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Benzothiadiazole, a Novel Class of Inducers of Systemic Acquired Resistance, Activates Gene Expression and Disease Resistance in Wheat

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Systemic acquired resistance is an important component of the disease resistance repertoire of plants. In this study, a novel synthetic chemical, benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), was shown to induce acquired resistance in wheat. BTH protected wheat systemically against powdery mildew infection by affecting multiple steps in the life cycle of the pathogen. The onset of resistance was accompanied by the induction of a number of newly described wheat chemically induced (WCI) genes, including genes encoding a lipoxygenase and a sulfur-rich protein. With respect to both timing and effectiveness, a tight correlation existed between the onset of resistance and the induction of the WCI genes. Compared with other plant activators, such as 2,6-dichloroisonicotinic acid and salicylic acid, BTH was the most potent inducer of both resistance and gene induction. BTH is being developed commercially as a novel type of plant protection compound that works by inducing the plant's inherent disease resistance mechanisms.

INTRODUCTION

As early as 1901, it was recognized that plants previously infected by a pathogen became resistant to further infection (Beauverie, 1901; Ray, 1901). Over the 30 years that followed these initial reports, many descriptive studies were published that, taken together, suggested the existence of an immune system in plants. In 1933, Chester questioned whether the "acquired immunity of the animal type occur(s) in plants." He concluded that there was substantial evidence supporting such a process and that we "must be awake to the possibility of distinct manifestations of acquired immunity in the plant realm" (Chester, 1933). In retrospect, it is clear that several independent processes were described by Chester, including viral cross-protection, bacterial and fungal antagonism, and a response later known as induced systemic resistance (ISR).

Over the past 60 years, both viral cross-protection and antagonism have received intense study. Cross-protection by attenuated, live viruses has been used successfully to control disease in several agricultural systems (Fulton, 1986). An understanding of the mechanisms underlying cross-protection has led to our ability to exploit this control mechanism by using virus-resistant, genetically engineered plants (Powell Abel et al., 1986; reviewed in Fitchen and Beachy, 1993; Wilson, 1993).

Fungal and bacterial antagonism also have been the focus of many years of research and have evolved into the discipline known as biocontrol (Cook and Baker, 1983). Although biocontrol has been deployed successfully, it is thought that a more effective use will result from a better understanding of the molecular basis for this mechanism of disease control (Lam and Gaffney, 1993).

A third phenomenon summarized by Chester was a plant response we now call ISR (Gianinazzi, 1984; Kloepper et al., 1992). This response is induced by a prior pathogen infection and can be distinguished both from cross-protection, in that the resistance is not specific, and from antagonism, in that an active plant process is involved in the resistance. Although there may be several types of ISR processes in plants, the one that has been studied extensively is systemic acquired resistance (SAR) (Ross, 1961a; Ryals et al., 1994). SAR is induced by pathogens that cause a necrotic reaction that can range from a hypersensitive response (Ross, 1961b) to a necrotic disease lesion (Kuc and Richmond, 1977). The resistance response is characterized by the induction of long-lasting, systemic resistance that is often effective against viral, bacterial, and fungal pathogens. SAR is characterized by the spectrum of both disease resistance and the genes activated in resistant tissue. For example, in tobacco, SAR provides a significant level of protection against tobacco mosaic virus, *Pseudomonas syringae* pv *tabaci*, *Cercospora nicotianae*, *Phytophthora parasitica*,

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Peronospora tabacina, and *Erwinia carotovora* (Vernooij et al., 1995; Friedrich et al., 1996).

In both tobacco and Arabidopsis, SAR is associated with the coordinate expression of a set of so-called SAR genes. The set of tobacco SAR genes is composed of a number of nonallelic genes that can be broadly classified as encoding pathogenesis-related (PR) proteins (e.g., Ward et al., 1991). The gene families that constitute the SAR genes apparently play an active role in the resistance process because their expression in transgenic plants can impart significant disease resistance (Broglie et al., 1991; Alexander et al., 1993; Liu et al., 1994; Logemann et al., 1994; Jach et al., 1995). In Arabidopsis, the SAR response involves the induction of a subset of the genes expressed in tobacco (Uknes et al., 1992), suggesting that the types of SAR genes expressed may be unique among species. Thus, although there may be several types of ISR responses, SAR can be distinguished by broad-spectrum, systemic resistance and the associated expression of a particular set of genes.

Although little is known about the signal transduction pathway leading to SAR, one step apparently involves the accumulation of salicylic acid (SA). SA levels are elevated at the onset of SAR in cucumber (Métraux et al., 1990), tobacco (Malamy et al., 1990), and Arabidopsis (Uknes et al., 1993). The exogenous application of SA to leaves of tobacco or Arabidopsis induces resistance against the same spectrum of pathogens and the same set of SAR genes, as with pathogen-induced SAR. Transgenic plants expressing a bacterially derived gene that encodes salicylate hydroxylase (*nahG*), an enzyme that converts SA to catechol, are unable to induce SAR (Gaffney et al., 1993). Taken together, this evidence strongly suggests that SA plays an important role in the signal transduction pathway leading to SAR. However, recent studies indicate that SA is not likely to be the systemically transmitted signal (Vernooij et al., 1994).

Although SAR was first described as a response to pathogen infection, it recently has been found that treatment of plants with the synthetic chemical 2,6-dichloroisonicotinic acid (INA) also can induce SAR (Métraux et al., 1991). In both tobacco and Arabidopsis, INA induces the same spectrum of pathogen resistance and gene expression as does pathogen infection (Ward et al., 1991; Uknes et al., 1992; Lawton et al., 1995; Vernooij et al., 1995). Because INA can restore resistance in tobacco and Arabidopsis plants expressing *nahG* and because both INA and SA fail to induce SAR in an Arabidopsis mutant blocked in the SAR pathway (Delaney et al., 1995), INA and SA most likely induce SAR via the same signal transduction pathway. INA initially showed promise as a plant protection compound but was insufficiently tolerated by some crops to warrant practical use.

Most of the studies on SAR have been conducted in dicotyledonous plants, and there is less known about SAR in monocots. SAR has been demonstrated in rice, where an inducing infection by *P. s.* pv *syringae* led to systemic protection against *Pyricularia oryzae* (Smith and Metraux, 1991), the causative agent of leaf blast, and in barley and wheat, where a prior infection by *Erysiphe graminis* led to enhanced protection against *E. graminis*, the causative agent of powdery mildew (Schweizer et al., 1989; Hwang and Heitefuss, 1992). Chemically induced resistance by INA has been described recently in barley (Kogel et al., 1994; Wasternack et al., 1994).

Here, we describe the mode of action in wheat of a novel SAR inducer, benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester, CGA-245 704 (BTH; Schurter et al., 1987). When this compound is sprayed on wheat plants at an early developmental stage, resistance is induced against *E. graminis*, *Puccinia recondita*, and *Septoria* spp. BTH treatment induces the accumulation of transcripts from a number of novel plant genes that also are shown to be induced during pathogen infection. A close relationship exists between the induction of these chemically induced wheat genes and the expression of resistance following SA, INA, or BTH treatment. SAR has been suggested to have great promise as a disease control measure in plants. However, in contrast to viral cross-protection and biocontrol, it has yet to be exploited in agricultural practice. Currently, BTH is being developed as a disease control compound and represents a novel disease control measure that works by inducing the plant's own pathogen defense mechanism. We believe that this new type of plant protection strategy will have a significant impact on agriculture by providing the farmer with a new option for disease control.

RESULTS

Characteristics of BTH-Induced Resistance against Powdery Mildew

Figure 1 shows the structure of BTH compared with those of SA and INA. When applied to leaves, BTH can protect diverse species of dicotyledonous plants, including tobacco and

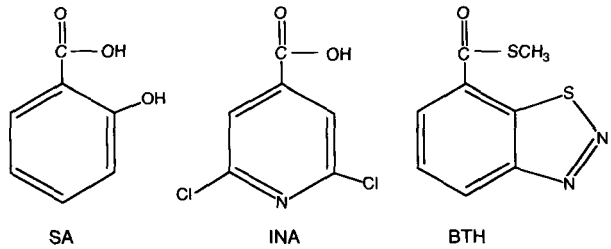

Figure 1. Structures of SAR-Inducing Compounds.

Comparison of the structures of three known plant activators: SA, INA, and BTH.

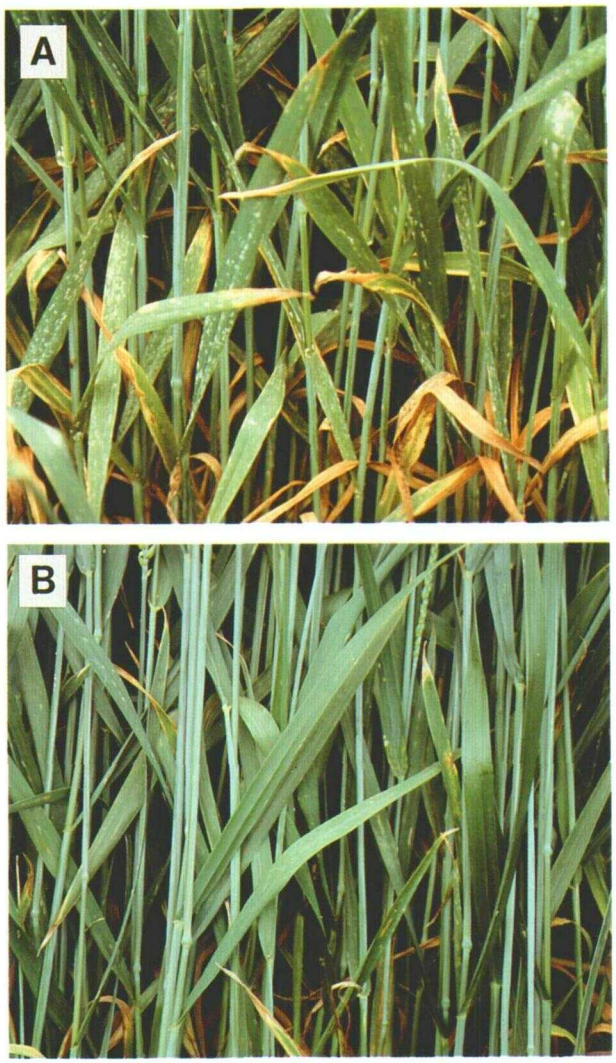

Figure 2. Phenotypic Expression of BTH-Induced Resistance.

The influence of BTH on the spread of *E. g. tritici* on wheat plants is shown. Field-grown plants were treated with 30 g of BTH per hectare. Plants were photographed 2 months after treatment.
(A) Powdery mildew development on untreated plants.
(B) Powdery mildew development on BTH-treated plants.

Arabidopsis, from diseases caused by various viral, bacterial, and fungal pathogens (Lawton et al., 1996). In addition, BTH treatment can protect monocotyledonous plants from disease. For example, BTH treatment of seedlings protects rice from the blast fungus *Magnaporthe grisea* for an entire season. Thus, BTH is a broad-spectrum plant protection compound with respect to both the diversity of protected plant species and the kind of pathogens against which it acts.

As demonstrated in Figure 2, BTH application to wheat before fungal infection resulted in a long-lasting resistance against powdery mildew caused by *E. g.* f sp *tritici.* Plants treated at the two-node stage were effectively protected against powdery mildew for the entire growing season (Figure 2B), in contrast to a field showing severe disease symptoms upon natural inoculation (Figure 2A). Efficient protection also was achieved against infection by the leaf rust fungus *P. recondita* and *Septoria* leaf spot. Treatment of wheat plants with 30 g of BTH per hectare in repeated field trials led to an average of 35% reduction in disease symptoms caused by either of these two wheat pathogens compared with the untreated control plants. BTH treatment of field-grown plants led to an increase in yield of ~18% relative to untreated control plants and compared with a 17% yield increase in plants treated with a combination of Tilt (125 g per hectare) and fenpropidin (500 g per hectare).

BTH treatment affects different steps of the life cycle of *E. g. tritici* on infected wheat plants (Figure 3). Although germination rate and appressorium formation of the fungus were not altered by BTH application, the penetration rate dropped to ~30% compared with control plants. One possible explanation for the unsuccessful fungal penetration attempts is an increase in the formation of effective papillae in BTH-treated tissue, as demonstrated in Figures 4C through 4F; this plant response is usually observed only to a minor extent in the compatible interaction of cereals with powdery mildew (Kogel et al., 1994). In addition, the formation of mature primary haustoria at successful penetration sites was reduced to ~50%, and only 50% of those went on to form secondary haustoria. Both reduced penetration and reduced haustoria formation could be caused partially by hypersensitive reactions of BTH-treated

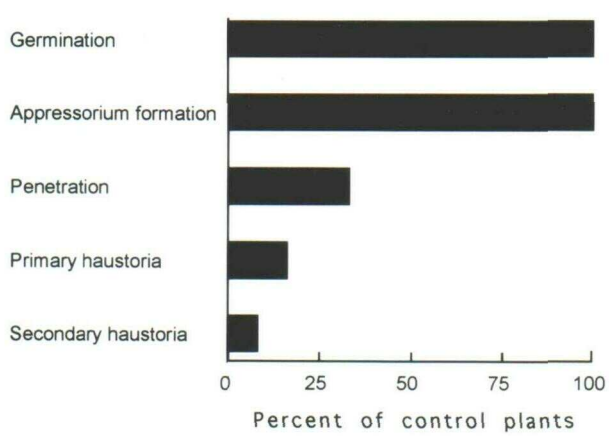

Figure 3. Reduction of Disease Progression in BTH-Treated Plants.

Germination rate, formation of appressoria, frequency of penetration, and formation of mature primary and secondary haustoria were determined for BTH-treated (0.3 mM) and control plants (set to 100%). Inoculation was performed 4 days after chemical treatment, and the development of 300 conidia was monitored.

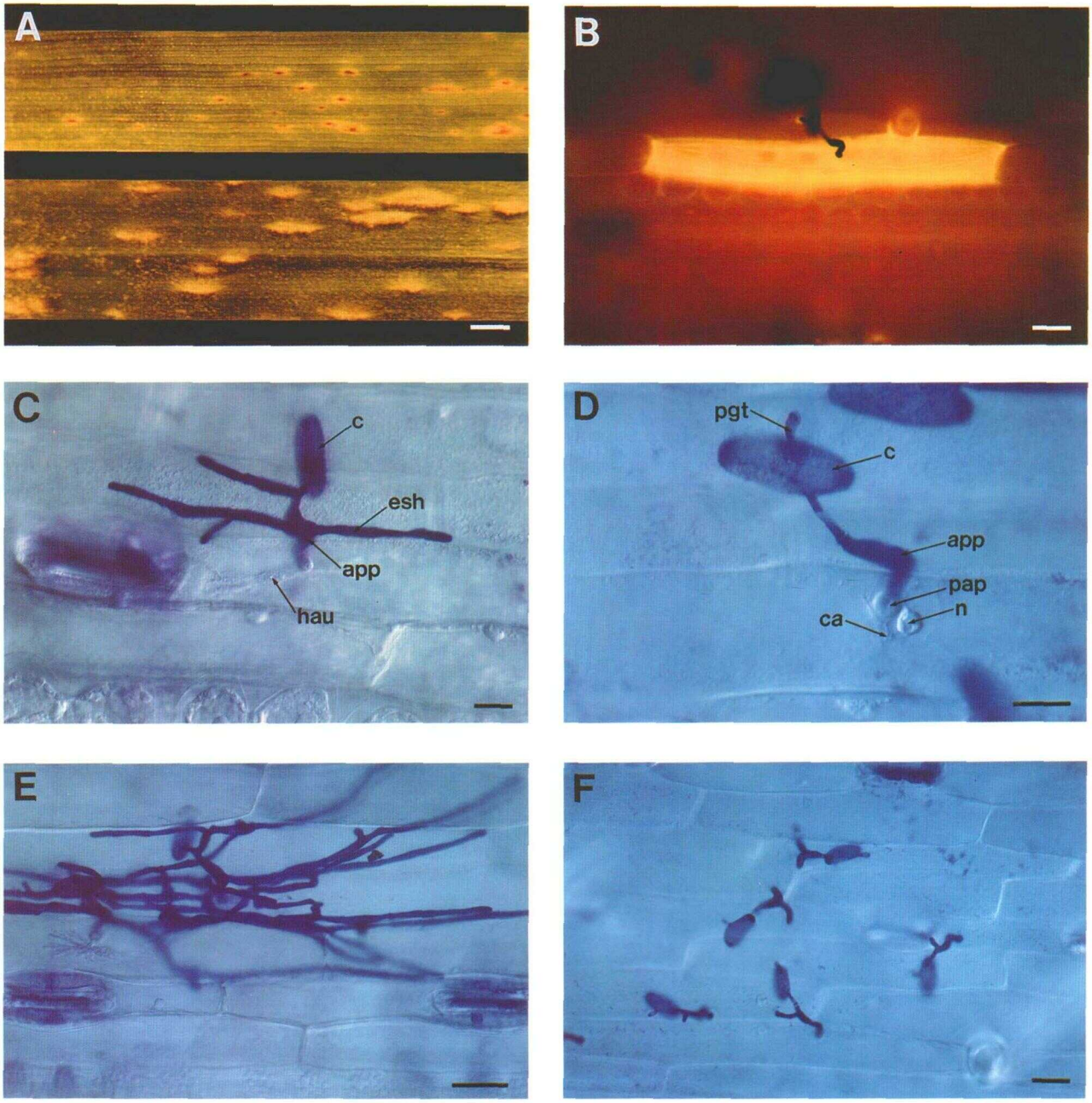

Figure 4. BTH-Mediated Resistance Responses.

(A) Infection phenotypes 10 days after inoculation with *E. g. tritici*. Plants were sprayed with BTH (top, 0.5 mM) or, as a control, were not sprayed (bottom).
(B) Autofluorescence of a BTH-mediated hypersensitive reaction of an attacked epidermal cell 48 hr after inoculation (UV light excitation, λ = 485 nm).
(C) and **(D)** Infection sites in control **(C)** and BTH-treated **(D)** leaves 48 hr after inoculation.
(E) and **(F)** Infection sites in control **(E)** and BTH-treated **(F)** leaves 72 hr after inoculation.
Formation of mature haustoria and secondary hyphae indicates a compatible interaction in the controls, whereas fungal development is restricted by cell wall appositions (papillae) and/or a hypersensitive reaction after BTH treatment. At later stages, mycelium with secondary haustoria develops into the epidermal layer of control plants. app, appressorium; c, conidia; ca, cytoplasmic aggregation; esh, elongated secondary hyphae; hau, haustorium; n, nucleus; pap, papilla; pgt, primary germ tube. Bars = 2 mm in **(A)**, 25 μm in **(B)**, **(E)**, and **(F)**, and 10 μm in **(C)** and **(D)**.

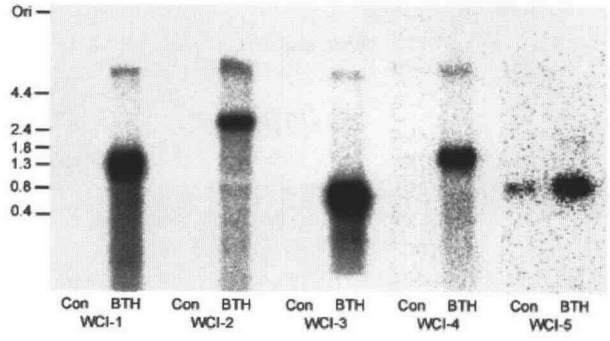

Figure 5. Induction of WCI Genes by BTH Treatment.

Two-week-old wheat plants were treated with water (Con) or 0.3 mM BTH (BTH). After 2 days, total RNA was isolated and subjected to gel blot analysis (2 μg per lane). Inserts of the isolated WCI clones were used as probes. Positions of length standards are at left, indicated in kilobases. Ori, origin.

cells (Figure 4B) upon attack of a fungal spore. This reaction occurred in ~40% of all infection sites in BTH-treated tissue, whereas it was not observed in untreated control tissue. As a result of the impaired development of the fungus, the total number of secondary haustoria formed in BTH-treated plants was <10% of the number formed in untreated control plants (Figure 3); this impairment was also reflected in a phenotypic difference in the disease development (Figure 4A). In addition, growth of mycelia, as measured by mycelia circumference, was strongly influenced by BTH treatment (data not shown). Similar effects on fungal pathogens are known from biologically induced systemic resistance in cucumber and tomato (Kováts et al., 1991a, 1991b).

No influence on a number of plant pathogens from different fungal classes could be observed in vitro by treatment with BTH. A series of in vitro assays was performed with fungi, including tests with *P. infestans* (Oomycetes), *Botrytis cinerea*, *Alternaria* spp, *Fusarium culmorum* (Hyphomycetes), *Ustilago maydis* (Hemibasidiomycetes), *S. nodorum* (Coleomycetes), and *Rhizoctonia solani* (Agonomycetes), without detection of any fungicidal activity of BTH (Friedrich et al., 1996). These data demonstrate that BTH does not act as an antibiotic in vitro.

Isolation of BTH-Inducible Genes from Wheat

In dicots, the onset of pathogen- or chemically induced SAR is accompanied by the accumulation of a number of mRNAs encoded by so-called SAR genes. To determine whether BTH-induced resistance in wheat also correlated with enhanced mRNA accumulation, we isolated cDNAs expressed predominantly in BTH-treated leaves. Using differential screening (St. John and Davis, 1979) and differential display (Liang and Pardee, 1992), we isolated five BTH-induced wheat cDNAs referred to as cWCI-1 through cWCI-5 (for wheat chemical induction) that were detected in wheat plants 4 days after BTH treatment. RNA gel blot analysis showed a strong differential accumulation of the corresponding transcripts in BTH-treated and control plants, as shown in Figure 5. Sequence analysis of the WCI clones revealed no homology with previously described SAR genes. Three encode polypeptides with limited homology with known proteins. Characteristics of the cWCI clones are summarized in Table 1. For all clones, except cWCI-2, the detected transcript size is consistent with the length of the cDNA clones, indicating that they are at least near full length (GenBank accession numbers are U32427 through U32431 for WCI-1 through WCI-5).

Characteristics of WCI Clones

The deduced amino acid sequence of cWCI-1 is shown in Figure 6. The longest open reading frame found in cWCI-1 starting with the first ATG encodes a protein of ~37 kD, and the sequence surrounding the proposed start codon fits the consensus for plant initiation codons (Lütcke et al., 1987). The C-terminal half of the postulated polypeptide (residues 192 to 344) has significant homology with two previously described rice proteins of unknown function, salT and COS9. The *salT* gene encodes a salt and drought stress–associated protein (Claes et al., 1990), and *COS9* is expressed specifically in roots (de Pater and Schilperoort, 1992). WCI-1 expression was not detected in organs of untreated wheat plants, including seedlings, roots, leaves, anthers, and pistils (data not shown). The N terminus (amino acids 1 to 191) of WCI-1 has no significant homology with known sequences or peptide motifs. Furthermore, it does not contain sequences similar to known protein targeting signals; thus, cWCI-1 may encode a soluble, cytosolic protein.

Table 1. Characteristics of Isolated WCI cDNA Clones

cDNA	Length (bp)	Detected Transcript Length (bp)	Properties and Functions of Encoded Proteins
cWCI-1	1209	~1300	Function unknown; C terminus homolog of rice stress gene
cWCI-2	1818	~2900	Lipoxygenase (C terminus)
cWCI-3	688	~750	Function unknown; cysteine-rich C terminus
cWCI-4	1353	~1400	Cysteine proteinase
cWCI-5	862	~950	Function unknown; no similar proteins known

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      1                                                          60
WCI-1 MSPQPTLATTTTPATGPNPSYYQTAPVSQESIQRTEVLLHLYAYQHTQGKPNANQTVIVD

      61                                                         120
WCI-1 PKLPACFGALAANDWTIYDGTCTNANLVAHAQGLHIGAGMTKENWFICFNMVFVDRRFMG

      121                                                        180
WCI-1 SSFKVMGDFRGNEGEWAIVGGTGEFAYAQGVITFNKTWSAQANVRELHVRALCLSFSKAP

      181                                                        240
WCI-1 ETPCSRTPRESSVTKIGPWGKISGEFLDVPTTPQRLECVTIRHGVVIDSLAFSFVDQAGG
salT            MTLVKIGLWGGNGGSAQDISVPPKKLLGVTIYSSDAIRSIAFNYIGVDGQ
COS9          MSTQLVKIGTWGGNGGGRVDLSVLPRSLKSVTIRSGAAIDAIAFTYIGTDGK

      241                                                        300
WCI-1 QHNVGPWGGPCGDNKDTIKLGPSEIVTEVSGTIG VFGAANVEYNAITSLTITTNVRTYG
salT  EYAIGPWGGGEG TSTEIKLGSSEHIKEISGTHGPVYDLADI    VTYLKIVTSANNTY
COS9  EHLAGPWGGGGG NPTTITLGSQEFVKGISGTFTNV          VTNLKIVTNVT TY

      301                                        344
WCI-1 PFGEPQCTRFSVPVQDKSSIVGFFVCARKYVEALGVYVCPPISN
salT  EAGVPNGKEFSIPLQDSGHVVGFFGRSGTLIDAIGIYVHP
COS9  NFGQGGGTAFSLPLQ SGSVVGFFGRAGALVDSIGVYVHI
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Figure 6. Deduced Amino Acid Sequence of cWCI-1 and Comparison with Known Proteins.

Primary structures of WCI-1 are compared with a root-specific (COS9) and a stress-induced (salT) protein from rice. Identical amino acids are double underlined.

The difference between the length of cWCI-2 and the detected transcript size (Table 1) suggests that cWCI-2 is not a full-length cDNA. Nevertheless, the deduced amino acid sequence (data not shown) was consistent with the C terminus of a lipoxygenase, showing ~80% similarity to lipoxygenases from barley (Vanmechelen et al., 1995) and rice (Ohta et al., 1992). Lipoxygenases have been shown to be involved in plant–pathogen interactions in a number of plant species (Siedow, 1991; Farmer, 1994). The size of the detected mRNA is in agreement with the expected size of a full-length lipoxygenase mRNA.

The deduced amino acid sequence of cWCI-3 is shown in Figure 7. cWCI-3 contains an open reading frame encoding a polypeptide of 9.8 kD preceded by two in-frame stop codons. The encoded protein contains two characteristic domains, a hydrophobic N terminus and a cysteine-rich C terminus. The N terminus, underlined in Figure 7, resembles a signal peptide found in protein precursors targeted to the endoplasmic reticulum (von Heijne, 1985). Two potential cleavage sites consistent with the requirement for small amino acids in positions −3 and −1 (von Heijne, 1986) are located at residues 26 and 31. The proposed mature protein of ~60 amino acids contains 10 cysteine residues, and the precursor contains an additional six methionine residues, classifying this protein as sulfur rich. WCI-3 shows high structural similarity to a thionin-like protein from petunia (Karunanandaa et al., 1994) in that it also is synthesized as a precursor protein and contains sulfur-rich amino acids at conserved positions (Figure 7). Endoplasmic reticulum retention signals were not evident in WCI-3, indicating that the mature protein is probably either secreted to the apoplast or localized in the lysosomal compartment, consistent with the localization of other sulfur-rich proteins from plants (Bohlmann, 1994).

The cWCI-4 clone may encode a cysteine proteinase, an enzyme class involved in activation and processing of other proteins. The primary structure as well as the deduced amino acid sequence of cWCI-4 are shown in Figure 8. The encoded polypeptide contains a hydrophobic presequence (Figure 8, double underlined) with homology with signal peptides found in protein precursors targeted to the endoplasmic reticulum (von Heijne, 1985), suggesting that WCI-4 is a secreted protein. In addition to this short presequence, WCI-4 contains an N-terminal extension of ~115 amino acids (Figure 8) also found in the propeptides of oryzains (Watanabe et al., 1991) and aleurain (Holwerda et al., 1990), suggesting a similar two-step processing to generate a mature cysteine proteinase. All active site residues characteristic for cysteine proteinases from plants and animals (Dufour, 1988) are conserved in the proposed mature protein encoded by cWCI-4 (Figure 8). A comparison of >30 known primary amino acid structures of plant proteinases shows a high degree of amino acid conservation surrounding these active residues (Figure 8). The homology of WCI-4 with a gibberellin-regulated thiol protease from wheat (Cejudo et al., 1992) is substantially lower (~20%) than for all other plant cysteine proteinases (between 32 and 46%), suggesting the existence of at least two different classes of cysteine proteinases in wheat.

cWCI-5 contains an open reading frame preceded by an in-frame stop codon that encodes a 25-kD polypeptide of unknown function. No homology with known protein sequences was evident. Like WCI-3 and WCI-4, WCI-5 contains an endoplasmic reticulum–targeting signal sequence (residues 1 through 23). The calculated isoelectric point of the proposed mature protein is 4.5, making vacuolar targeting less probable than targeting to the apoplast.

Induction of WCI Genes by Pathogens

At the onset of acquired resistance in tobacco (Ward et al., 1991) and Arabidopsis (Uknes et al., 1992, 1993), coordinately regulated sets of genes are induced both biologically and chemically. A number of these genes represent PR genes, which

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      1                                                          50
WCI3  MASGAQMKMVAVGMMLAIMFIAAAHAEPAPAETCIDKTEKVGLATDCICS
PPT   MGRSIRLFATFFLIAMLFLSTEMGPMTSAEARTCESQSHRF     HGTCV
                                          *          *

      51                                                         95
WCI3  KNCACAGKCILEGGEGGEIQKCFVECVLKNDCNCNAKHHSAAAPQ
PPT   RESNCASVCQTEGFIGGNCRAFRRRCFCTRNC
        *   *                 *     *
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Figure 7. Deduced Amino Acid Sequence of cWCI-3 and Comparison with a Thionin-like Protein of Petunia.

Primary structures of WCI-3 are compared with a γ-thionin–like protein of petunia (PPT). Putative, hydrophobic signal peptides are underlined, identical amino acids are in boldface letters, and conserved cysteine residues are indicated with asterisks.

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ATCCACCTCCATCCAGCCATGCTGTCCTCCAAGTCTTCTTTACATGTTGCTCTAATCCTT  60
                M  L  S  S  K  S  S  L  H  V  A  L  I  L

GTGGTCACGCTGACAGATGTGCTACCGCTGGTATCTTCTTCACGTGGCCTCGTTGCGGTA 120
V  V  T  L  T  D  V  L  P  L  V  S  S  S  R  G  L  V  A  V
                                              ↑

CCCACTGATGGGGCACTGGAGGACTCTCTGCTGATGATGGAGAGGTTCCATGGCTGGATG 180
P  T  D  G  A  L  E  D  S  L  L  M  M  E  R  F  H  G  W  M

GCAAAGCATGGCAAGTCGTATGCGGGAGTCGAGGAGAAACTGCGGCGGTTTGACATATTC 240
A  K  H  G  K  S  Y  A  G  V  E  E  K  L  R  R  F  D  I  F

CGCAGGAACGTAGAGTTCATCGAGGCGGCGAACCGAGATGGCAGGCTCTCGTACACCCTC 300
R  R  N  V  E  F  I  E  A  A  N  R  D  G  R  L  S  Y  T  L

GGGGTGAACCAGTTCGCCGACCTCACCCACGAGGAGTTCCTTGCCACGCACACCAGCCGC 360
G  V  N  Q  F  A  D  L  T  H  E  E  F  L  A  T  H  T  S  R

CGTGTGGTGCCGTCAGAGGAGATGGTGATTACAACTCGCGCTGGCGTTGTTGTCGAGGGT 420
R  V  V  P  S  E  E  M  V  I  T  T  R  A  G  V  V  V  E  G

GCCAATTGTCAGCCGGCGCCAAATGCTGTGCCTCGTAGCATCAATTGGGTGAATCAAAGC 480
A  N  C  Q  P  A  P  N  A  V  P  R  S  I  N  W  V  N  Q  S
                         ↑

AAAGTCACCCCAGTCAAAAATCAAGGAAAAGTATGCGGGGCTTGCTGGGCTTTTTCTGCC 540
K  V  T  P  V  K  N  Q  G  K  V  C  G  A  C  W  A  F  S  A
                  *                       *

GTGGCCACGATCGAAAGCGCCTACGCGATCGCCAAGCGAGGCGAGCCGCCGGTTCTGTCC 600
V  A  T  I  E  S  A  Y  A  I  A  K  R  G  E  P  P  V  L  S

GAGCAGGAGCTCATCGACTGTGACACATTCGACAGAGGCTGCACGAGCGGCGAGATGTAC 660
E  Q  E  L  I  D  C  D  T  F  D  R  G  C  T  S  G  E  M  Y

AATGCCTATTTCTGGGTCTTGAGGAACGGCGGCATCGCCAACAGCTCAACGTACCCCTAC 720
N  A  Y  F  W  V  L  R  N  G  G  I  A  N  S  S  T  Y  P  Y

AAAGAGACTGACGGCAAGTGCGAGAGAGGGAAACTGCAGGAACACGCGGCCACGATCAGG 780
K  E  T  D  G  K  C  E  R  G  K  L  Q  E  H  A  A  T  I  R

GACTACAAATTCGTCAAACACAACTGCGAGGAGCAGCTCATGGCAGCAGTGGCGGTGCGA 840
D  Y  K  F  V  K  H  N  C  E  E  Q  L  M  A  A  V  A  V  R

CCCGTCGCCGTCGGGTTCGACTCCAACGACGAATGCTTCAAGTTCTACCAAGCTGGTTTG 900
P  V  A  V  G  F  D  S  N  D  E  C  F  K  F  Y  Q  A  G  L

TACGACGGCATGTGCATCAAGCACGGGGAATACTTTTGGCCCGTGCTCGTCCAACGACCGC 960
Y  D  G  M  C  I  K  H  G  E  Y  F  G  P  C  S  S  N  D  R

ATCCACTCCTTGGCCATTGTCGGGTACGCCGGCAAGGGGGGCGACAGGGTCAAGTACTGG 1020
I  H  S  L  A  I  V  G  Y  A  G  K  G  G  D  R  V  K  Y  W
   *

ATCGCCAAGAACTCGTGGGGCGAGAAGTGGGGAAAGAAGGGCTACGTCTGGCTGAAGAAG 1080
I  A  K  N  S  W  G  E  K  W  G  K  K  G  Y  V  W  L  K  K
         *     *

GATGTTGATGAGCCGGAAGGCCTCTGCGGCCTTGCAATTCAGCCGGTATATCCTATAGTC 1140
D  V  D  E  P  E  G  L  C  G  L  A  I  Q  P  V  Y  P  I  V

TGATCTGATCTGACGAGATCGACTGCACTGGGCGTGCATGAAACCTACGGAAATGGCATT 1200
*

CACCTATATTTTGGGTTGCTCTGTATGCATGGATGCGCCTACTATATTTTACTACATATA 1260

TATTCATCTCCCGCCAATAAAACTATATGTCCTTGTATCCATTTATGCACGTTTATGCAT 1320

ATCTTGAATAAATTGGACGGATTGGTTATCCCC(A)n 1353
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Figure 8. Primary Structure of cWCI-4 and Its Deduced Amino Acid Sequence.

The nucleotide sequence and the deduced amino acid sequence of cWCI-4 are shown. Amino acid residues, conserved in >90% of published sequences for plant cysteine proteinases (GenBank and EMBL Sequence Data Libraries, issued July 1995), are in boldface letters. Highly conserved regions in the vicinity of the residues involved in formation of the catalytic site (marked by asterisks) are underlined. The hydrophobic presequence is double underlined. The arrows indicate proposed processing sites of the putative preproprotein, determined by comparison with the known N terminus of the mature oryzain α.

also are induced during plant–pathogen interactions (Alexander et al., 1994). An incompatible interaction often is accompanied by a hypersensitive response, the induction of local resistance or SAR, and the rapid induction of PR genes. These genes also can be induced in compatible interactions but at later time points, as shown, for example, for chitinase and lipoxygenase from bean (Meier et al., 1993). To test whether the isolated WCI genes exhibit an expression pattern common to genes that encode PR proteins, we infected wheat plants with the compatible pathogen *E. g. tritici*. We compared the expression of the WCI genes with the induction of a wheat PR-1 gene encoding a protein with 96% identity to a PR-1a protein from barley (Bryngelsson et al., 1994), because PR-1 is a reliable marker for pathogen infection in different plant species (Ward et al., 1991; Uknes et al., 1993; Mouradov et al., 1994). RNA gel blot analysis presented in Figure 9 shows that PR-1 and WCI-5 are strongly induced 9 days after inoculation with *E. g. tritici* (Figure 9), whereas WCI-1, WCI-2, and WCI-3 are induced to a lesser extent. Induction of WCI-2 precedes the other genes but peaks along with WCI-1 and WCI-3 by day 12. Expression of WCI-4 was extremely low; its induction at day 12 was very weak and barely detectable above background.

Kinetics of BTH-Induced Resistance and WCI Gene Induction by Different Plant Activators

Because BTH seems not to interact directly with *E. g. tritici* to inhibit its pathogenic development on wheat plants, it was

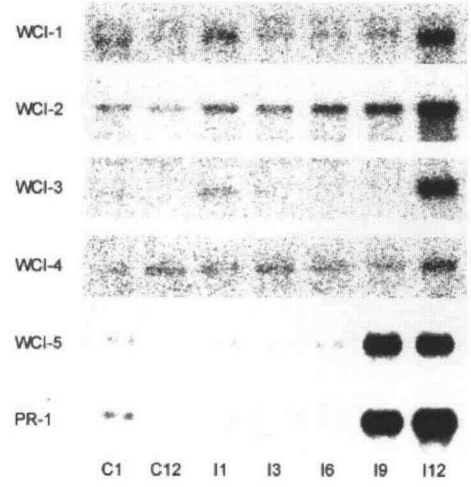

Figure 9. Response of WCI and PR-1 Genes to *E. g. tritici* Infection.

Wheat plants (2 weeks old) were infected with spores of *E. g. tritici*. Total RNA was isolated 1, 3, 6, 9, and 12 days after infection (I1 through I12) and from untreated plants (C1 and C12). RNA gel blot analysis (2 μg per lane) was performed using inserts of the cWCI clones and a cDNA encoding wheat PR-1 as probes. The corresponding stages of disease development are as follows: secondary haustoria and mycelium formed 3 days after infection (also see Figure 4); 10, 20, and 35% infected leaf area 6, 9, and 12 days after infection, respectively.

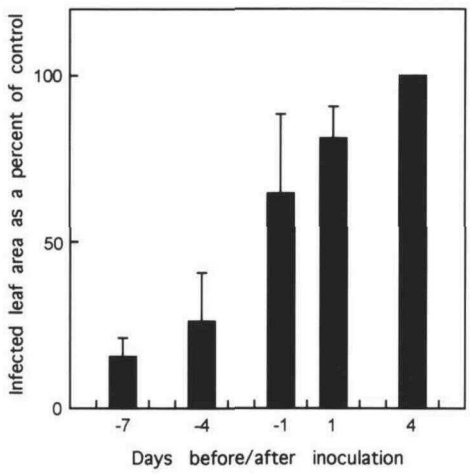

Figure 10. Kinetics of Resistance Development after BTH Treatment.

Wheat plants were treated with 0.3 mM BTH or water at different time points before and after infection with *E. g. tritici* at day 0. Development of disease was scored by determination of infected leaf areas at 14 or 18 days after inoculation. Infected leaf areas of control plants were set to 100% (corresponding to 55 ± 5% infected leaf area after 14 days and 80% infected leaf area after 18 days). Bars represent standard deviations of eight measurements.

unclear when plant activation must occur relative to pathogen infection for induced resistance to be effective. To address this issue, we treated wheat plants with BTH at different time points before and after pathogen inoculation and monitored the development of disease symptoms. The results presented in Figure 10 show that BTH treatment of wheat plants after infection with *E. g. tritici* had little impact on the progress of the disease. Effective protection could be achieved by pretreatment of the plants 4 to 7 days before pathogen inoculation. These data are consistent with the proposed activity of BTH as a plant activator.

If the WCI genes are suitable biochemical markers for plant activation, their induction by BTH should be correlated with the onset of effective plant protection. We investigated the induction kinetics of WCI-1 through WCI-4 by RNA gel blot analysis, as shown in Figure 11. All four genes showed a rapid induction with elevated expression 6 to 12 hr after application of the chemical. This expression was transient, with elevated levels evident 10 days after BTH treatment. Maximal induction was reached between days 2 and 4 for all genes. Furthermore, the timing of induction of WCI-2 clearly preceded the induction of the other genes as already observed for the induction of the WCI genes by *E. g. tritici*. The maximal gene induction observed several days after application of BTH is consistent with the induction of resistance several days after BTH treatment.

To compare the WCI gene activation capacity of BTH with that of other plant activators, we treated wheat plants with the SAR-inducing compounds SA and INA. RNA dot blot analysis was performed with plant material harvested during a 2-week time period after chemical application (0.3 mM BTH or INA and 3 mM SA); autoradiographic signals were quantified using a PhosphorImager. Data for the time course of WCI gene induction are presented in Figure 12 and Table 2. All three SAR-inducing compounds were activators of the WCI genes, and maximal induction was almost identical for the INA- and BTH-treated tissue, whereas SA showed a substantially lower efficacy (Table 2). Independent of the activating chemical, induction factors were highest for WCI-2, followed by WCI-3 and

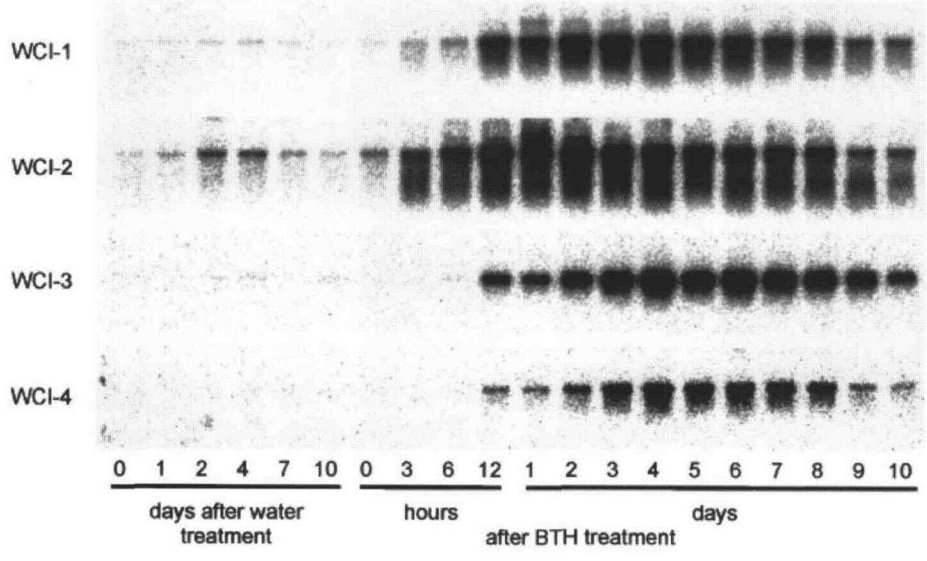

Figure 11. Time Course of WCI Gene Induction by BTH.

Wheat plants (2 weeks old) were treated with water (first six lanes) or 0.3 mM BTH (all other lanes). Tissue was harvested at different time points (0, time of treatment). RNA was isolated and subjected to gel blot analysis by using the cWCI clones as probes.

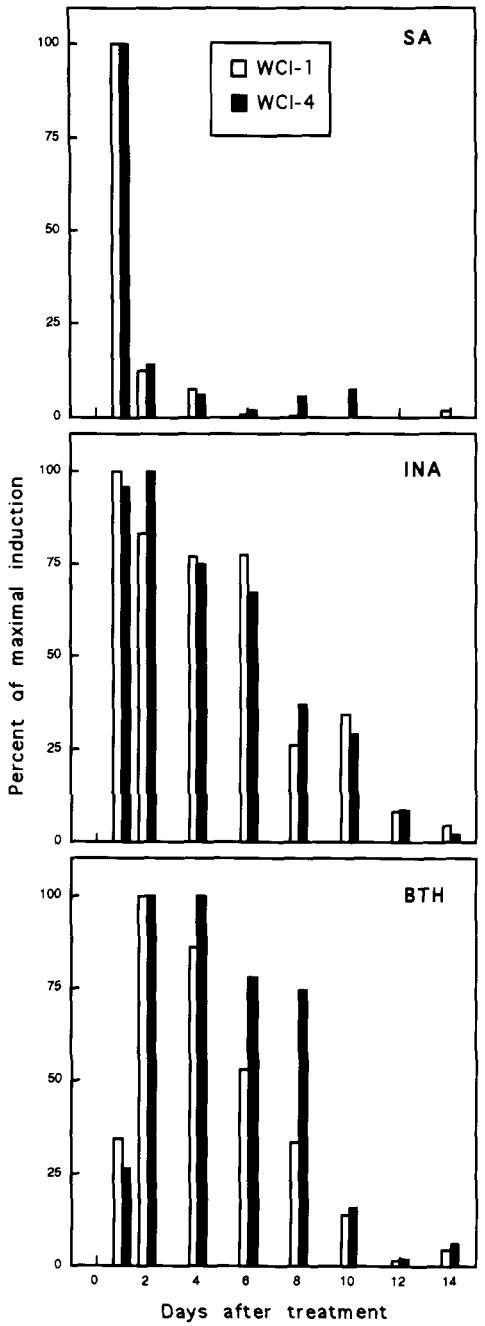

Figure 12. Comparison of Induction of WCI Genes by Different SAR-Inducing Compounds.

Wheat plants (2 weeks old) were treated with 3 mM SA, 0.3 mM INA, or 0.3 mM BTH. Tissue was harvested at given time points, and RNA was isolated and subjected to dot blot analysis by using the cWCI clones as probes. Blots were exposed, and signal strength was determined using a PhosphorImager. For each probe and treatment, maximal signal strength was determined and relative induction values were calculated.

WCI-1, giving a qualitatively similar induction pattern for all three plant activators with respect to the different WCI genes.

The time course of gene induction by SA, INA, and BTH showed distinct characteristics for each treatment. Time courses for induction of WCI-1 and WCI-4 are shown in Figure 12; the data for WCI-2 and WCI-3 are similar. Although transcript levels of the different WCI genes were similarly affected by each respective plant activator treatment, each chemical provokes a different induction kinetic. The transient induction by SA was short, reaching its maximum after 1 day. However, after 6 days, transcript levels were already reduced to background. In contrast, INA and BTH treatments led to more prolonged gene induction. For INA, maximal induction levels were reached after 1 to 2 days and started to drop ~4 days after chemical application. BTH caused a slower induction, reaching maximal levels at day 2 and maintained until day 4. Taken together, SA represents the weakest gene activator of the compounds tested, even if applied at high concentrations, and induced a comparably short and transient expression of the WCI genes. Both INA and BTH showed similar qualitative and quantitative activities.

Correlation of WCI Gene Induction and Development of Resistance

Because the ability to activate WCI gene expression differs for the three compounds investigated, we tested whether this difference is correlated with the plant protection effectiveness of SA, INA, and BTH. First, we investigated the induction of the WCI genes 4 days after chemical treatment with increasing concentrations of the plant activators, as shown in Figure 13A. For each treatment, transcript levels of the genes were determined by quantitation of RNA blot autoradiographic signals. The expression of each gene was calculated relative to the highest level of expression. To avoid induction of the genes by pathogens, the plants were grown and treated under sterile conditions. All four WCI genes exhibited a strikingly similar

Table 2. Maximal Induction Factors of WCI Genes

	Treatment[a]		
Probe	SA	INA	BTH
WCI-1	9	262	270
WCI-2	18	427	431
WCI-3	12	391	395
WCI-4	6	135	136

[a] Plants were treated for different times with SA (3 mM), INA (0.3 mM), or BTH (0.3 mM). RNA gel blot analysis was performed for each treatment, and signal strengths were determined for each gene probe. Maximal induction factors were calculated by comparison of the strongest signal for each WCI gene with the signal strength of water-treated control tissue. Maximal induction was reached at day 1 for SA, day 1 or 2 for INA, and day 2 to 4 for BTH (also see Figure 12).

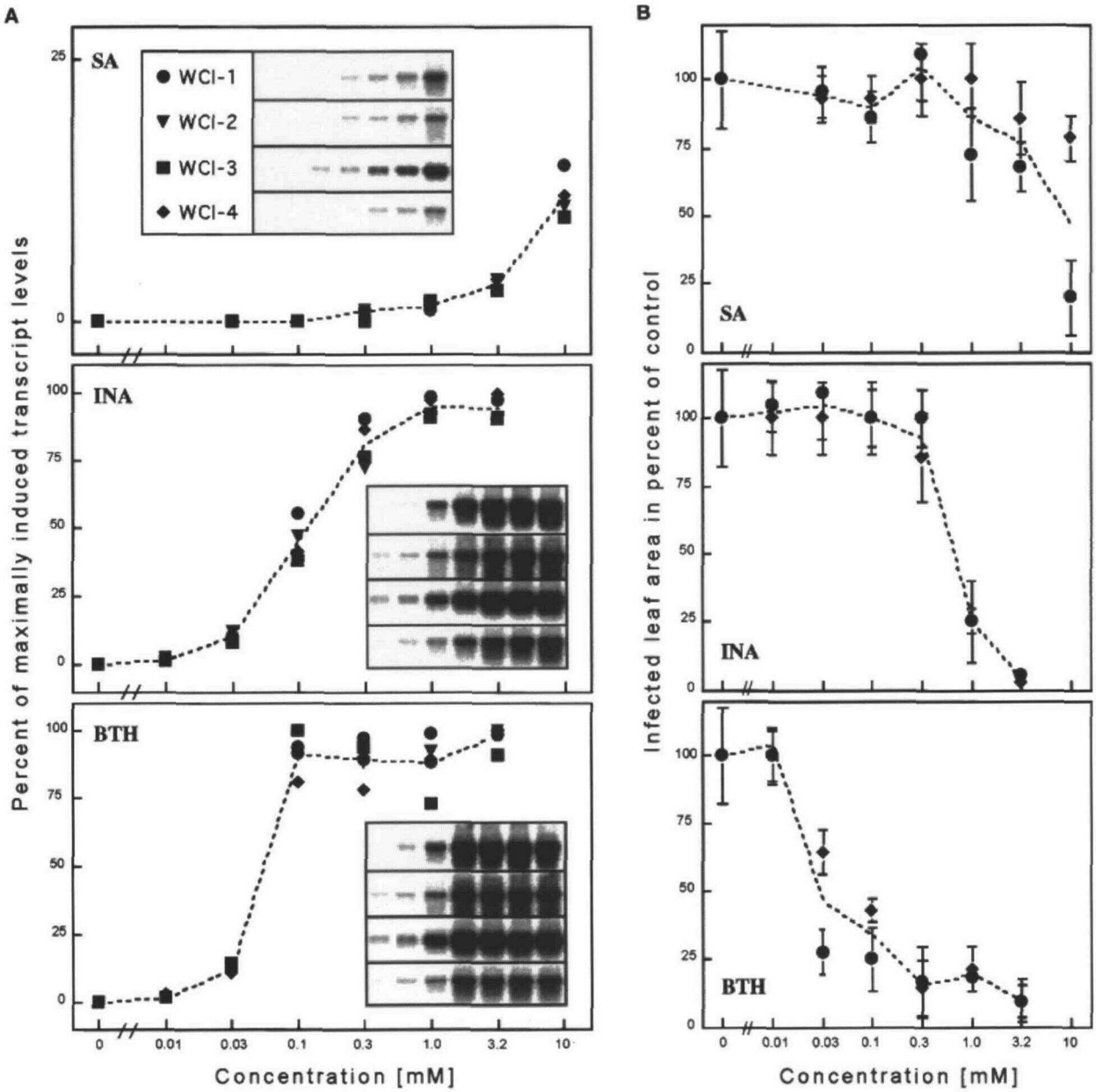

Figure 13. WCI Gene Induction and Induction of Resistance by Different Plant Activators.

(A) Dose–response curves of WCI genes to plant activator treatment. Wheat plants (2 weeks old) were treated with water or different concentrations of SA, INA, or BTH. RNA was isolated after 4 days and subjected to gel blot analysis by using the cWCI clones as probes (autoradiographs shown as insets; lanes correspond to increasing concentrations of the respective plant activator). Maximal signal strength was determined for each gene by using a PhosphorImager, and relative induction levels were calculated.

(B) Dose–response curves of resistance against powdery mildew by different plant activators. Wheat plants (2 weeks old) were treated with water or different concentrations of SA, INA, or BTH. After 4 days, plants were inoculated with spores of *E. g. tritici*. Development of disease was scored by determination of infected leaf areas at 14 days (●) or 18 days (◆) after inoculation. Infected leaf areas of control plants were set to 100%. Bars represent standard deviations of measurements of 10 plants.

response toward different concentrations of the plant activators (Figure 13A). As previously observed, SA showed the lowest induction, and significant induction was observed only for concentrations >1 mM. Treatment with 10 mM SA resulted in phytotoxic effects on the wheat leaves. In contrast, INA and BTH caused substantial increase of gene expression at concentrations <3 mM. Although maximal induction of the WCI genes was found at INA concentrations of 1 to 3 mM, BTH induced these genes to the same extent at concentrations as low as 0.1 mM.

In Figure 13B, the influence of increasing concentration of activator on the development of powdery mildew disease symptoms is illustrated. Four days after chemical treatment, the plants were inoculated with *E. g. tritici*, and the infected leaf area was estimated 14 and 18 days later. Disease development was significantly reduced by 0.1 mM BTH, and the infected leaf area was <10% of the control after application of 3 mM (Figure 13B). Significant protection also was achieved by treatment with 1 to 3 mM INA, reducing the infected leaf area to <25% of the control (Figure 13B). Compared with the control plants, treatment with SA had almost no influence on the disease development. Treatments with concentrations of ≥1 mM led to a slight delay in the progress of fungal growth that was overcome after ~3 weeks. We never observed effective long-term protection of wheat plants by treatment with SA (data not shown). Figure 13 shows a good correlation between the concentration and efficacy of chemical activators, the extent of WCI gene expression, and the development of powdery mildew resistance.

DISCUSSION

In wheat, BTH induces significant resistance against *Septoria* spp, *P. recondita*, and *E. graminis*. A set of genes is induced in BTH-treated wheat plants, and this set constitutes the WCI genes (described here) and a PR-1a homolog from wheat (J. Görlach, S. Volrath, E. Ward, and J. Ryals, unpublished results). The induction of resistance by BTH correlates with the expression of the WCI genes with respect to both timing and dose–response of the chemical.

Because a biologically induced SAR response in wheat has not been previously described, we cannot determine conclusively that BTH induces SAR in wheat. Evidence supporting the fact that BTH induces SAR comes from a separate study in which we show that BTH induces resistance against the same spectrum of pathogens and induces the same spectrum of SAR gene expression as with SAR in tobacco (Friedrich et al., 1996). Furthermore, BTH induces PR-1 gene expression by using the same promoter elements as either TMV or SA and induces PR-1–dependent reporter gene expression in the same cell types as TMV, SA, and INA. Similar to INA, BTH induces SAR in transgenic tobacco that expresses the bacterial salicylate hydroxylase enzyme (Gaffney et al., 1993). Finally, BTH induces SAR in Arabidopsis, and this induction is blocked by a mutation in *nim1* (for noninducible immunity; Delaney et al., 1995) but not in transgenic *nahG* plants (Lawton et al., 1996). Thus, BTH induces the SAR response in tobacco and Arabidopsis, according to all of the criteria that currently define SAR. Because both BTH and INA induce SAR in dicots, it is likely that the resistance and gene activation response in wheat are SAR-like responses.

BTH is the third well-characterized chemical inducer of SAR along with INA and SA. All three compounds may bind to the same receptor, but this is unclear. All three plant activators share common structural features, including an aromatic ring system with a substituted carboxyl group. This close structural relationship also is reflected in the finding that SA as well as INA bind and inactivate a catalase isozyme (Chen et al., 1993; Conrath et al., 1995). However, activator binding and inactivation of catalase do not appear to be involved in SAR signal transduction (Bi et al., 1995; León et al., 1995; Neuenschwander et al., 1995; Summermatter et al., 1995). Nonetheless, the function of BTH and INA as activators of plant defense and gene expression in both dicot and monocot plants indicates a high similarity between receptor specificity and signal transduction in both plant classes.

Although the basic mechanisms of chemically induced SAR seem to be highly conserved, every plant species exhibits specific responses linked to SAR. For example, the set of genes induced in tobacco, Arabidopsis, and wheat during the onset of chemically induced resistance are different; this distinction is reflected also in the nature and spectrum of defense responses against different pathogens in the different plant species. Neither lipoxygenases nor cysteine-rich proteins have been described as SAR genes in tobacco or Arabidopsis, although they are highly induced in wheat. At the same time, the WCI genes as well as the dicot SAR genes represent PR genes. Irrespective of the mechanism of chemically induced SAR, BTH-induced resistance in wheat is manifested at various stages of the plant–pathogen interaction.

BTH-induced genes from wheat comprise both resistance-related and pathogenesis-response genes, although the corresponding cDNAs were isolated from plants in the absence of pathogens. A clear differentiation between pathogenesis response and resistance-related genes is a major problem in the identification of bonafide resistance-conferring genes from pathogen-infected tissue. The use of resistance-inducing compounds such as INA and BTH for the isolation of resistance-related genes provides a tool that facilitates distinction between disease resistance–related genes and pathogenesis-response genes. Furthermore, SAR-inducing compounds provide a unique opportunity to investigate induced resistance mechanisms in plants in the absence of biological model systems, as described in this article. The identification of molecular and biochemical elements involved in systemic resistance signal transduction, such as biological receptors and systemic

signals, whether common or different in both monocot and dicot systems, may be greatly augmented by the use of this class of plant activators.

A single application of 30 g of BTH per hectare (~12 g per acre) can protect wheat against powdery mildew in the field for an entire season, combining long-lasting protection with an ecologically desirable, low-application rate. The plants appear unaffected by the treatment, and the yield is indistinguishable from that of plants treated with standard fungicides. Thus, BTH represents a novel commercially attractive disease control compound that activates the plant's inherent disease control mechanisms. We believe that BTH and analogous compounds will have an impact on future agricultural practices by providing the farmer with new options for disease management in respect to both application rate and frequency.

METHODS

Plant Materials

The wheat cultivar Kanzler was used in all experiments. Plants were grown in growth chambers in soil at 20°C. For experiments under sterile conditions, plants were grown in boxes on water agar including 2.15 g/L Murashige and Skoog microsalts (Gibco BRL).

Pathogen Treatment

Erysiphe graminis f sp *tritici* was maintained on 3- to 4-week-old plants. For inoculation, freshly produced conidia from heavily infected plants were used. Infected leaf areas were determined by eye. To determine germination, appressorium formation, penetration, and colony growth, leaf samples were taken 2, 3, and 4 days after inoculation and cleared by the vapor phase exchange method with isopropanol and water (Gerlach, 1969). Cleared segments of 1 cm^2 were mounted on slides and stained with 0.05% trypan blue in lactophenol–ethanol (1:2) under careful heating. Samples were analyzed by light microscopy.

For observation of haustorium formation, inoculated leaves were embedded in 20% gelatin. After solidification of the gelatin, the leaves were torn off and cleared, and trypan blue staining was performed in a boiling-water bath, as described above. After 24 hr, samples were mounted on slides and analyzed by light microscopy.

Chemical Treatment

Chemicals were applied by spraying suspensions of formulations with wettable powders in distilled water; controls were treated with wettable powder in water.

Molecular Techniques

The basic molecular techniques were performed as described previously (Maniatis et al., 1982; Ausubel et al., 1987).

Isolation of WCI cDNA clones

Wheat plants were treated with 1 mM benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) WP 25 (wettable powder, 25% active ingredient), and RNA was extracted 4 days after treatment by using a standard phenol extraction–lithium chloride precipitation method (Lagrimini et al., 1987). Poly(A)$^+$ RNA was purified by passage over oligo(dT) cellulose columns (Stratagene) and used to create a cDNA library in the bacteriophage vector λZapII (Stratagene), following the manufacturer's instructions.

For the isolation of cWCI-1, cWCI-2, and cWCI-3, the library was screened with radiolabeled (Feinberg and Vogelstein, 1983) first-strand cDNA from BTH-treated and control plants. Plaques hybridizing preferentially to the cDNA of chemically treated tissue were purified, and cDNA clones were excised in vivo (Stratagene). The cDNA inserts were used to probe total RNA gel blots of control and BTH-treated tissue. Clones showing differential expression were selected, and their sequences were determined by the chain termination method (Sanger et al., 1977) by using fluorescent dyes (Applied Biosystems, Foster City, CA).

For the isolation of cWCI-4 and cWCI-5, polymerase chain reaction differential display of cDNAs was performed essentially as described previously (Liang and Pardee, 1992). Candidate fragments were used to probe total RNA gel blots of control and BTH-treated tissue. Fragments showing differential expression were selected and used to screen the cDNA library described above. Positive clones were plaque-purified and excised in vivo according to the protocol of the manufacturer (Stratagene), and their sequences were determined.

Analysis of RNA

Total RNA was isolated from leaf tissue by using a standard phenol extraction–lithium chloride precipitation method (Lagrimini et al., 1987). For gel blot analysis, 2 μg of total RNA was separated on formaldehyde agarose gels (Maniatis et al., 1982) and transferred to nylon membranes (GeneScreen Plus; Du Pont–New England Nuclear, Boston, MA). For dot blot analysis, 1 μg of RNA was dotted onto the same membrane as described previously (Görlach et al., 1994). Membranes were hybridized with α-^{32}P-dATP–labeled (Feinberg and Vogelstein, 1983) inserts of the respective cDNA clones, washed under stringent conditions (Church and Gilbert, 1984), and autoradiographed. For determination of transcript levels, signals of the blots were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Induction factors were calculated by dividing the signal strengths by the average signal strengths of the respective controls.

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