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Expression of the *TaCR1* Gene Induced by Hessian Fly Larval Infestation in Wheat Carrying a *H21* Gene.

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ABSTRACT : The Hessian fly, *Mayetiola destructor* (Say), is known to be one of the major insect herbivores of wheat worldwide. In order to provide molecular events on interactions of the NIL with *H21* and larvae of Hessian fly biotype L, the *TaCR1* gene, *Triticum aestivum* cytokinin repressed 1, was isolated through the suppression subtractive hybridization, which was constructed using stems of the NIL with *H21* at 6 days after infestation as tester and stems of the recurrent parent Coker797 without *H21* at 6 days after infestation as driver. Transcript levels of *TaCR1* mRNA in the NIL with *H21* were highest at 6 days after infestation but in the Coker797 without *H21* until 8 days were similar with those of non-infested plants. Expression of the *TaCR1* gene was decreased at early time and then recovered after wounding or H_2O_2 treatment as well as 6-BAP treatment. Transcripts levels of the *TaCR1* gene was changed after MeJA, SA, ethephone, or ABA treatment. In drought treatment, the *TaCR1* gene were increased at early stage of stress and then decreased at late stage. Expression of the *TaCR1* gene was continued to decrease through 24 h in the cold treatment. Although the *TaCR1* gene is increased through infestation in NIL with *H21*, further study was required to elucidate a role on resistance against larvae of Hessian fly. However, the *TaCR1* gene could be used as marker gene on response of plants against abiotic stresses as well as application of plants with several hormones.

Keywords: *H21*, Hessian fly, infestation, *TaCR1*

Plants must defend many aggressive biotic agents, such as insects, bacteria, fungus, and nematodes, during their lifetime. Recently, researchers have elucidated the mechanisms of direct and indirect plant defense. Plants against insect attack respond to direct defenses, which make the plant more resistant to further herbivore. Hermsmeier *et al.* (2001) reported characterization of the transcriptional reorganization that occurred after insect attack and verified differential expression for 27 cDNAs. Van de Ven *et al.* (2000) reported that two genes were differentially expressed in local and systemic changes in squash to silverleaf whitefly feeding.

2BS/2RL wheat-rye translocation lines contain the *H21* gene, a resistant gene of Hessian fly, which is one of the major insect herbivores of wheat (Seo *et al.*, 1997). Seo et al. (1997, 2001) developed the molecular markers such as RAPD, AFLP, and STS associated with the *H21* gene through near-isogenic lines (NILs). Expressed sequence tags (ESTs) were analyzed from healthy leaves of young seedlings (Jang *et al.*, 1999) and from underside of leaf sheathes infested with larvae of Hessian fly biotype L in NIL carrying *H21* (Jang *et al.*, 2003a). Jang *et al.* (2002) reported two cDNAs encoding lipid transfer protein, which were induced by drought treatment, in the NIL.

The resistant wheat varieties were known to have tough stems with a large amount of hemicellulose for preventing normal feeding of Hessian fly larvae (Refai *et al.*, 1955). The larvae secret the pectinase for degradation of plant cell walls to access plants sap without complete disruption of cell walls (Grover *et al.*, 1988). Williams *et al.* (2002) reported that a lectin-like gene was differentially expressed by infestation of larvae of Hessian fly between resistant plants and susceptible plants. However, little is known on the molecular interactions of NIL carrying *H21* gene and biotype L of larvae of Hessian fly.

Cross talk of several signaling elicitors, e.g. jasmonate (JA) or methyl jasmonate (MeJA), salicylic acid (SA), and ethylene are regulated in plant defense against pathogen attack (Kunkel & Brooks, 2002). Chen *et al.* (1985) suggested the SA plays an important signaling role in local hypersensitive responses and systemic acquired resistance against many plant pathogens. JA or MeJA acts as a transmissible wound signal as plant defense against herbivore attack (Li *et al.*, 2002). In the report, we characterized a gene, *TaCR1*, induced by infestation of larvae of biotype L of Hessian fly in NIL carrying the *H21* gene. Regulation of the gene would be elucidated through treatments of several elicitors such as MeJA, SA, and ethephon, as well as H_2O_2 and wounding. Also, we examined expression mode of the gene on abiotic stresses such as drought and cold.

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MATERIALS AND METHODS

Plant materials and Infestation of larvae

Development of NILs for *H21* was described as Seo et al. (1997). An NIL with the *H21* gene and a recurrent parent Coker797 without *H21* were grown for 3 weeks at 25/18 (day/light) in a growth chamber. Biotype L of Hessian fly flax seeds was kindly provided by Drs. Roger H. Ratcliffe and Sue E. Cambron (Purdue Univ.). Bulk population of biotype L of Hessian fly flaxseeds stored in cold chamber was transferred to growth chamber at 24°C for emergence of adults. Periodic misting was applied to assure adequate moisture for a good emergence of flies. Seedlings grown for 3 weeks were transferred to Hessian fly emerging growth chamber when the insect population reached maximum. After inspection for eggs on the leaves, plants were transferred to Hessian fly free growth chamber set 24/18°C (day/night) with adequate moisture (over 60%). After infestation of larvae of Hessian fly, stem of the plants were harvested at 5, 6, 7, and 8 days. At 6 days, plant tissues were harvested from leaves, stems, and roots.

Suppression subtractive hybridization

In order to isolate differentially expressed genes between NILs by infestation of larvae of Hessian fly, suppression subtractive hybridization (SSH) were carried out with a PCR-Select™ cDNA Subtractive Kit according to commercial protocol (Clontech). Total RNAs were isolated from stems of the NIL with *H21* at 6 days after infestation as tester and stems of the recurrent parent Coker797 without *H21*. Two micrograms of mRNAs from both tissues were used for construction of subtractive cDNA library. After two hybridizations, two PCRs were performed to enrich and amplify the differentially expressed genes in the stems of infested NIL with *H21*. The subtracted cDNAs were ligated into pGEM-T Easy Vector (Promega) and transformed into *E. coli*.

Elicitor treatments

For treatment of several elicitors leaves of 3 week-old plants were sprayed with aqueous solution of 0.05% Tween 20 containing each of the following: 100 μM 6-benzylaminopurine (BAP), 100 μM methyl jasmonate (MeJA), 5 mM salicylid acids (SA), 10 mM ethephon, and 10 mM H_2O_2 and placed in a growth chamber set 25/18°C (day/night) and 16/8-h (day/night) photoperiods for 48 h. For treatments of MeJA, ethephon, and H_2O_2, the treated plants were covered by a transparent plastic bag. For the mock treatment, healthy plants were also covered by a plastic bag for 48 h and then harvested. The leaves of treated plants were harvested at 2, 6, 12, 24, and 48 h after the each treatments. For wounding treatment, leaves of the plants were inflicted with a surface-sterilized needle, about 0.3 mm in diameter. The leaves of wounded plants were harvested each at 0.5, 1, 3, 6, and 12 h after the treatment.

Abiotic stress treatment

Four week-old NILs carrying 2RL were transferred to a growth chamber (constant 4°C) and incubated for 24 h on a 12 h photoperiod for cold treatment. For ABA treatment, a NIL carrying 2RL were sprayed with a 100 μM solution of ABA containing 0.05% (v/v) Tween 20 and placed in a growth chamber at 25°C/18°C (day/night) for 24 h on a 12 h photoperiod. Tissues from the cold- and ABA-treated plants were harvested at 0, 6, 12, 18, and 24 hours after the treatment initiation and were immediately frozen in liquid nitrogen. For the drought treatment, the NIL carrying 2RL was grown for 4 weeks under a normal watering regime, and subsequently exposed to drought stress (without applications of water). The plants were harvested 1, 2, 3, 4, and 5 days after the drought treatment initiation.

RNA isolation and Northern blot analysis

Total RNAs of individual samples were extracted with Trizol, according to the manufacturers instructions (InVitrogen). The extracted RNAs (20 μg) were separated on 1% formaldehyde agarose gel and transferred to a positive nylon membrane (MSI). cDNAs were labeled with Biotin-incorporated dCTP by PCR. Hybridization and detection methods were performed as described by Jang *et al.* (2003b).

RESULTS

Isolation of the differential expressed gene, *TaCR1*

In order to provide molecular events on interactions of the NIL with *H21* and biotype L of Hessian fly larvae, the subtractive library was constructed using stems of the NIL with *H21* at 6 days after infestation as tester and stems of the recurrent parent Coker797 without *H21* at 6 days after infestation as driver. One hundred clones were randomly selected from each subtractive library. Several clones were isolated using differential hybridization methods from the subtractive library (data not shown). A partial clone showed homology with a cytokinin-repressed gene in cucumber termed CR9 (Teramoto *et al.*, 1993) Unfortunately, we did not isolate any full-length clone in cDNA library constructed from stems of

infested NIL with *H21* (Jang *et al*, 2003a). The SSH partial clone was designed as *TaCR1* (*Triticum astivum* Cytokinin Repressed gene1) because it possessed high similarity with C-terminal region of CR9 (data not shown).

Gene expression of the *TaCR1* gene by infestation of larvae of Hessian fly

In order to examine gene expression induced by larval infestation, stems of the NIL with *H21* and the Coker797 without *H21* were harvested at 5, 6, 7, and 8 days after infestation. As shown in Northern blot, the *TaCR1* gene was differentially expressed between the NIL with *H21* and the Coker797 without *H21* (Fig. 1). Transcript levels of *TaCR1* in the NIL with *H21* were highest at 6 days after infestation, but in the Coker797 without *H21* the transcript levels were similar with that of non-infested control plants.

Northern blot was carried out in three tissues such as leaves, stems, and roots of NIL with *H21* at 6 days after infestation. *TaCR1* was expressed in leaves and stems but not in roots (Fig. 2). The expression levels of *TaCR1* were highest in leaves of the NIL with *H21*.

Response on treatments of 6BAP, MeJA, SA or ethephon

The *TaCR1* gene induced by infestation of larvae of Hessian fly were examined on regulation by several elicitors such as 6BAP, MeJA, SA, and ethylene in NIL with *H21* (Fig. 3). Transcripts of the *TaCR1* gene were decreased 2 h, increased 6 and 12 h and then again decreased from 24 h after the 6-BAP treatment. In treatment of MeJA, expression of *TaCR1* gene was slightly increased at 24 h after treatment and then continued to increase until 48 h. SA treatment dramatically changed responses of the *TaCR1* genes. Expression of *TaCR1* gene was gradually increased from 2 through 48 h after treatment. In order to examine responses to ethylene, ethephon that is a chemical releasing gaseous ethylene was applied. For mock treatment, we also treated hydrochloric acid (HCl) and phosphonic acid (H_3PO_3) because ethephon was known to produce HCl and H_3PO_3 as breakdown products for ethylene releasing. However, treatment of HCl and H_3PO_3 did not affect expression of the *TaCR1* genes. The *TaCR1* gene reached highest expression at 6 h after ethephon treatment.

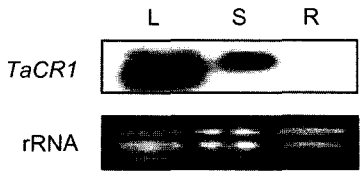

Fig. 2. Northern blot hybridization of the *TaCR1* gene from different tissues of the NIL with *H21* infested larvae of biotype L of Hessian fly. Tissues were harvested from the plants at 6 days after infestation of larvae of biotype L of Hessian fly. L; leaves, S; stems, R; roots.

Response to the treatments of wounding or H_2O_2

Response to wounding or H_2O_2 treatment was represented in Fig. 4. In the wounding treatment, *TaCR1* gene was rapidly decreased from 0.5 h after treatment and then rapidly increased from 3 h. For the H_2O_2 treatment, transcripts of *TaCR1* gene were rapidly decreased at 2 h after treatment and then increased from 6 h after the treatment.

Expression of *TaCR1* on ABA, drought, or cold treatment

In order to study expression of the *TaCR1* gene on abiotic stresses, NIL with *H21* was suffered by drought, and cold treatment, or exogenous ABA application (Fig. 5). Transcripts of the *TaCR1* gene were increased 1 day to 2 days and then decreased until 5 days after the non-irrigation. In the cold treatment, expression of the *TaCR1* gene was continued to decrease through 24 h. The *TaCR1* gene was increased 6 h, decreased until 18 h and then recovered 24 h after ABA application.

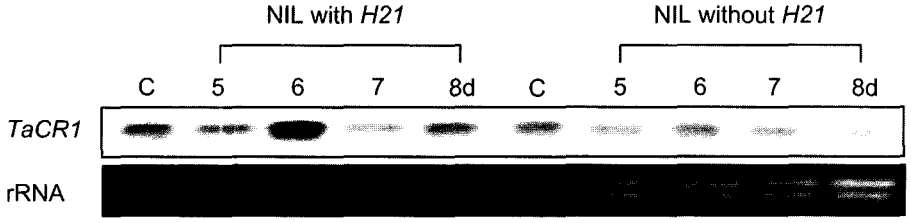

Fig. 1. Northern blot hybridization of the *TaCR1* gene from stems of the NIL carrying *H21* gene and the Coker797 infested larvae of biotype L of Hessian fly. Stem materials were harvested from the plants infested larvae of biotype L of Hessian fly at 5, 6, 7,and 8 days. d; days.

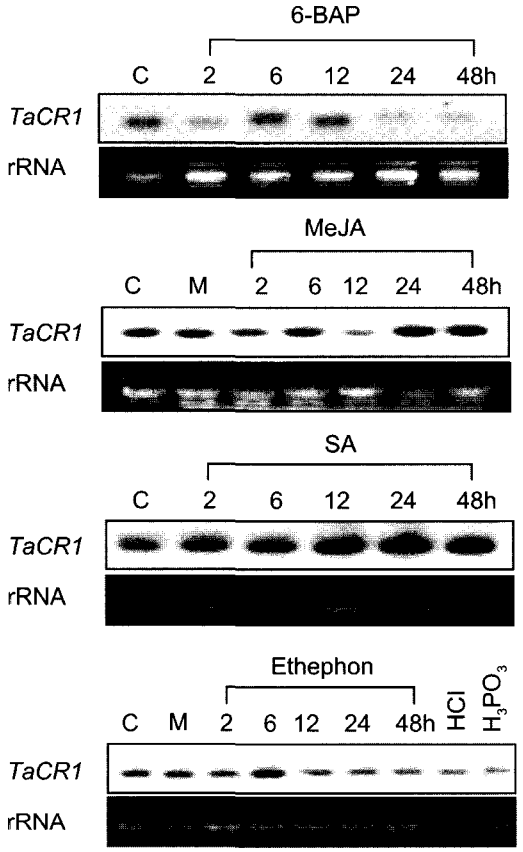

Fig. 3. Northern blot hybridization of the *TaCR1* gene in the leaves of the NIL with *H21*, which had been treated with 6-benzylaminopurine (BAP), methyl jasmonate (MeJA), salicylic acid (SA), or ethephone. The total RNA of tissues was fractionated on a 1% denaturing agarose gel. Leaf materials were harvested from plants sprayed with each solution of 6-BAP (100 μM), MeJA (100 μM), SA (5 mM), or ethephon (10 mM) with 0.05% (v/v) Tween 20 at 2, 6, 12, 24, and 48 h. Healthy plants were also covered with a transparent plastic bag for 48 h. To treat HCl and H_3PO_3, plants were sprayed with solutions of HCl (10 mM) or H_3PO_3 (10 mM) containing 0.05% (v/v) Tween 20 and the leaf materials were harvested 24 h after treatment. C, healthy plants, M: mock; h: hours.

DISCUSSION

After mating of Hessian fly, oviposition and larval infestation take 3-5 days and death of larvae occurs 2-3 days in the resistant plants. Therefore, transcription changes by infestation of larvae were expected to begin at 4-6 days after establishment. We performed SSH analysis at 6 days after emergence of Hessian fly. The *TaCR1* gene was induced at 6 days after oviposition in the NIL with *H21* but not in the Coker797 without *H21*. Our other results were similar to expression of the *TaCR1* gene. The *TaCR1* gene was increased from 5 days after oviposition in NIL with *H21* but not in Coker797

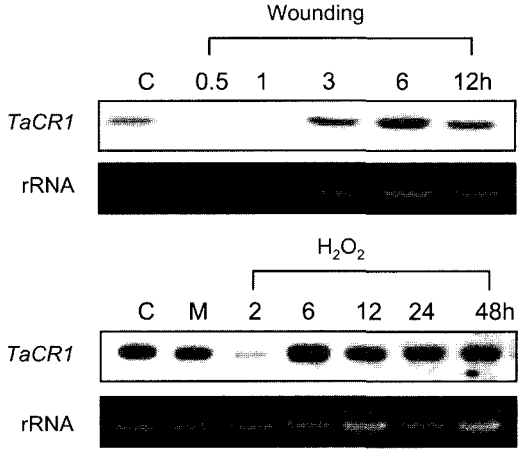

Fig. 4. Northern blot hybridization of the *TaCR1* gene from leaves of NIL with *H21* that has been treated with wounding or H_2O_2. Leaf materials were harvested from plants wounded with a sterilized needle (diameter 0.3 mm) at 0.5, 1, 3, 6, and 12 hrs after wounding. Plants were sprayed with a solution of H_2O_2 containing 0.05% (v/v) Tween 20, and leaf materials were harvested at 2, 6, 12, 24, and 48 h after treatment. Healthy plants were also covered with a transparent plastic bag for 48 h. C, healthy plants; M, mock, h, hours.

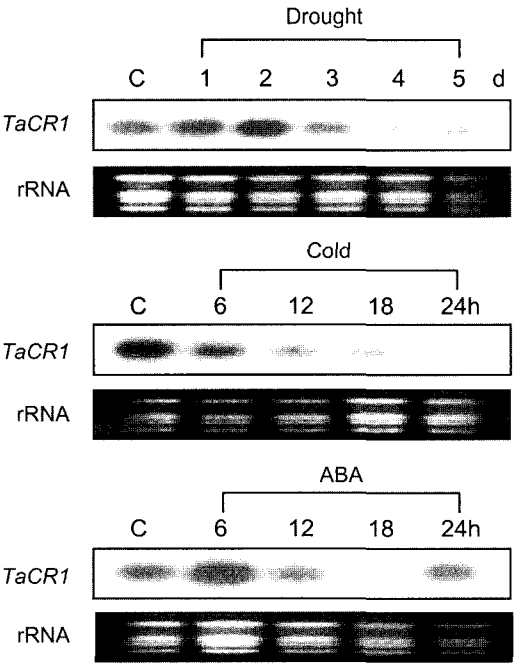

Fig. 5. Northern blot hybridization of the *TaCR1* gene in leaves of NIL with *H21* treated with drought, cold or ABA. The leaf material was harvested from the plant with drought treatment for 1, 2, 3, 4, and 5 days. The leaf material was harvested from the plant incubated at 4°C for 6, 12, 18, and 24 hours. The leaf material was harvested from the plant sprayed with 100 μM ABA with 0.3% tween 20 for 6, 12, 18, and 24 hours. C : control, d: day, h : hour.

without *H21* (unpublished data). Williams *et al.* (2002) reported transcripts of lectin-like gene were increased in leaf blades of the wheat cv. Iris for about 48 h after infestation.

The *TaCR1* gene showed high similarity with cytikinin-repressed gene, CR9, in the cucumber (Teramoto *et al.*, 1993). Transcripts of CR9 mRNAs were decreased tenfold within 4 hrs after N^6-benzyladenine (BA) treatment, but not by adenine or 2,4-dichlorophenoxyacetic acid (2,4-D). Furthermore, levels of the *CR9* gene decreased during the early phase of greening and soon after wounding of cotyledon (Teramoto *et al.*, 1993, 1995). Cytokinins are essential plant hormones that are related to shoot meristem and leaf formation, cell division, chloroplast biogenesis and senescence. Also, the roots are expected to be the major site of cytokinin synthesis in plant (Chen *et al.*, 1985). The *TaCR1* gene is highly expressed in leaves and stems than roots. This fact was expected to inhibit expression of the *TaCR1* gene in the roots, which are site of cytokinin synthesis. As shown in Northern blotting, the *TaCR1* gene was rapidly decreased 2 h after 6-BAP treatment. Also, this result was coincided with the nature of the *TaCR1* gene, which is cytokinin-repressed gene.

Expression mode of *TaCR1* gene was similar with that of the *CR9* gene in that decreasing of transcripts after wounding treatment. Also, transcripts *TaCR1* gene were rapidly decreased after H_2O_2 treatment. Cytokinin level is increased through wounding treatment in plant. Decreasing of the *TaCR1* gene after wounding or H_2O_2 treatments was speculated to produce endogenous cytokinin derived from cell wall damage.

Plant response to Hessian fly larval infestation could be cross-talking among several signal elicitors, such as MeJA, SA, and ethylene. In our results on responses to MeJA, SA, or ethephon, *TaCR1* gene was slightly increased after treatments. Therefore, theses facts suggested that transcript levels of the *TaCR1* gene were regulated by cross talking of MeJA, SA or/and ethylene produced by infestation of larvae of Hessian fly in the NIL with *H21*.

Abiotic stress typically resulted in changes of transcripts of stress related genes and in the accumulation of several hormones such as ABA or SA. ABA accumulation is expected to provide plants with a tolerance to abiotic stresses (Chandler and Robertson, 1994). SA plays an important role not only in plant defense mechanism to pathogen attack, but also in the tolerance to water stress, such as drought and high concentration of NaCl (Senaratna *et al.*, 2000) Transcripts of the *TaCR1* gene were increased through ABA or SA treatment, although differences of increasing time. In drought treatment, the *TaCR1* gene was increased in 1 day to 2 days and then decreased until 5 days. In the previous report, Jang *et al.* (2002) reported the *TaLTP1* and *TaLTP2* genes were increased 3 days, in which the plants showed initiation of visually detectable symptoms, after drought treatment. Also, expression of the *TaCR1* gene was gradually decreased from 6 to 24 h after cold stress. The results suggested that expression of the *TaCR1* gene was increased at early stage of stress treatment and then decreased from late stage.

In this report, we observed expression mode of the *TaCR1* gene, which is differentially expressed between the NILs to infestation of larvae of Hessian fly. Although the *TaCR1* gene is increased through infestation in NIL with *H21*, further study was required to elucidate a role on resistance against larvae of Hessian fly. However, the *TaCR1* gene could be used as marker gene on response of plants against abiotic stresses as well as application of plants with several hormones.

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