Productivity and biochemical properties of green tea in response to full-length and functional fragments of $HpaG_{Xooc}$, a harpin protein from the bacterial rice leaf streak pathogen *Xanthomonas oryzae* pv. *oryzicola*

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Harpin proteins from plant pathogenic bacteria can stimulate hypersensitive cell death (HCD), drought tolerance, defence responses against pathogens and insects in plants, as well as enhance plant growth. Recently, we identified nine functional fragments of $HpaG_{Xooc}$, a harpin protein from *Xanthomonas oryzae* pv. *oryzicola*, the pathogen that causes bacterial leaf streak in rice. Fragments $HpaG_{1–94}$, $HpaG_{10–42}$, and $HpaG_{62–138}$, which contain the $HpaG_{Xooc}$ regions of the amino acid sequence as indicated by the number spans, exceed the parent protein in promoting growth, pathogen defence and HCD in plants. Here we report improved productivity and biochemical properties of green tea (*Camellia sinensis*) in response to the fragments tested in comparison with $HpaG_{Xooc}$ and an inactive protein control. Field tests suggested that the four proteins markedly increased the growth and yield of green tea, and increased the leaf content of tea catechols, a group of compounds that have relevance in the prevention and treatment of human diseases. In particular, $HpaG_{1–94}$ was more active than $HpaG_{Xooc}$ in expediting the growth of juvenile buds and leaves used as green tea material and increased the catechol content of processed teas. When tea shrubs were treated with $HpaH_{Xooc}$ and $HpaG_{1–94}$ compared with a control, green tea yields were over 55% and 39% greater, and leaf catechols were increased by more than 64% and 72%, respectively. The expression of three homologues of the expansin genes, which regulate plant cell growth, and the *CsCHS* gene encoding a tea chalcone synthase, which critically regulates the biosynthesis of catechols, were induced in germinal leaves of tea plants following treatment with $HpaG_{1–94}$ or $HpaG_{Xooc}$. Higher levels of gene expression were induced by the application of $HpaG_{1–94}$ than $HpaG_{Xooc}$. Our results suggest that the harpin protein, especially the functional fragment $HpaG_{1–94}$, can be used to effectively increase the yield and improve the biochemical properties of green tea, a drink with medicinal properties.

[Wu X, Wu T, Long J, Yin Q, Zhang Y, Chen L, Liu R, Gao T and Dong H 2007 Productivity and biochemical properties of green tea in response to the intact molecule and functional fragments of $HpaG_{Xooc}$, a harpin protein from the bacterial rice leaf streak pathogen *Xanthomonas oryzae* pv. *oryzicola*; *J. Biosci.* **32** 1119–1131]

Keywords. Green tea; $HpaG_{Xooc}$; productivity; biochemical properties

Abbreviations used: CsCHS, tea (*Camellia sinensis*) chalcone synthase; CsDFR, tea dihydroflavonol 4-reductase; CsEXPL, tea expansin-like protein; CsSAMS, tea s-adenosylmethionine synthase; EVP, empty vector preparation; GRM, glycine-rich motif; HCD, hypersensitive cell death; TCS1, tea caffeine synthase

1. Introduction

HpaG proteins are recognized as a peculiar asset of harpin proteins in bacteria of the genus *Xanthomonas* (Zhu *et al* 2000). $HpaG_{Xoo}$ and $HpaG_{Xooc}$ of *X. oryzae* pv. *oryzae* (Li *et al* 2004 Peng *et al* 2004a) and *X. oryzae* pv. *oryzicola* (Liu *et al* 2006), which cause bacterial leaf blight and bacterial streak of rice, respectively, have been characterized for their functions in plants. Similar proteins were found in *X. axonopodis* pv. *glycines* (Kim *et al* 2003) and *X. campestris* pv. *vesicatoria* (Noel *et al* 2002). The *hpaG* genes and HpaG proteins are unique in several respects. Reported *hpaG* genes are homologous with each other but not with other harpin genes (Zhu *et al* 2000; Li *et al* 2004). HpaG proteins contain two to four copies of the glycine-rich motif (GRM), which is characteristic of harpin proteins (Wei *et al* 1992; Kim and Beer 2000). $HpaG_{Xooc}$ is composed of 138 amino acids, is 15.18 kD in size, and contains two GRMs that span 72 to 77 and 107 to 112 amino acid sequences (Liu *et al* 2006). Moreover, all HpaG proteins contain cysteine (Wei *et al* 1992; Li *et al* 2004; Liu *et al* 2006), which is absent in other harpins such as $HrpN_{Ea}$ of *Erwinia amylovora* (Wei *et al* 1992). The cysteine residue is located at site 47 in the $HpaG_{Xooc}$ sequence (Liu *et al* 2006). Despite these distinctions, HpaG proteins affect plants in a similar manner as other harpins. When applied to plants or produced in transgenic plants, HpaG proteins can promote plant growth, induce hypersensitive cell death (HCD), and induce defence responses (Li *et al* 2004; Peng *et al* 2004a; Liu *et al* 2006; Ren *et al* 2006a, b).

The multiple effects are attributable to the functions of particular internal regions of harpin proteins. We found that plant growth, HCD and pathogen defence induced by $HpaG_{Xooc}$ were inhibited by GRM and cysteine; deleting either of the elements increased the effects (Liu *et al* 2006). Fragments created by truncating $HpaG_{Xooc}$ are more effective than the full length protein in inducing plant growth, HCD and defence responses based on their abilities to activate specific promoters engineered with plants (Peng *et al* 2004b). The *X. axonopodis* pv. *glycines* HpaG region covering 35–50 amino acid residues is critical for the induction of tobacco HCD (Kim *et al* 2004). This region is homologous with those containing 23 and 27 amino acids, respectively, in the HrpW harpin domains identified in *Pseudomonas* (Charkowski *et al* 1998) and *Erwinia* (Kim and Beer 1998) species. Several fragments of $HrpN_{Ea}$ are responsible for the induction of HCD and a set of beneficial effects (Wei *et al* 2005; Laby *et al* 2006). Enhanced growth, and resistance to pathogens and insects have been achieved by using the fragments in a number of ways, including transgenic expression in plants (Wei *et al* 2005). Thus, the importance of functional regions is common to harpin proteins.

We have generated nine fragments of $HpaG_{Xooc}$; the fragments $HpaG_{1–94}$, $HpaG_{10–42}$ and $HpaG_{62–138}$, which contain $HpaG_{Xooc}$ amino acid sequence spans as indicated by the numbers, differ from the parent protein in the ability to affect plants (Chen *et al* 2007a). In tobacco, $HpaG_{10–42}$ fails to induce evident HCD; $HpaG_{62–138}$ is 2.45-fold more active while $HpaG_{1–94}$ is 25% less active than $HpaG_{Xooc}$ in producing this effect (Chen *et al* 2007a). Compared with $HpaG_{Xooc}$, $HpaG_{1–94}$ and $HpaG_{10–42}$ provide 1.5-fold greater levels of plant growth enhancement. $HpaG_{1–94}$ is similar to the parent protein while $HpaG_{10–42}$ induces 7.5-fold greater resistance in rice to bacterial blight, rice blast and sheath blight (Chen *et al* 2007a). When applied to grower's plantings, $HpaG_{10–42}$ is more effective than $HpaG_{Xooc}$ in impeding several significant diseases and increases the grain yield of rice (Chen *et al* 2007b). Testing the differential functions of the fragments in different crops in accordance with the product orientation of crops is necessary for agricultural use.

Tea production has strict requirements for the growth, management and processing of tea leaves, which are used as a drinking material. In daily life, drinks are as important as food in their unique, often indispensable way. With a long history of origin in China, green tea (*Camellia sinensis*), a popular variety of tea, has increasingly become a global favourite (Graham 1992). Extracts from green tea contain a full spectrum of amino acids, which enrich human nutrition, and peculiar sets of catechins and flavonoids (Ashihara and Crozier 2001; Punyasiri *et al* 2004), which have a potential role to play in the prevention and treatment of cancers and cardiovascular disorders (Shibata *et al* 2000; Cooper *et al* 2005; Stangl *et al* 2006; Friedman *et al* 2007). The biochemical properties of green tea are important for achieveing its beneficial effects. In a cooked tea solution, a high concentration of catechols has medicinal relevance, high levels of anthocyanins and amino acids are supposed to enhance enjoyable colours and provide nutrient merits (Friedman *et al* 2007), but proper rather than high levels of theines are required to have a beneficial effect on neuronal excitability and cough suppression (Usmani *et al* 2005). An optimized combination of these properties is difficult to achieve and is believed to depend on the modulation of enzyme activities during tea leaf growth (Wang 1981; Misako and Kouichi 2004; Punyasiri *et al* 2004). Tea chalcone synthase (CsCHS) and dihydroflavonol 4-reductase (CsDFR) play essential roles in the biosynthesis of anthocyanins and catechols (Takeuchi *et al* 1994), respectively. Tea caffeine synthase (TCS1) and s-adenosylmethionine synthase (CsSAMS) dominantly control the production of caffeine and other types of theines (Kato *et al* 1999, 2000). It is unclear if biotic elicitors, such as harpin proteins, affect these biochemical properties of tea.

Here we describe the effects of $HpaG_{Xooc}$, $HpaG_{1–94}$, $HpaG_{10–42}$ and $HpaG_{62–138}$ on green tea. Based on the replicate

results obtained from two-year experiments conducted in a tea garden, optimal increase in yield and optimal improvement in the biochemical character of green tea are provided by $HpaG_{1–94}$, rather than $HpaG_{10–42}$ although it is effective in impeding disease development and increasing the grain yield of rice. We provide evidence that germinal leaf growth and expression of the tea genes essentially involved in plant growth and biosynthesis of catechols are induced consistently with an increase in the yield and catechols of processed tea.

2. Materials and methods

2.1 *Protein preparation*

The pET30a(+) vector (EMD Biosci. Inc., Darmstadt, Germany) was used to clone genes encoding $HpaG_{Xooc}$ and the fragments $HpaG_{1–94}$, $HpaG_{10–42}$ and $HpaG_{62–138}$ (Chen *et al* 2007a); recombinant vectors were transferred into cells of *Escherichia coli* BL21 (Liu *et al* 2006; Chen *et al* 2007a). Previous methods were used to produce proteins from BL21 (Chen *et al* 2007a, b) and determine protein concentrations (Bauer *et al* 1995; Dong *et al* 1999; Liu *et al* 2006). An empty vector preparation (EVP) was produced by BL21 cells containing pET30a(+) only and used as a control; the EVP was determined to not have bioactivity (Chen *et al* 2007a, b). $HpaG_{Xooc}$ and the three fragments contain a histidine tag (Chen *et al* 2007a) and were thus purified by nickel chromatography with the HisTrap HP Kit (Amersham Biosci. Corp., Piscataway, NJ, USA). Purified proteins were analysed by tricine–sodium dodecyl sulphate–plolyacrylamide (T-SDS-PAGE) (Schagger *et al* 1987).

Properly treated and concentrated formulations of proteins were prepared for use in tea. The histitine tag was excised by treating proteins with the Novagen Enterokinase Cleavage Capture Kit (EMD Biosci. Inc., Darmstadt, Germany). Concentrations of proteins were adjusted to 200 μg/ml in an aqueous formula along with 50 μg/ml phenylmethylsulfonyl fluoride (PMSF, prepared in dimethyl formamide), a protease inhibitor that protects proteins from decomposition by proteases (Wei *et al* 1992). EVP was supplemented with bovine serum albumin (BSA) if required (Bauer *et al* 1995). The surfactant Silwet-77 also was added to the concentrated protein solutions at a concentration of 0.3%, which resulted in a final concentration of about 0.03% in field applications. The protein formulate was maintained at 4 °C and diluted with tap water immediately before application to rice seedlings in nurseries and transplanting fields. With the lowest dilution used, none of the BSA (Bauer *et al* 1995; Dong *et al* 1999), the enterokinase and PMSF (Liu *et al* 2006; Chen *et al* 2007a, b) impaired the effects of the proteins based on assays for HR induction.

2.2 *Field experiments*

Our experimental site was The 916 Tea Guard, a nationally reputed tea farm situated in Shucheng City (31.45 °N, 116.95 °E), Anhui Province, China (figures 1A and 1B). Standard plot experiments (figure 1C) were conducted during spring tea production cycles in 2005 and 2006. The guard is near a scenic area with a climate favouring green tea production in accordance with the sanitary requirements of the crop (Wang 1981). The noted green tea variety YuHuaCha ShuChengZao was tested when it was 5 and 6 years old. Concentrated protein formulations of $HpaG_{Xooc}$, the three fragments and EVP were adjusted to 12 μg/ml by dilution with clear tap water; protein solutions were applied separately to plants by spraying plant tops 10 days after manicure as a part of routine management. Each protein treatment was tested in 3 replicates distributed randomly as 3 survey plots; each plot occupied 10 m^2. Agronomic management was regular and as per growers' schedules; fungicides and other chemicals were not applied.

Tea was picked at intervals based on the conventional standard for judging maturation (Wang 1981). Intervals of tea-picking were similar in 2005 and 2006 (table 1), and the actual dates were 5 days earlier in 2005 than 2006. A stem bud and three juvenile top leaves picked together were regarded as a tea-picking unit. The times of picking and numbers of tea-picking units were monitored. The fresh weight of tea-picking units and dry weight of processed tea were determined. Processed tea was sorted into commercial classes by the conventional standards (Wang 1981); proportions of classes were scored.

Data were analysed separately by year owing to variations in manicure and management. Each survey plot was treated as an experimental unit. Distribution patterns of observed values were justified with Microsoft Excel graph tools. One-way *F* tests were used to determine if the application of proteins had significant effects on the production and biochemical properties of tea.

2.3 *Biochemical and molecular studies*

Biochemical assays were done with three samples of processed tea from each survey plot. Previous methods were used to determine the content of amino acids (Dey and Harborne 1991) and catechols (Aucamp *et al* 2000) in processed and unsorted tea. To determine the expression of tea genes, 5-month-old tea seedlings growing in pots were sprayed separately with solutions of the proteins tested. Five days later, RNA was isolated from the buds and two youngest leaves as described (Liu *et al* 2006). Gene expression was determined by reverse transcriptase polymerase chain reaction (RT-PCR) protocols and confirmed by RNA gel-blotting analysis. In RNA gel-blotting analysis, 10 μg of

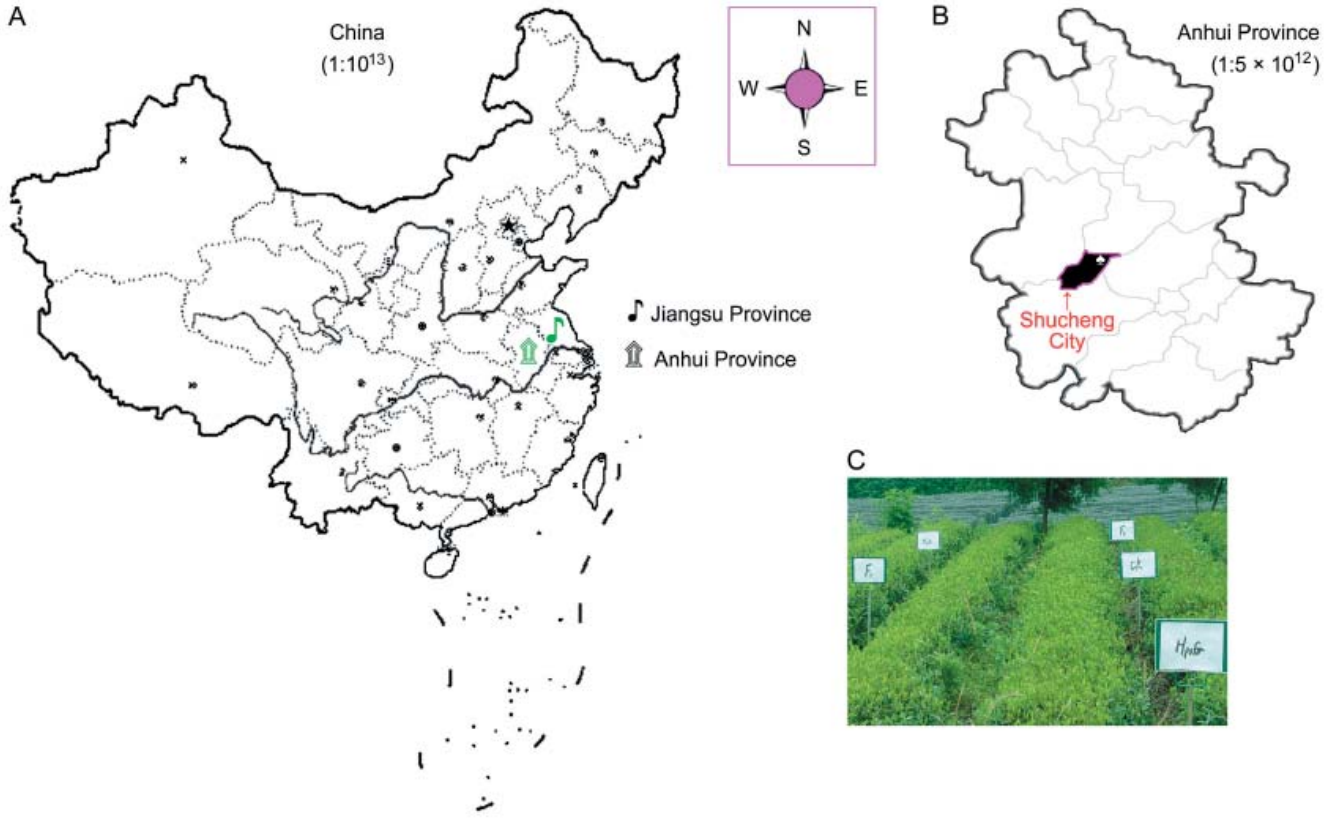

Figure 1. Experimental locations. **(A)** A map of China with localization of two study sites. Situated in the middle-east part of the map, the music stuff and house symbol indicate the localizations of Jiansu Province and Anhui Province, where proteins were produced and tested, respectively. **(B)** A map of Anhui Province with the experimental area highlighted. Our experimental site, The 916 Tea Garden, is located at the central part of the play card club symbol in white, in Shucheng City territory. (**C**) Shows individual plots. The functional fragments of $HpaG_{Xooc}$ (HpaG) were coded arbitrarily in the experiments. F7, for example, represents $HpaG_{10–42,}$ which contains the $HpaG_{Xooc}$ amino acid sequence span as indicated by the numbers.

Table 1. Schedule of tea-picking in field experiments in 2005

Protein applied	Date of tea-picking				No. of times picked
	1st	2nd	3rd	4th	
EVP	10 March	28 March	18 April	No	3
$HpaG_{Xooc}$	5 March	18 March	2 April	16 April	4
$HpaG_{1–94}$	5 March	18 March	2 April	16 April	4
$HpaG_{10–42}$	7 March	22 March	5 April	20 April	3
$HpaG_{62–138}$	10 March	28 March	18 April	No	3

Tea plants were manicured on 18 February. On 25 February and 15 March, the proteins were applied separately in a 12 μg/ml aqueous solution to growing tea plants. Stem tops, called a tea-picking unit here, included buds and the three youngest leaves, and were picked artificially as compared with the conventional standard. In 2006, the data of tea-picking were collected 5 days later but the intervals were similar.

RNA was resolved by electrophoresis, blotted onto a Nelon membrane, and probed with digoxigenin-labelled cDNA of the genes evaluated. RT-PCR protocols were performed using RT-PCR Beads (Amersham Pharmacia Biotech. Inc., Piscataway, NJ), as per the manufacturer's protocol. The *EF1α* gene, which is highly conserved and constitutively

expressed in eukaryotes (Gallie *et al* 1998; Huang *et al* 2000), was used as a standard (Peng *et al* 2003; Liu *et al* 2006). Specific primers were synthesized according to reported sequences. The RT-PCR protocol has been proven valid for estimating gene expression levels (Liu *et al* 2006; Ren *et al* 2006a, b). Genes were amplified for 25 cycles; products were cloned into the pGEM®-T Easy Vector (Promega), sequenced (Takara Biotech. Co., Ltd., Dalian, China), and confirmed by Blast searches. RT-PCR products were visualized by staining with ethidium bromide in agarose gels after electrophoresis. The relative level of gene expression was quantified by scanning the ethidium bromide staining density in an area measuring horizontally 7 mm × vertical 4 mm with a gel documentation system (Molecular Imager Gel Doc XR System and Quantity One 1-D analysis software, Alfred Nobel Drive Hercules, California, USA).

Primers specific for the genes studied and sizes (bp) of the gene products are as follows: *CsEXPL1*, 5′-ACATTCTACGGTGGTGGTGATGC-3′, 5′-CACTCTTCCTAACGTAGCTGCGC-3′, 665; *CsEXPL*, 5′-CCGCTACAAACTTCTGCCCGCCAAAT-3′, 5′-ACCGAGCCCTCGACGCATCACTCT-3′, 574; *CsEXPL18*, 5′-TGTCGTCGCCTAACTGCTACTAC-3′, 5′-TGGTCTGACGAGTGGTGTAAGAG-3′, 435; *CsDFR*, 5′-GATTCATCGGCTCGTGGCTCGTCAT-3′, 5′-ACTCAGTGGGGACATTGTACTCG-3′, 795; *TCS1*, 5′-GAACAGAGGAGAAGGAGAAAGT-3′, 5′-TTCTACGCTATCAAGATCAAAC-3′, 859; *CsSAMS*, 5′-TGCGAAAGAATGGAACTTGCCCCTG-3′, 5′-GCCAAAATGTCCATAGGCAGCAGTC-3′, 653; *CsCHS*, 5′-ACAAAGGCAATCAAAGAATGGGGTC-3′, 5′-AAACAACACGCACGCACTTGACATA-3′, 702; EF1α, 5′-AGACCACCAAGTACTACTGCAC-3′, 5′-CCACCAATCTTGTACACATCC-3′, 495.

Sequence data from this article have been deposited with the GenBank data libraries under accession numbers U30476 (*CsEXPL1*), AF229437 (*CsEXPL2*), AF332444 (*CsEXPL18*), AB018686 (*CsDFR*), AB031280 (*TCS1*), AB041534 (*CsSAMS*), AY169403 (*CsCHS*), and AF120093 (EF1α).

3. Results

3.1 *Protein production*

$HpaG_{Xooc}$ and the three fragments excised from it have been cloned in the pET30a(+) vector as translated to include a histidine tag (His-tag), which facilitates protein purification (Subramanian *et al* 2002; Chen *et al* 2007a). After production by recombinant *E. coli* cells, proteins were purified and subjected to T-SDS-PAGE. Figure 2 indicates that the proteins were produced uniformly in the correct sizes. The EVP contained inactive proteins but neither $HpaG_{Xooc}$ nor its variants (Peng *et al* 2004a; Liu *et al* 2006); no proteins were seen on the gel when the EVP was subjected to a purification protocol similar to the one for $HpaG_{Xooc}$ and the fragments (figure 2). Therefore, EVP was used properly in the form of a cell-free preparation as a control during subsequent experiments.

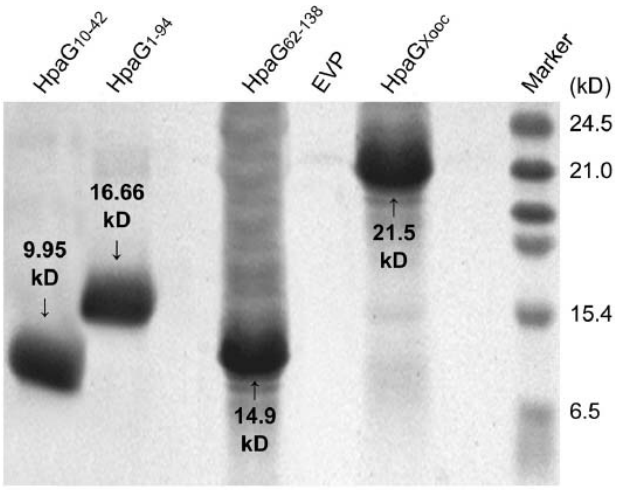

Figure 2. T-SDS-PAGE of $HpaG_{Xooc}$ and its fragments compared with the empty vector preparation (EVP) containing a mixture of inactive proteins but neither $HpaG_{Xooc}$ nor the variants. Proteins were purified by nickel chromatography and loaded at 12 μg per panel. Similar results were obtained when the experiments were repeated 3 times.

3.2 *Germinal palingenesis of tea buds and leaves*

Green tea is harvested annually in three cycles during spring, summer and autumn; high-quality tea is produced in the first cycle. Standard plot experiments were done during spring production cycles in 2005 and 2006 with the nationally noted green tea variety ShuChengZao (figure 1C; figure 3A). After each of the protein solutions was applied by spraying the shrub tops, production characters crucial to tea yield were studied. In comparison with the control, all the proteins except $HpaG_{62–138}$ evidently expedited palingenesis and the growth of juvenile buds and leaves. As shown in figure 3B and table 2, $HpaG_{1–94}$ and $HpaG_{10–42}$ produced similar and greater increase in tea-picking units than $HpaG_{Xooc}$ (one-way F tests, P <0.01), but $HpaG_{62–138}$ produced results that were no different from the control ($F < F_{0.05}$). During the spring season, germinal palingenesis occurred and juvenile buds and the top three leaves were picked 3 times in the control and $HpaG_{62–138}$-treated plants as usual, but 4 times in plants treated with any of the other three proteins, i.e. picking time was 3–5 days earlier for these plants (table 1). The enhancement in growth of the tea buds and leaves was

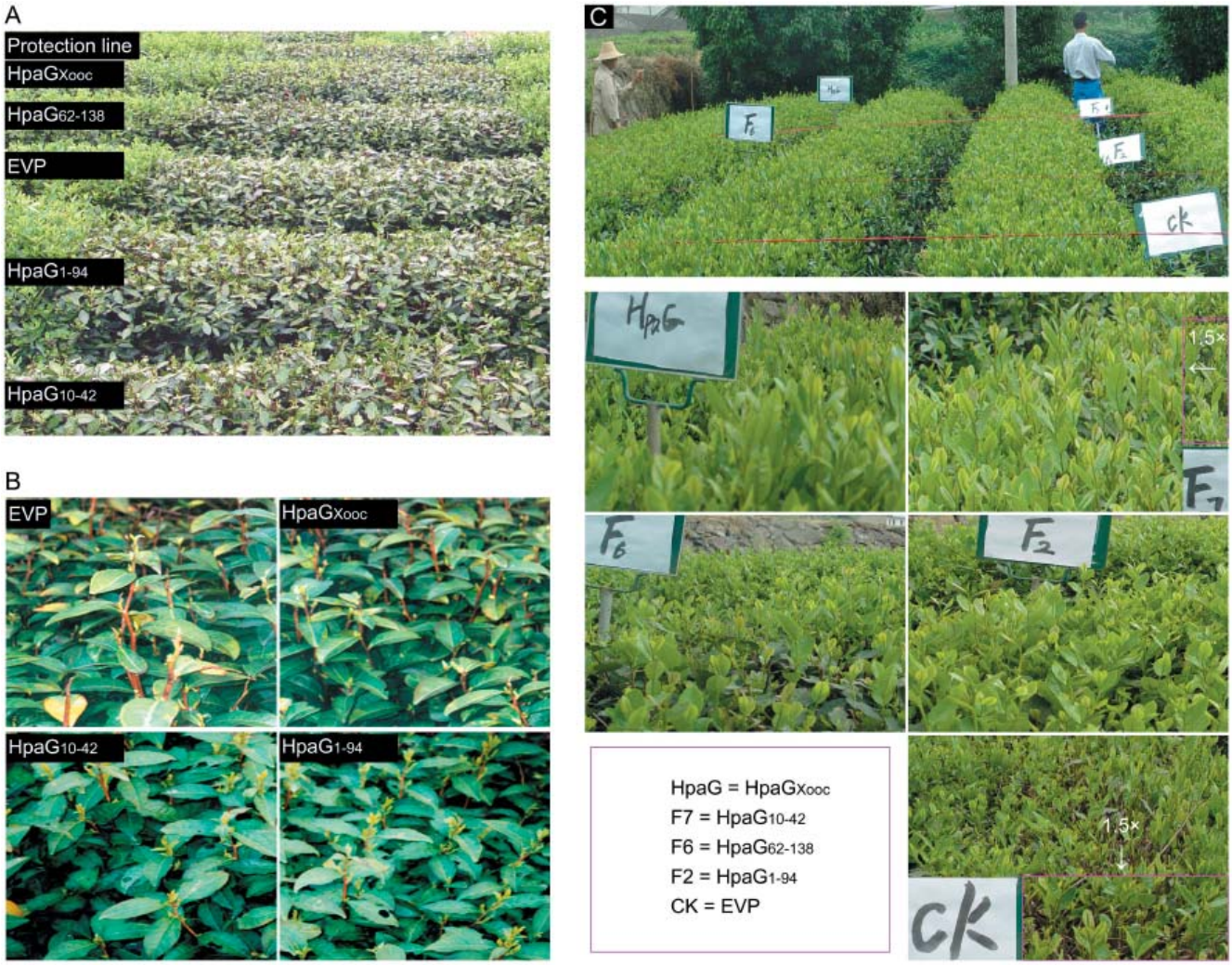

Figure 3. Parts of the plot in which experiments were conducted in 2005. **(A)** Distribution of the 5 plots showing plant growth 7 days after treatment with the indicated proteins. **(B)** A close view of juvenile buds photographed 5 days after treatment. **(C)** Views of five plots on the day of tea-picking. The photo at the top shows plot distribution in one of the three replicates. The two scientists localized plots of the same size for tea-picking in different treatments. The lower panels provide close-up views of the leaves to be picked in the plots. Codes on label plates correspond to the names of the proteins.

clear to the eyes in experimental plots (figure 1C). As a result (table 2), the number of tea-picking units increased by more than 36% (P <0.01) following the application of $HpaG_{Xooc}$, $HpaG_{1–94}$, or $HpaG_{10–42}$, in contrast to the control. Inversely, $HpaG_{62–138}$ caused a 5.3% decrease in effect compared with the control (P <0.05). The results of the tests in 2006 were similar to those in 2005 (tables 1, 2; figure 2).

3.3 *Biological productivity*

As shown in table 2, the fresh weight of tea-picking units was increased by 37.3%, 50.8% and 38.8%, respectively, in response to $HpaG_{Xooc}$, $HpaG_{1–94}$ and $HpaG_{10–42}$ compared with the control (P <0.01). By contrast, $HpaG_{62–138}$ did not significantly differ from the control in its effect ($F < F_{0.05}$). To reveal the molecular basis for plant growth enhancement, we studied tea (*Camellia sinensis*) expansin-like (*CsEXPL*) genes because expansins regulate growth of the cell and the plant (Link and Cosgrove 1998; Cosgrove 2000; Nieuwland *et al* 2005) in response to a harpin protein (Dong *et al* 2004; Ren *et al* 2006a, b). After tea plants growing in a greenhouse were treated with EVP or $HpaG_{1–94}$, production of new buds and leaves (figure 4A) was coincident with the expression of three *CsEXPL*s, particularly *CsEXP10* (figure 4B). The relative levels of gene expression as a function of $HpaG_{Xooc}$ (data not shown) and $HpaG_{1–94}$ (figure 4B), respectively, were

Table 2. Densities of tea-picking units surveyed in field experiments in 2005

Protein applied	Number of tea-picking units per m^2						*F* tests
	Plot 1	Plot 2	Plot 3	Total	Mean	% increase	
EVP	300	352	366	1018	339		A
$HpaG_{Xooc}$	401	527	456	1384	461	36.1	B
$HpaG_{1–94}$	445	543	419	1407	469	38.3	B
$HpaG_{10–42}$	479	536	393	1408	469	38.3	B
$HpaG6_{2–138}$	337	338	289	964	321	–5.3	C

Treatments were the same as in table 1. Tea buds were counted when picked. One-way *F* tests were done based on mean numbers of tea-picking units; the same letters denote insignificance in differences among treatments; different letters indicate significance.

Table 3. Bioproduction of tea buds and leaves surveyed in field experiments in 2005

Protein applied	Fresh weight (g/100 tea-picking units)						*F* tests
	Plot 1	Plot 2	Plot 3	Total	Mean	% increase	
EVP	55.5	55.5	57.0	168.0	56.0		A
$HpaG_{Xooc}$	80.0	73.6	77.1	230.7	76.9	37.3	B
$HpaG_{1–94}$	87.6	75.3	90.0	252.9	84.3	50.5	C
$HpaG_{10–42}$	76.7	80.0	76.4	233.1	77.7	38.8	B
$HpaG6_{2–138}$	50.5	60.0	62.9	173.4	57.8	3.2	A

Tea buds were weighed after picking. Treatments, *F* tests, and significance denotation were the same as in table 2.

Table 4. Tea yield surveyed in field experiments in 2005

Protein applied	Dry weight of processed tea (g/10 m^2)						*F* tests
	Plot 1	Plot 2	Plot 3	Total	Mean	% increase	
EVP	149.9	177.8	185.6	513.3	171.1		A
$HpaG_{Xooc}$	229.1	261.6	239.9	730.6	243.5	42.3	B
$HpaG_{1–94}$	266.3	273.5	260.0	799.8	266.6	55.8	C
$HpaG_{10–42}$	247.3	290.3	247.5	785.1	261.7	53.0	C
$HpaG6_{2–138}$	153.5	182.5	165.0	501.0	167.0	–2.8	A

Weight of tea buds was determined after stir-frying. *F* tests were similar to those in table 2.

1.8-fold to 8-fold, and 2.3-fold to 25-fold greater compared with the control. This result was confirmed by RNA gel-blotting analysis. *CeEXPL10* was expressed markedly in response to $HpaG_{1–94}$ treatment in contrast to the control, in which no evident expression of the gene was detected under the experimental conditions (figure 4C).

3.4 *Green tea yields and quality*

After picked buds and juvenile leaves were processed as spring tea, dry yields were determined. As shown in tables 4 and 5, tea yields were increased by over 39%, 54% and 44%, respectively, following treatment with $HpaG_{Xooc}$, $HpaG_{1–94}$ and $HpaG_{10–42}$ compared with the control ($P < 0.01$) in two spring seasons. $HpaG_{62–138}$, however, caused insignificant decrease in tea production relative to that of the control ($F < F_{0.05}$). After processed tea was sorted into the best class (c1) and lesser classes (c1–c4) by commercial standards (figure 5A), it was found that the proportion of top class tea (c1 and c2) was increased by the application of $HpaG_{1–94}$ compared with the control (figure 5B). When evaluated against a conventional standard for tea cooking, the floating time in boiled water of tea from plants treated with $HpaG_{Xooc}$ or $HpaG_{1–94}$ was longer compared with the control (figure 5C). Because a proper duration of tea floating and a brilliant green colour after cooking with boiled water are regarded as desirable physical properties of green tea (Wang 1981), $HpaG_{1–94}$ was found to be better than $HpaG_{Xooc}$ in its effect (figure 5C). Consistently, the expression level of the *CsDFR* gene involved in the biosynthesis of anthocyanins (Xie *et al* 2003) conspicuously increased in the germinal leaves

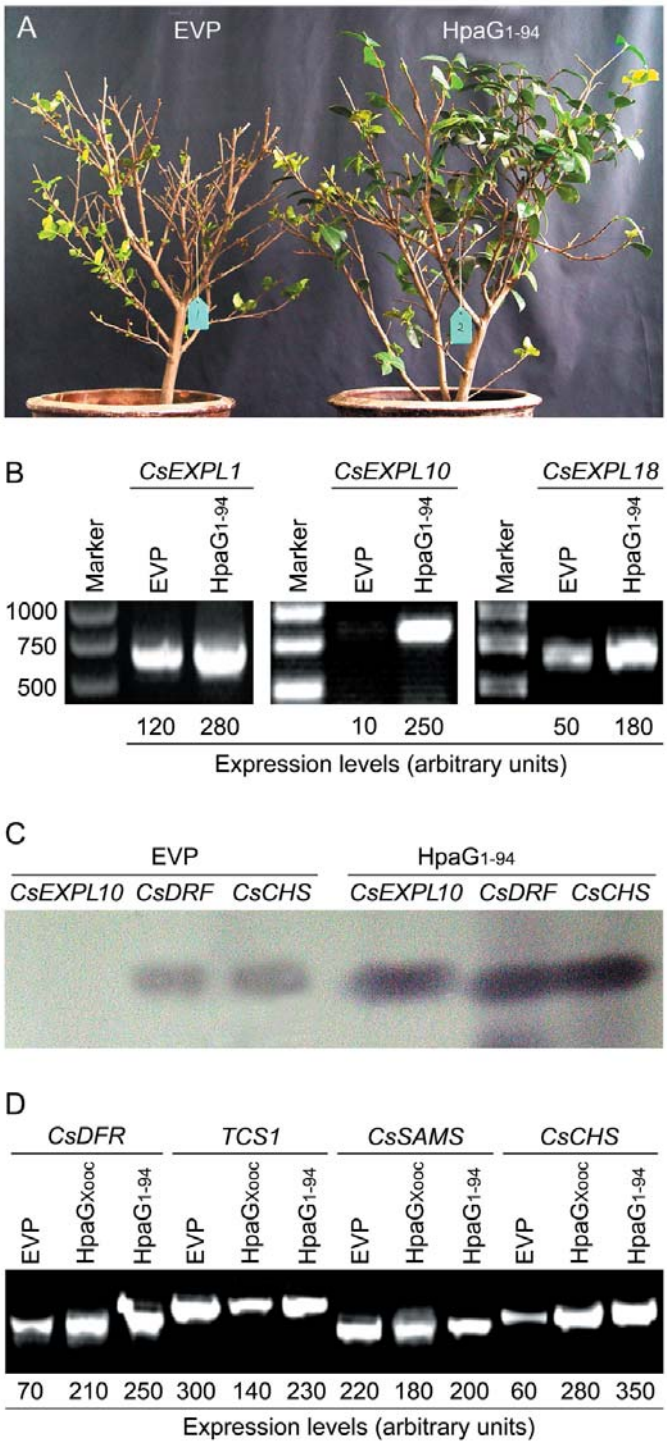

Figure 4. Laboratory assays for expression of relevant genes in tea plants. **(A)** Phenotypic performance of plants used for gene expression. The photo represents 9 plants observed 15 days after treatment as indicated. **(B)** RT-PCR analysis of the expansin genes *OsEXPL*s. **(C)** RNA gel-blotting analysis of selected genes. **(D)** RT-PCR analysis of genes related to the biochemical properties of green tea. *CsDFR*, *TCS1*, *CsSAMS* and *CsCHS* are involved in the biosynthesis of anthocyanins, caffeine, theines and catechols, respectively. To determine gene expression, RNA was isolated from buds and the top two leaves 5 days after treatment. In RT-PCR protocols, the standard gene *EF-1α* was expressed at similar levels irrespective of treatments; the gel is similar to those in our previous studies (Peng *et al* 2003; Dong *et al* 2005; Liu *et al* 2006; Ren *et al* 2006) and is not shown. RNA gel blot was probed with digoxigenin-labelled cDNA of the indicated genes. All experiments were done 3 times with similar results.

of plants treated with $HpaG_{1-94}$ compared with the control, based on RNA gel-blotting (figure 4C) and RT-PCR (figure 4D) analyses.

3.5 *Biochemical properties*

Concentrations of amino acids in processed tea indicate the quality of the nutrients but have no biological and medicinal effects distinct from those from other sources (Wang 1981). The leaf content of amino acids was significantly (P <0.01) reduced by all the proteins tested in comparison with the control (tables 6, 7). However, the content of catechols is more characteristic of green tea as these are critical compounds with relevance in the prevention and treatment of human diseases (Punyasiri *et al* 2004; Shimizu and Weinstein 2005). In our tests, tea catechols were markedly increased by the application of the three proteins compared with the control (P <0.01). Levels of

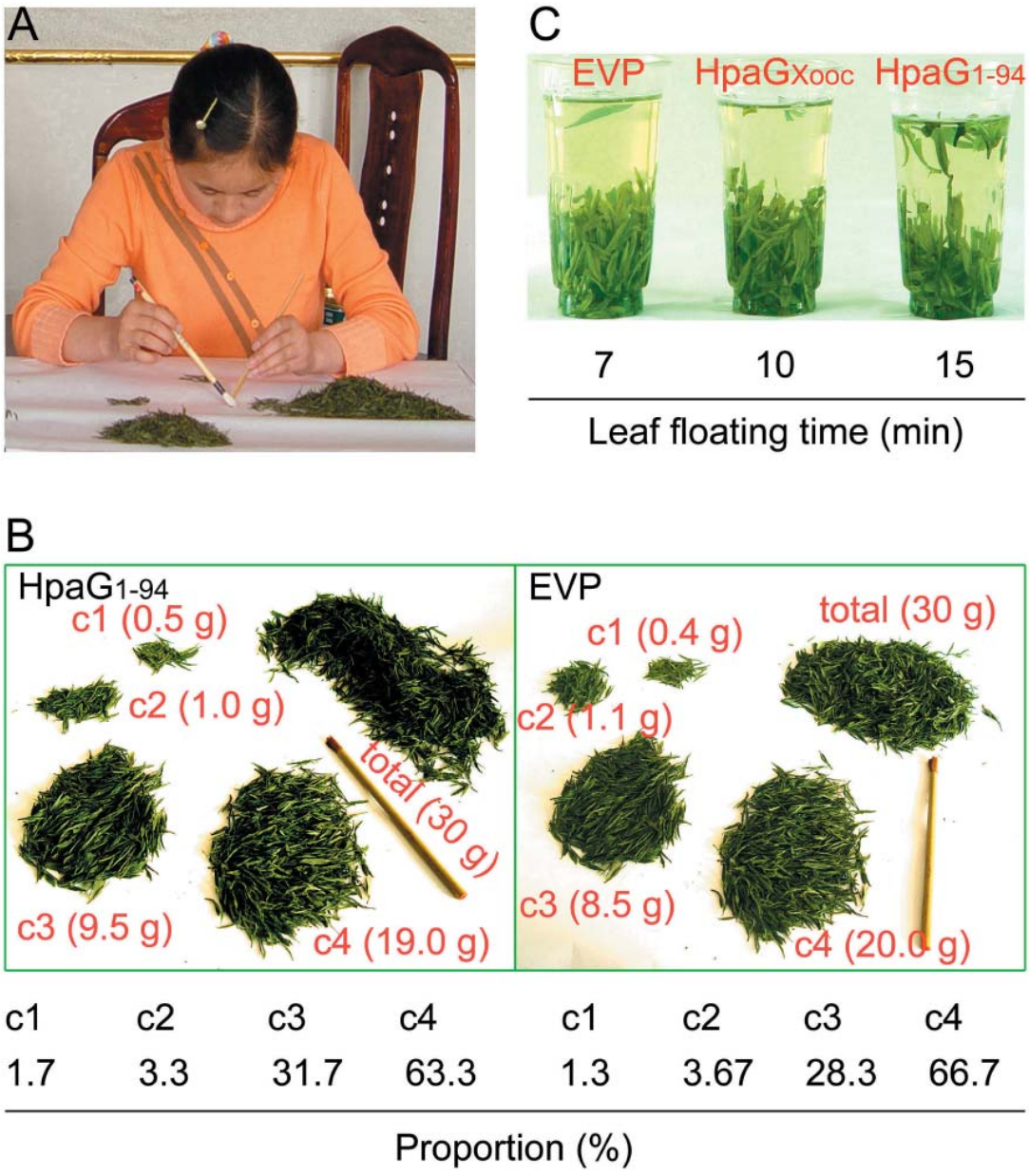

Figure 5. Assortment of tea by class and a rough evaluation of cooking. **(A)** Artificial assortment. **(B)** Proportion of the best class (c1) and c2–c4. **(C)** Tea cooking process showing flotation in water. The tea was obtained from plants treated with the indicated proteins; 2 g processed and unsorted tea was cooked by soaking in boiled water. All the processed tea was classified. Cooking experiments were done 3 times with similar results.

Table 5. Tea yield surveyed in field experiments in 2006

Protein applied	Dry weight of processed tea (g/10 m^2)						*F* tests
	Plot 1	Plot 2	Plot 3	Total	Mean	% increase	
EVP	166.5	195.4	208.6	570.5	190.2		A
$HpaG_{Xooc}$	240.8	290.7	263.6	794.8	264.9	39.3	B
$HpaG_{1–94}$	292.5	307.2	282.7	882.4	294.1	54.6	C
$HpaG_{10–42}$	275.2	321.6	225.3	822.1	274.0	44.1	B
$HpaG6_{2–138}$	170.2	202.8	181.8	554.8	184.9	–2.8	A

The study site was the same as in 2005; plant management and treatments were similar but data were collected 5 days later than those in Table 1 (2005). *F* tests were similar to those in table 2.

Table 6. Free amino acids and catechols tested in field experiments in 2005

Protein applied	% free amino acids		% catechol		*F* tests	
	Mean	% decrease	Mean	% increase	Amino acids	Catechol
EVP	1.395		17.556		A	A
$HpaG_{Xooc}$	1.150	17.6	28.875	64.5	B	B
$HpaG_{1–94}$	1.331	4.5	30.325	72.7	C	B
$HpaG_{10–42}$	1.278	8.4	37.500	113.6	C	C
$HpaG_{62–138}$	1.220	12.5	30.586	74.2	B	B

Contents of both compounds were determined based on dry weight of processed tea buds. *F* tests were done as in table 2.

Table 7. Free amino acids and catechols tested in field experiments in 2006

Protein applied	% free amino acids		% catechol		*F* tests	
	Mean	% decrease	Mean	% increase	Amino acids	Catechol
EVP	1.423		17.422		A	A
$HpaG_{Xooc}$	1.172	17.6	29.130	67.2	B	B
$HpaG_{1–94}$	1.345	5.5	30.327	74.1	B	B
$HpaG_{10–42}$	1.281	10.0	38.117	118.8	C	C
$HpaG_{62–138}$	1.219	14.3	31.648	81.7	B	B

Studies were the same as in table 6.

catechols were increased by over 64%, 72%, one-fold, and 74%, respectively, with $HpaG_{Xooc}$, $HpaG_{1–94}$, $HpaG_{10–42}$ and $HpaG_{62–138}$ (tables 6, 7). Therefore, these proteins can all affect the important medicinal properties of green tea; of these, $HpaG_{1–94}$ is the most effective.

To reveal the molecular basis for changes in the biochemical properties of green tea, the *TCS1*, *CsSAMS*, and *CsCHS* genes were studied because TCS1 and CsSAMS play a critical role in the production of theines (Kato *et al* 1999, 2003) and CsCHS is a key enzyme that catalyses the biosynthesis of catechols (Takeuchi *et al* 1994) in growing tea plants. The expression of the genes is presented in figure 4D. Whereas expression levels of *TCS1* were evidently decreased in the germinal leaves of tea plants treated with $HpaG_{Xooc}$ or $HpaG_{1–94}$ compared with the control, *CsSAMS* expression was similar irrespective of treatment. In contrast, induced expression of *CsCHS* was markedly increased; expression levels were approximately 4.7-fold and 5.8-fold higher, respectively, in plants treated with $HpaG_{Xooc}$ and $HpaG_{1–94}$ relative to the control. These results suggest that $HpaG_{1–94}$ consistently stimulates *CsCHS* expression in germinal leaves which has the effect of increasing the content of catechols in processed tea.

4. Discussion

Public concern about the use of pesticides and other agronomic chemicals in crops has stimulated an increasing number of studies on bioactive natural products from various sources, including plant pathogens, aimed at secure protection and production of crops (Dixon 2001; Stuiver and Custers 2001; Zasloff 2002). The public requirement for harmless production of tea calls for greater rigour because tea is processed from buds and leaves, which are sensitive areas for the access of chemicals to the plant. The

effects of $HpaG_{Xooc}$ and its functional fragments on green tea provide an alternative to the organic planting system that integrates multiple measures in watering, fertilizing, and control of diseases and insects into a procedure of management applied throughout the farming season (Geldermann and Kogel 2002; Sauerborn 2002). Moreover, using proteins as the active ingredients of spraying mixtures (Fontanilla *et al* 2005a, b) is simpler, and more acceptable by the public in contrast to the recalcitrance regarding some other approaches, such as genetic engineering (Stuiver and Custers 2001; Jang *et al* 2006).

Our study with green tea implies an expansion of protein use to industrial crops besides food crops (Ren *et al* 2006a, b; Chen *et al* 2007a, b). Modifying $HpaG_{Xooc}$ created nine functional fragments with optimized ability to elicit defence and promote productivity of plants but with nullified capacity of inducing cell death (Chen *et al* 2007a). In rice, $HpaG_{10–42}$ is similar to $HpaG_{1–94}$ in promoting plant growth but can induce an optimal level of disease resistance, and is most vigorous in increasing grain yield (Chen *et al* 2007b). In contrast, $HpaG_{62–138}$, identified as most active in eliciting plant HCD (Chen *et al* 2007a), does not affect disease resistance and plant growth (Chen *et al* 2007b). This study compares the effects of $HpaG_{1–94}$, $HpaG_{10–42}$, $HpaG_{62–138}$ and $HpaG_{Xooc}$ on green tea. $HpaG_{1–94}$, and not $HpaG_{10–42}$, was found to be the most effective in promoting the growth of tea buds and young leaves used as tea materials, increasing tea yield and commercial quality, in comparison with $HpaG_{Xooc}$ and $HpaG_{62–138}$. Therefore, the practical merits of the use of proteinaceous fragments in agriculture vary with the types of crops.

The different responses of the tea genes tested to $HpaG_{Xooc}$ and $HpaG_{1–94}$ are implicated in the molecular bases that underlie the changes in physical and biochemical properties of green tea. Figure 6 proposes experimental linkages between induced or suppressed expression of the genes with ultimate alternations in the yield and quality of processed tea. Induced expression of the three expansin genes indicates that cell wall expansion and cell growth may be contributing to facilitated growth of germinal tea leaves, similar to the enhanced growth of *Arabidopsis* plants treated with $HrpN_{Ea}$ (Dong *et al* 2004). The induced expression of *CsCHS* in germinal leaves is consistent with the elevation in catechol levels in processed tea, indicating that the use of $HpaG_{Xooc}$ or $HpaG_{1–94}$ plays a role in modulating catechol biosynthesis, which is essentially regulated by CsCHS activity (Kato *et al* 2000; Misako and Kouichi 2004; Punyasiri *et al* 2004). It can be assumed that either of the proteins applied to growing tea plants could subsequently increase the medicinal properties of green tea through the biochemical effects of the compounds (Shibata *et al* 2000; Cooper *et al* 2005; Shimizu and Weinstein 2005). The concomitant decrease in transcripts of *TCS1* and *CsSAMS* suggest that theine synthesis may be downregulated by the application of $HpaG_{Xooc}$ or $HpaG_{1–94}$. In growing tea leaves, TCS1 (Ashihara and Crozier 2001; Misako and Kouichi 2004) and CsSAMS (Koshiishi *et al* 2001) function to control the biosynthesis of caffeine and other theines (Kato *et al* 1999, 2000, 2003), which belong to a group of plant metabolites with effects on neuronal excitability and cough suppression (Birrell *et al* 2005). A purpose of modulating growing tea leaves is to control theines at moderate and correct levels in processed tea (Tressl and Voubrecht 1998). In fact, we were not clear about the medicinal functions of the compounds until browsing the literature in an attempt to explain the observed differences in expression levels of several genes. Moreover, the content of amino acids in processed tea decreases significantly subsequent to treating plants with $HpaG_{Xooc}$ and $HpaG_{1–94}$. Actually, amino acids may be available from many other sources richer than tea. These results suggest that the use of $HpaG_{Xooc}$ and $HpaG_{1–94}$ holds promise in a combination that optimizes the biochemical properties of green tea.

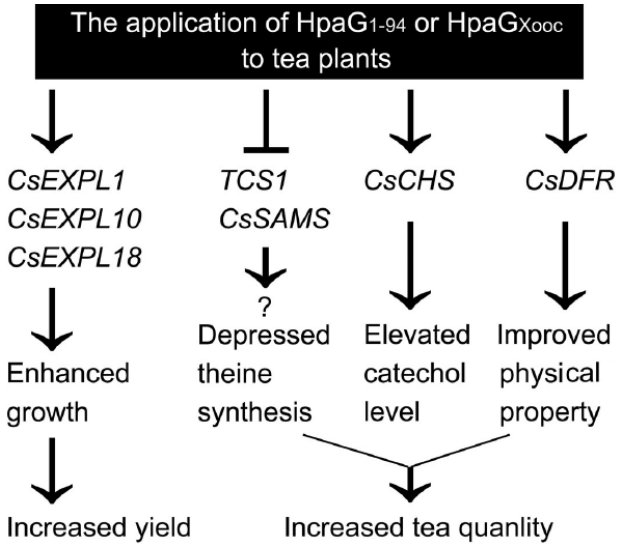

Figure 6. Schematic summary of the effects of $HpaG_{Xooc}$ and $HpaG_{1–94}$ on green tea. After tea plants are treated with the proteins, expression of the genes is activated (arrows) or suppressed (bar). Subsequently, biochemical and productive properties are altered. All responses shown here have been determined except theine production.

In conclusion, the application of $HpaG_{1–94}$ or $HpaG_{Xooc}$ causes an increase in yield and improvement in the biochemical properties of green tea. Several studies are required to characterize the mechanisms that underlie the experimental relationships between the altered expression of several genes and induced phenotypes in tea plants.

Acknowledgements

We thank Miss Junzi Dong and the two anonymous reviewers for comments on the manuscript. This study was supported by the National Science Foundation for Distinguished Young Scholars of China (30525088), National Development Plan of Key Basic Scientific Studies (The 973 Plan) of China Project 2 (2006CB101902), and Ministry of Education of China Century-Across Talent Award (2002-48).

References

Ashihara H and Crozier A 2001 Caffeine: a well known but little mentioned compound in plant science; *Trends Plant Sci*. **6** 407–413

Aucamp J P, Hara Y and Apostolides Z 2000 Simultaneous analysis of tea catechins, caffeine, gallic acid, theanine and ascorbic acid by micellar electrokinetic capillary chromatography; *J. Chromatogr. A* **876** 235–242

Bauer D W, Wei Z M, Beer S V and Collmer A 1995 *Erwinia chrysanthemi* harpin$_{Ech}$: an elicitor of the hypersensitive response that contributes to soft-rot pathogenesis; *Mol. Plant–Microbe Interact.* **8** 484–491

Birrell M A, Korbonits M, Korbonits D and Barnes P J 2005 Theobromine inhibits sensory nerve activation and cough; *FASEB J.* **19** 231–233

Charkowski A O, Alfano J R, Preston G, Yuan J, He S Y and Collmer A 1998 The *Pseudomonas syringae* pv. tomato HrpW protein has domains similar to harpins and pectate lyases and can elicit the plant hypersensitive response and bind to pectate; *J. Bacteriol.* **180** 5211–5217

Chen L, Qian J, Qu S, Long J, Yin Q, Zhang C, Wu X, Sun F, Wu T, Beer S V, and Dong H 2007a Identification of specific fragments of HpaG$_{Xooc}$, a harpin protein from *Xanthomonas oryzae* pv. *oryzicola*, that induce disease resistance and enhanced growth in rice. *Phytopathology* (in press)

Chen L, Zhang S-S, Qu S, Long J, Yin Q, Qian J, Sun F, Zhang S-J, Chunling Zhang, Wang L, Wu X, Wu T, Zhang Z, Cheng Z, Beer S V and Dong H 2007b A selected fragment of HpaG$_{Xooc}$, a harpin protein from *Xanthomonas oryzae* pv. *oryzicola*, affects disease reduction and grain yield of rice in extensive grower plantings; *Phytopathology* (in press)

Cooper R, Morre D J and Morre D M 2005 Medicinal benefits of green tea: part II. Review of anticancer properties; *J. Altern. Complement. Med.* **11** 639–652

Cosgrove D J 2000 New genes and new biological roles for expansins; *Curr. Opin. Plant Biol.* **3** 73–78

Dey P M and Harborne J B 1991 *Methods in plant biochemistry. Volume 5. Amino acids, proteins and nucleic acids* (New York: Academic Press)

Dixon R A 2001 Natural products and plant disease resistance; *Nature* **411** 843–847

Dong H P, Peng J, Bao Z, Meng X, Bonasera J M, Beer S V and Dong H 2004 Downstream divergence of the ethylene signaling pathway for harpin-stimulated *Arabidopsis* growth and insect defense; *Plant Physiol.* **136** 3628–3638

Dong H P, Yu H, Bao Z, Guo X, Peng J, Yao Z, Chen G, Qu S and Dong H 2005 The *ABI2*-dependent abscisic acid signalling controls HrpN-induced drought tolerance in *Arabidopsis*; *Planta* **221** 313–327

Dong H, Delaney T P, Bauer D W and Beer S V 1999 Harpin induces disease resistance in *Arabidopsis* through the systemic acquired resistance pathway mediated by salicylic acid and the *NIM1* gene; *Plant J.* **20** 207–215

Fontanilla J M, Montes M and De Prado R 2005a Induction of resistance to the pathogenic agent *Botrytis cinerea* in the cultivation of the tomato by means of the application of the protein "harpin" (messenger); *Commun. Agric. Appl. Biol. Sci.* **70** 35–40

Fontanilla M, Montes M and De Prado R 2005b Effects of the foliar-applied protein "harpin$_{Ea}$" (messenger) on tomatoes infected with *Phytophthora infestans*; *Commun. Agric. Appl. Biol. Sci.* **70** 41–45

Friedman M, Mackey B E, Kim H J, Lee I S, Lee K R, Lee S U, Kozukue E and Kozukue N 2007 Structure–activity relationships of tea compounds against human cancer cells; *J. Agric. Food Chem*. **55** 243–53

Gallie D R, Le H, Caldwell C and Browning K S 1998 Analysis of translation elongation factors from wheat during development and flowering heat shock; *Biochem. Biophys. Res. Commun.* **245** 295–300

Geldermann U and Kogel K H 2002 Nature's concept. The 'new agriculture' amidst ecology, economy and the demythologization of the gene; *J. Agron. Crop. Sci.* **188** 368–375

Graham H N 1992 Green tea composition, consumption, and polyphenol chemistry; *Prev. Med.* **21** 334–350

Huang J R, Takano T and Akita S 2000 Expression of α-expansin genes in young seedlings of rice (*Oryza sativa* L.); *Planta* **211** 467–473

Jang Y S, Sohn S I and Wang M H 2006 The *hrpN* gene of *Erwinia amylovora* stimulates tobacco growth and enhances resistance to *Botrytis cinerea*; *Planta* **223** 449–456

Kato M, Mizuno K, Crozier A, Fujimura T and Ashihara H 2000 Caffeine synthase gene from tea leaves; *Nature* **406** 956–957

Kato M, Mizuno K, Fujimura T, Iwama M, Irie M, Crozier A and Ashihara H 1999 Purification and characterization of caffeine synthase from tea leaves; *Plant Physiol*. **120** 579–586

Kato M, Gyoten Y, Sakai Kato K and Toyo'oka T 2003 Rapid analysis of amino acids in Japanese green tea by microchip electrophoresis using plastic microchip and fluorescence detection; *J. Chromatogr. A* **1013** 183–189

Kim J F and Beer S V 1998 HrpW of *Erwinia amylovora*, a new harpin that contains a domain homologous to pectate lyases of a distinct class; *J. Bacteriol*. **180** 5203–5210

Kim J F and Beer S V 2000 *hrp* genes and harpins of *Erwinia amylovora*: a decade of discovery; in *Fire blight and its causative agent*, *Erwinia amylovora* (ed.) J L Vanneste (Wallingford*:* CAB International) pp 141–162

Kim J G, Jeon E, Oh J, Moon J S and Hwang I 2004 Mutational analysis of *Xanthomonas* harpin HpaG identifies a key functional region that elicits the hypersensitive response in nonhost plants; *J. Bacteriol*. **186** 6239–6247

Kim J G, Park B K, Yoo C H, Jeon E, Oh J and Hwang I 2003 Characterization of the *Xanthomonas axonopodis* pv. *glycines* HpaG pathogenicity island; *J. Bacteriol.* **185** 3155–3166

Koshiishi C, Kato A, Yama S, Crozier A and Ashihara H 2001 A new caffeine biosynthetic pathway in tea leaves: utilisation of adenosine released from the S-adenesyl-L-methionine cycle; *FEBS Lett.* **499** 50–54

Laby R J, Wei Z M and Beer S V 2006 *Hypersensitive response elicitor fragments eliciting a hypersensitive response and uses thereof*; United States Patent 7132525

Li P, Lu X, Shao M, Long J and Wang J 2004 Genetic diversity of harpins from *Xanthomonas oryzae* and their activity to induce hypersensitive response and disease resistance in tobacco; *Sci. China C Life Sci.* **47** 461–469

Link B M and Cosgrove D J 1998 Acid-growth response and α-expansins in suspension cultures of bright yellow 2 tobacco; *Plant Physiol.* **118** 907–916

Liu F, Liu H, Jia Q, Wu X, Guo X, Zhang S, Song F and Dong H 2006 The internal glycine-rich motif and cysteine suppress several effects of $HpaG_{Xooc}$ in plants; *Phytopathology* **96** 1052–1059

Misako K and Kouichi M 2004 Caffeine synthase and related methyltransferases in plants; *Front Biosci.* **9** 1833–1842

Nieuwland J, Feron R, Huisman B A, Fasolino A, Hilbers C W, Derksen J and Mariani C 2005 Lipid transfer proteins enhance cell wall extension in tobacco; *Plant Cell* **17** 2009–2019

Noel L, Thieme F, Nennstiel D and Bonas U 2002 Two novel type III-secreted proteins of *Xanthomonas campestris* pv. *vesicatoria* are encoded within the HpaG pathogenicity island; *J. Bacteriol*. **184** 1340–1348

Peng J, Bao Z, Dong H, Ren H and Wang J 2004a Expression of $harpin_{Xoo}$ in transgenic tobacco induces pathogen defense in the absence of hypersensitive cell death; *Phytopathology* **94** 1048–1055

Peng J, Bao Z, Ren H, Wang J and Dong H 2004b $Harpin_{Xoo}$ and its functional domains activate pathogen-inducible plant promoters in *Arabidopsis*; *Acta Bot. Sinica* **46** 1083–1090

Peng J, Dong H, Dong H P, Delaney T P, Bonasera B M and Beer S V 2003 Harpin-elicited hypersensitive cell death and pathogen resistance requires the *NDR1* and *EDS1* genes; *Physiol. Mol. Plant Pathol.* **62** 317–326

Punyasiri P A N, Abeysinghe I S B, Kumar V, Treutter D, Duy D, Gosch C, Martens S, Forkmann G and Fischer T C 2004 Flavonoid biosynthesis in the tea plant *Camellia sinensis*: properties of enzymes of the prominent epicatechin and catechin pathways; *Arch. Biochem. Biophys.* **431** 22–30

Ren H, Gu G, Long J, Qian J, Wu T, Song T, Zhang S, Chen Z and Dong H 2006a Combinative effects of a bacterial type-III effector and a biocontrol bacterium on rice growth and disease resistance; *J. Biosci.* **31** 617–627

Ren H, Song T, Wu T, Sun L, Liu Y, Yang F, Chen Z, Dong H 2006b Effects of a biocontrol bacterium on growth and defence of transgenic rice plants expressing a bacterial type-III effector; *Ann. Microbiol.* **56** 281–287

Sauerborn J 2002 Site productivity, the key to crop productivity; *J. Agron. Crop. Sci.* **188** 363–375

Schagger H and von Jagow G 1987 Tricine–sodium dodecyl sulphate–polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa; *Anal. Biochem.* **166** 368–379

Shibata K, Moriyama M, Fukushima T, Kaetsu A, Miyazaki M and Une H 2000 Green tea consumption and chronic atrophic gastritis: a cross-sectional study in a green tea production village; *J. Epidemiol.* **10** 310–316

Shimizu M and Weinstein I B 2005 Modulation of signal transduction by tea catechins and related phytochemicals; *Mutat. Res.* **591** 147–160

Stangl V, Lorenz M and Stangl K 2006 The role of tea and tea flavonoids in cardiovascular health; *Mol. Nutr. Food Res.* **50** 218–228

Stuiver M H and Custers J H H V 2001 Engineering disease resistance in plants; *Nature* **411** 865–868

Subramanian S, Kondaiah P and Adiga P R 2002 Expression, purification, and characterization of minimized chicken riboflavin carrier protein from a synthetic gene in *Escherichia coli*; *Protein Expres. Purif.* **26** 284–289

Takeuchi A, Matsumoto S and Hayatsu M 1994 Chalcone synthase from *Camellia sinensis*: isolation of the cDNAs and the organ-specific and sugar-responsive expression of the genes; *Plant Cell Physiol*. **35** 1011–1018

Tressl R and Voubrecht H R 1998 Health effects of decaffeinated tea; *Tea Coffee Trade* **170** 52–58

Usmani O S, Belvisi M G, Patel H J, Crispino N, Birrell M A, Korbonits M, Korbonits D and Barnes P J 2005 Theobromine inhibits sensory nerve activation and cough; *FASEB J.* **19** 231–233

Wang Z N 1981 *Tea, biochemical properties* (Chaye, Shenghua Yuanli) (in Chinese) (Bejing: Agricultural Publishing House [*Nongye Chubanshe*])

Wei Z M, Fan H, Stephens J J, Beer S V and Laby R J 2005 *Hypersensitive response elicitor fragments which are active but do not elicit a hypersensitive response*; United States Patent 6,858,707

Wei Z M, Lacy R J, Zumoff C H, Bauer D W, He S Y, Collmer A and Beer S V 1992 Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*; *Science* **257** 85–88

Xie D Y, Sharma S B, Paiva N L, Ferreira D, Dixon R A 2003 Role of anthocyanidin reductase, encoded by BANYULS in plant flavonoid biosynthesis; *Science* **299** 396–399

Zasloff M 2002 Antimicrobial peptides of multicellular organisms; *Nature* **415** 389–395

Zhu W G, Magbanua M M and White F F 2000 Identification of two novel *hpaG*-associated genes in the *hpaG* gene cluster of *Xanthomonas oryzae* pv. *oryzae*; *J. Bacteriol.* **182** 1844–1853

MS received 6 December 2006; accepted 29 June 2007

*e*Publication: 30 July 2007

Corresponding editor: Imran Siddiqi