の発現を解析するためには、顕微鏡下で感染過程を連続的に観察し、感染を受けている特定細胞において遺伝子発現を検出する細胞レベルの解析系が必須となる。そこで本実験では、顕微鏡下で標的とした単一細胞からマイクロピペットを用いて細胞内容物を吸引し、そこに存在するmRNAを鋳型とした単一細胞RT-PCR/nested PCR法を適用することとした。実験材料としては、うどんこ病菌の感染過程が顕微鏡下で観察可能なオオムギ子葉鞘の内表皮細胞を供試した。まず、既に報告されているオオムギの構成発現遺伝子を検索し、Vacuolar ATPase B subunit(ATP-B)、Cytosolic triosephosphate isomerase(cTPI)、Glycolytic glyceraldehyde-3-phosphate dehydrogenase(gGAPDH)について、その塩基配列をもとにプライマーを構築した。また、核由来の増幅産物とmRNA由来の産物を識別するため、増幅産物にイントロン配列が含まれる位置にプライマーを設計し、スプライスの有無からその由来を判別することとした。顕微鏡下で標的細胞から細胞内容物を吸引する際には、細胞内に核を残存させ、核を含まない内容物を鋳型としてRT-PCR/nested PCRを行った。その結果、イントロン配列がスプライスされた増幅産物のみが検出され、核を含まない細胞質を吸引することにより実際に発現している遺伝子のPCR検出が可能となった。次に、病原菌の感染によって発現が誘導される遺伝子を検索し、Chitinase 2(CHI2)、β-1,3-glucanase(GLU)、について、前述と同様にイントロン配列を含む位置にプライマーを設計した。誘導型遺伝子の発現を厳密に評価するため、PCRを行う際に先の構成的発現遺伝子を増幅するプライマーを混合し、指標遺伝子として同時に増幅することにより、誘導型遺伝子の発現検出を行った。その結果、病原菌の感染を受けていない細胞およびその感染を受けている両者の細胞において指標とした構成的発現遺伝子は検出された。誘導型遺伝子として使用したCHI2遺伝子およびGLU遺伝子は病原菌の感染を受けていない細胞においてその発現が検出され、本菌の感染過程において抑制される傾向にあった。