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Role of Physical Barriers and Chitinase in Conferring Blister Blight Resistance to *Camellia sinensis* (L.) O. Kuntze*

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Abstract: The role played by certain physical barriers and chitinase enzyme in conferring blister blight resistance to tea was investigated. The blister blight resistance of the tea clone SA-6 was due to higher amounts of epicuticular wax and increased thickness of cuticle/epidermal layer, functioning as physical barriers to hyphal penetration of *Exobasidium vexans*. Higher quantum of chitinase in intercellular spaces of palisade tissues was observed in the resistant clone SA-6 through immunolocalization study. Both chitinase assay as well as western blotting studies confirmed that the constitutive level of chitinase expression was higher in the resistant clone when compared with the susceptible tea clone.

Key words: Tea, inter-cellular localization, constitutive expression, inducible expression, disease resistance

Introduction

The blister blight disease of tea is by far the most serious disease of tea (Arulpragasam, 1992). A distinct relationship exists between the extent of disease incidence and amount of crop lost due to blister blight disease (Venkataram, 1968).

The pathogen *Exobasidium vexans* infects tender leaves and stems of tea. The spread of the disease is highly dependent upon weather conditions (Agnihothrudu and Chandramouli, 1990; Agnihothrudu and Chandramouli, 1991). The disease was favoured by relative humidity in the range of 60-100% (Prernkumar, 1996). Recently, Sugha (1997) reported that *E. vexans* could survive on necrotic blister during offseason. To restrain the disease within the economic threshold level, protectant and eradicant fungicides are sprayed at regular intervals.

Certain clones of tea plants in south India were known to manifest resistance to blister blight. Use of resistant varieties is one of the acknowledged components of Integrated Pest Management (IPM). The nature and basis of resistance in tea clones to the disease is not known. Debnath and Paul (1994) attempted to correlate some anatomical and morphological characters of 17 clones with disease severity, but could not find any significant correlation. Even low levels of resistance are valuable since the need for chemical control measures can be limited. Effective infection of the host plant by the pathogen follows a complex phenomenon involving a series of events that enable or deter the pathogen to effectively cause infection. Pre- and post infectional biochemical and physical changes in the host plants play a vital role in influencing the events that impart resistance to the disease.

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Chitinases (EC 3.2.1.14) catalyze the hydrolysis of β-(1,4)-linkages between N-acetylglucosamine (2-acetamido-2-deoxyglucopyranoside) residues in the linear homopolymer, chitin. They are widely distributed enzymes found in microorganisms, plants and animals. A role for these enzymes in plant defense mechanism against fungal attack is suggested by the absence of chitin in higher plants (Abeles *et al*., 1970), its presence in fungal cell walls (Bartnicki-Garcia, 1968) and the finding that the plant chitinases inhibit *in vitro* spore germination and mycelial growth of certain fungi (Roberts and Selitrennikoff, 1988). Thus, these enzymes have the ability to hydrolyze the chitin present in the fungal cell wall and prevent the entry of fungal pathogen into leaf tissue. This enzyme is called b-protein or pathogenesis-related protein (Tuzun *et al*., 1989).

The objective of this research is to investigate the role of certain physical barriers and the PR-protein, chitinase on conferring blister blight resistance. Besides, cellular-localization and western blotting of tea leaf chitinase were also carried out.

Materials and Methods

This work was carried out at UPASI Tea Research Foundation, Valparai during 2003. The tea clones used in this study include a blister blight resistant clone (SA-6) and a blister blight susceptible clone (TES-34). The tolerance/susceptibility of these tea clones are based on unpublished data accumulated by the Plant Pathology Division, UPASI Tea Research Foundation, Valparai 642 127, India. In all the experiments, third leaves of crop shoots were harvested from the field and analyzed afresh. The appearance of the blister blight lesions in the leaf of susceptible tea clone is shown in Fig. 1.

Analysis of Physical Barriers

Transverse sections of tea leaves from SA-6 and TES-34 clones were made and used for observing the anatomical characters such as thickness of cuticle, thickness of epidermis, stomatal length, stomatal breadth and palisade tissue. The morphological characters examined were frequency of stomata and trichome besides quantification of epicuticular wax. For the study of these parameters, a microscope (Carl Zeiss) equipped with a camera, was used. There were ten replicates and the data were statistically analyzed following Duncan's Multiple Range Test.

Cellular-Localization of Chitinase

Transverse sections taken from the tea leaves of SA-6 and TES-34 were washed with TBS (100 mM Tris-HCl, 150 mM NaCl, pH 7.5). The sections were then incubated with anti-barley chitinase antibody (gift from Prof. S. Muthukrishnan, Kansas State University, Kansas 66506) diluted to 1:50,000 in TBS for 12 h at room temperature. Unbound antibody was removed with washes of TBS and the sections were incubated for 2 h at room temperature in goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Sigma, USA) diluted to 1:1,000 in TBS. Unbound secondary antibody was removed with washes of TBS. Bound alkaline phosphatase activity was detected on the sections using BCIP/NBT solution (Sigma, USA). The sections were observed under microscope and photographed.

Western Blotting of Chitinase

The infected and uninfected leaf tissues from SA-6 and TES-34 were used for protein extraction. Equal amount of protein was applied to a 10% PAGE minigel (Hoefer model SE250). The gel was run

under denaturing conditions, equilibrated in Tris-glycine buffer, pH 8.3 and then electro blotted (45 mA, constant current, 2 h) onto nitrocellulose membrane using a semi-dry type transfer system. After electroblotting, the membrane was stained for total protein using 0.2% (w/v) Ponceau S in 3% (w/v) TCA and the molecular weight markers recorded in pencil on the blot. The blot was destained with several 20 mL washes of TBS (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) and then blocked with 5% nonfat dry milk in TBS for 2 h at room temperature with gentle agitation. The blot was then probed with anti-barley chitinase antibody diluted to 1:50,000 in 5% nonfat dry milk in TBS for 12 h at room temperature. Unbound antibody was removed with four 20 mL washes of TBS and the blot was incubated for 2 h at room temperature in goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Sigma, USA) diluted to 1: 1,000 in 5% nonfat dry milk in TBS. Unbound secondary antibody was removed with four, 20 mL washes of TBS. Bound alkaline phosphatase activity was detected on the blot using BCIP/NBT solution (Sigma, USA). The blot was scanned with UMAX scanner (Amersham Biosciences) and analyzed using Image Master Total Lab Software, Ver 1.0.

Chitinase Activity Assay

Each sample was ground immediately to a fine powder in liquid nitrogen with a mortar and pestle. The powders were suspended in an extraction buffer consisting of 100 mM acetate buffer (pH 5.0) containing 1% (W/V) ascorbic acid and 1% (W/V) PVP. After centrifugation at 16,700 g for 30 min, the supernatant was saved and designated as crude extract. Commercial deacetyl glycol chitin (Sigma) was used as a substrate. A volume of 0.1 mL crude extract was added to 1.0 mL of 0.05% deacetyl glycol chitin in 50 mM acetate buffer (pH 5.0) at room temperature to start the enzyme reaction. After 30 min, the reaction was stopped by heating at 100°C for 5 min. The increased reducing ends, which were released by tea leaf chitinase, were determined according to the method of Imoto and Yagishita (1971). The preheated crude extracts (100°C for 5 min) were used as controls. One unit of chitinase activity was defined as an amount capable of releasing reducing ends corresponding to 1 μg GlcNAc (N-acetylglucosamine) from deacetyl glycol chitin at pH 5.0 in 1 h. The GlcNAc (5-40 μg) was used to plot the standard curve. There were five replicates and the data were statistically analyzed following Duncan's multiple range test.

Results and Discussion

Generally, the lesions do not appear in the tolerant clone, SA-6. Under favourable environmental conditions, the lesions enlarged fast in the susceptible clone (Fig. 1) while they were restricted in the tolerant ones. It is evident from Table 1 that SA-6 is the most resistant clone to blister blight infection while the clone TES-34 is susceptible to blister blight disease. Anatomical data showed that the third leaf of SA-6 clone had higher amount of epicuticular wax i.e., 42 μg cm^{-2} when compared to the other susceptible clone. The thickness of the upper and lower cuticle in SA-6 was also the highest. The susceptible clone TES-34 had only fewer trichomes and a less thickened epidermis and cuticle when compared to the resistant clone. The stomatal frequency was higher in TES-34 than the other clone examined.

The present study contradicts with the findings of Debnath and Paul (1994) who reported that leaf characters did not have any correlation with blister blight resistance of tea cultivars. The average stomatal frequency on both the leaf surfaces was higher in the susceptible than in the resistant clone; this finding was similar to that reported earlier by Basru *et al*. (1985). Since most of the infections in

Table 1: Physical barriers to the blister blight fungus *Exobasidium vexans* in tea leaves

	Upper surface		Lower surface	
Characters	SA 6	TES 34	SA 6	TES 34
Epicuticular wax on whole leaf (μg cm^{-2})	42±5.5^{a}	38±2.0^{b}	-	-
Thickness of cuticle (μm)	1.36±0.1^{a}	1.01±0.1^{b}	1.15±0.1^{ab}	0.94±0.0^{b}
Thickness of upper epidermis (μm)	14.34±1.9^{a}	10.56±1.4^{b}	-	-
Thickness of lower epidermis (μm)	-	-	11.78±0.7^{a}	9.64±1.5^{b}
Stomatal frequency (mm^{-2})	164.54±5.8^{a}	175.24±5.1^{b}	181.31±4.0^{b}	18934±27
Stomatal length (μm)	16.23±1.8^{a}	21.22±0.5^{b}	18.54±1.1^{ab}	24.15±1.9
Stomatal breadth (μm)	4.89±0.3^{a}	5.64±0.0^{b}	6.24±0.1^{b}	6.41±0.2
Trichome frequency (mm^{-2})	-	-	52.34±3.5^{a}	45.21±2.9
Average number of palisade cells (μm^{-1})	74.32±2.2^{a}	65.14±2.0^{b}	-	-

Values are means (followed by standard deviations) of 10 independent plant samples. Means denoted by the same letter did not differ significantly at $p<0.05$ based on Duncan's multiple range test

Table 2: Chitinase activity in infected and uninfected leaves of blister blight resistant and susceptible tea clones

Tea clones	Portion of leaf	Chitinase (Unit/g FW)
SA 6	Uninfected	151±7.8^{b}
	Infected	532±12.4^{c}
TES 34	Uninfected	034±5.6^{a}
	Infected	519±11.3^{c}

Values are means (followed by standard deviations) of 5 to 7 independent plant samples. Means denoted by the same letter did not differ significantly at $p>0.05$ based on Duncan's multiple range test

Fig. 1: Multiple lesions of blister blight infection in susceptible (TES-34) tea clone

tea took place through stomata, the reduction in the stomatal number and size should therefore, help in reducing the entry of the pathogen. The reduction in the number of stomata would essentially reduce the number of sites structurally suitable for penetration. The lesser number of stomata and their smaller size in the resistant varieties may enhance the physical/structural resistance against pathogen (Woloshuk *et al*., 1983).

The palisade cells were more compact in the resistant clone SA-6. But they were loosely arranged with greater intercellular space in the susceptible clone TES-34, thereby facilitating the rapid and easy spread of the pathogen, which confirms the observations by Godoy *et al*. (1985). The compactly arranged palisade tissue may account for the slower rate of blister formation on the leaves of the resistant clones. The resistant clone SA-6 had higher frequency of trichomes, more epicuticular wax content and increased epidermis-cum cuticle thickness. TES-34 had only a moderate number/content of all the said parameters. The results indicated that some of these characters might have cumulative effects in conferring resistance against blister blight infection (Gupta *et al*., 1992).

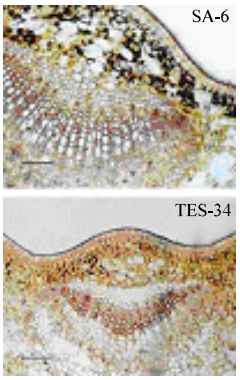

Fig. 2: Cellular localization of chitinase in SA-6 and TES-34 (bar = 50 μm)

Transvers section of leaves from tea clones such as SA-6 and TES-34 were taken for intercellular localization of chitinase using anti-barley chitinase antibody. Regions of chitinase activity were found to be dark to violet in colour (Fig. 2). Higher quantum of chitinase in region below upper epidermis i.e., near palisade tissue, was observed. The enzyme was found to be intercellular. Cellular-localization study helped in visualizing higher quantum of chitinase in between the palisade tissues of the tolerant clone SA-6. Since this enzyme is intercellular, it is expected that it would help in preventing the spread of fungal hyphae in intercellular spaces in the leaves of tolerant clones. Higher expression of chitinase in SA-6 and lower expression in TES-34 explain their relative tolerance/susceptibility to blister blight.

Densitometric study (Fig. 3) of the western blot (Fig. 4) revealed that uninfected leaves of SA-6 possessed three chitinases with molecular weights of 50.6, 41.6 and 34.7 kDa. In the infected leaves of SA-6, two more chitinases with molecular weights of 40.0 and 23.0 kDa were observed. Two chitinases with molecular weights of 50.6 and 41.6 kDa were observed in the healthy leaves of TES-34. The 50.6 kDa chitinase vanished in the infected leaves of TES-34 and four newer chitinases with molecular weights of 48.5, 40.0, 34.7 and 23.0 kDa were noticed in the infected leaves. Data on chitinase activity (Table 2) has also supported our finding that constitutive expression of chitinase was higher in the resistant clone.

Swegle *et al*. (1992) noticed five proteins with chitinase activity in barley (*Hordeum vulgare* L.) seeds. Two of these (CH2 and CH3) had amino terminal sequences resembling a portion of the chitin-binding domain of lectins and other plant defense proteins. All chitinases exhibited antifungal activity and inhibited the mycelial growth of some species of *Trichoderma* and *Fusarium*, *in vitro*.

Chitinases have been isolated from barley leaves infected with fungi (Kragh *et al*., 1990). They are constitutively expressed at low levels in leaves (Shinshi *et al*., 1987). Increased levels of gene expression or enzymic activity have been observed in leaves after inoculation with fungi (Roby *et al*., 1990).

The constitutive form of chitinase with molecular weight 34.7 kDa noticed in SA-6 was found to be not expressed constitutively in TES-34. Although both the cultivars were able to produce upon infection five types of chitinases, the constitutive level of expression was found to be higher in the tolerant clone, SA-6. As high levels of constitutive expression of chitinase is required to avoid the initial penetration by fungal hyphae, the tea clone such as SA-6 which maintains more chitinase activity at all times should be desired for planting in areas that are prone to blister blight infection and

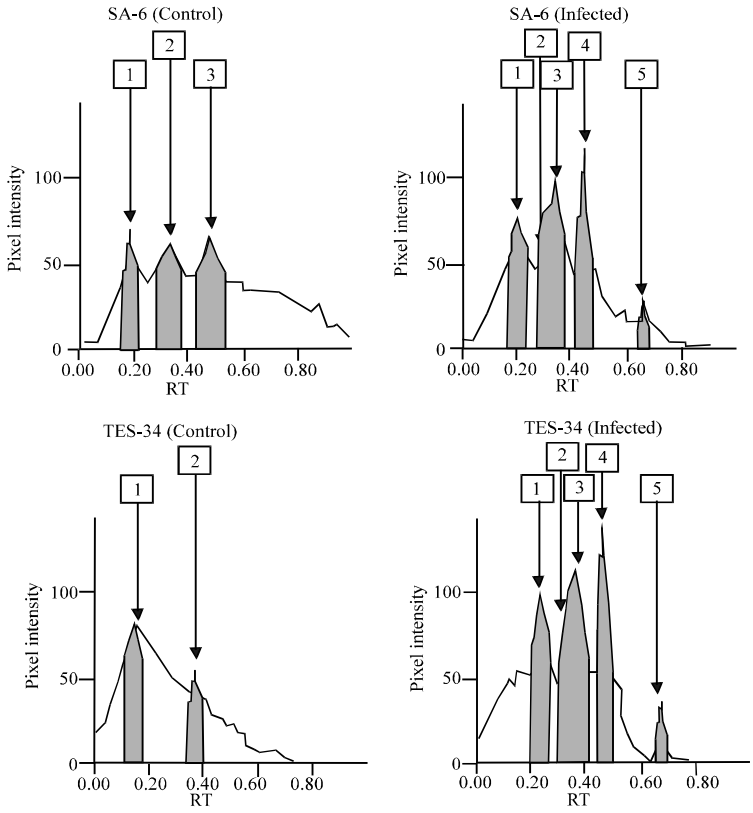

Fig. 3: Densitrometric scan of the western blot

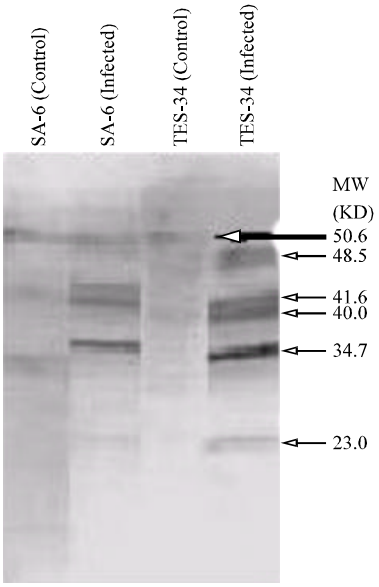

Fig. 4: Western blotting of tea leaf chitinase

for plant improvement programmes. It is suspected that in the susceptible clone, TES-34, the chitinase with molecular weight of 50.6 kDa in control (uninfected) leaves would have given rise to the formation of chitinase with molecular weight of 48.5 kDa in infected leaves.

The present study necessitated the importance of both physical barriers and b-protein such as chitinase in preventing the penetration of fungal hyphae. Since, resistance is a mechanism that involves a combination of more than one biochemical process, it would be rather unrealistic to think that a single DNA fragment with all genetic information for fungal resistance could be transferred into tea plants. Hence, it is necessary to generate further basic information about the factors conferring resistance to tea plants against the fungus, *Exobasidium vexans* before attempting to develop transgenic plants with blister blight resistance.

References

Abeles, F.B., R.P. Bosshart, L.E. Forrence and W.H. Habig, 1970. Preparation and purification of glucanase and chitinase from bean leaves. Plant Physiol., 47: 129-134.

Agnihothrudu, V. and B. Chandramouli, 1990. Blister blight of tea and its control. Planters' Chronicle 85: 205-217.

Agnihothrudu, V. and B. Chandramouli, 1991. Blister Blight of Tea, its Control and Future Line of Research. In: Yamanishi, T. (Ed.), Proceeding of the International Symposium on Tea Science. Shizuoki, Japan, pp: 655-659.

Arulpragasam, P.V., 1992. Disease Control in Asia. In: Wilson, K.C. and M.N. Clifford (Eds.), Tea Cultivation to Consumption. London, UK. Chapman and Hall, pp: 353-374.

Bartnicki-Garcia, S., 1968. Cell wall chemistry, morphogenesis and taxonomy of fungi. Ann. Rev. Microbiol., 22: 87-108.

Basru, K.K., S. Kaur and M. Dhillon, 1985. Anatomical and biochemical studies of the resistance and susceptibility of groundnut varieties Cercospora leaf spot. Ann. Biol., 1: 77-82.

Debnath, S. and A.K. Paul, 1994. Susceptibility of tea cultivars to blister blight disease and some of their anatomical and morphological characters. Two and a Bud., 41: 48-49.

Godoy, R., O.D. Smith, R.A. Taber and R.E. Petit, 1985. Anatomical traits associated with pod rot resistance in peanut. Peanut Sci., 12: 77-82.

Gupta, S.K., P.P. Gupta, C.D. Kasushik and H.K.L. Chawla, 1992. Metabolic changes in groundnut leaf due to infection by the leaf spot pathogens. Ind. Phytopathol., 45: 434-438.

Imoto, T. and K. Yagishita, 1971. A simple activity measurement of lysozyme. Agric. Biol. Chem., 35: 1154-1156.

Kragh, K.M., S. Jacobsen and J.D. Mikkelsen, 1990. Induction, purification and characterization of barley leaf chitinase. Plant Sci., 71: 55-68.

Premkumar, R., 1996. Forecasting blister blight disease of tea. Ann. Rep. UPASI Sci. Dept., 70: 47-48.

Roberts, W.K. and C.P. Selitrennikoff, 1988. Plant and bacterial chitinases differ in antifungal activity. J. Gen. Microbiol., 134: 169-176.

Roby, D., K. Broglie, R. Cressman, P. Biddle, I. Chet and R. Broglie, 1990. Activation of a bean chitinase promoter in transgenic tobacco plants by phytopathogenic fungi. Plant Cell, 2: 999-1007.

Shinshi, H., D. Mohnen and F. Meins, 1987. Regulation of a plant pathogenesis-related enzyme: Inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. Proc. Natl. Acad. Sci. USA., 84: 89-93.

Swegle, M., K.J. Kramer and S. Muthukrishnan, 1992. Properties of barley seed chitinases and release of embryo-associated isoforms during early stages of imbibition. Plant Physiol., 99: 1009-1014.

Sugha, S.K., 1997. Perpetuation and seasonal build up of *Exobasidium vexans*, causal agent of blister blight of tea in Himachal Pradesh. Trop. Sci., 37: 123-128.

Tuzun, S., M.N. Rao, U. Vogeli, C.L. Schardl and J. Kuc, 1989. Induced systemic resistance to blue mold: Early induction and accumulation of β-1, 3-glucanases, chitinases and other pathogenesis-related proteins (b-proteins) in immunized tobacco. Phytopathology, 79: 979-983.

Venkataram, C.S., 1968. Development of spraying technique and usage of fungicides against diseases of tea. Ann. Rep. UPASI Sci. Dept. Tea. Sect., 1967 68: 31-56.

Woloshuk, C.P., H.D. Sisler and E.L. Vigil, 1983. Actions of the antipenitrant, tricyclazole, on appresoria of *Pyricularia oryzae*. Physiol. Plantpathol., 22: 5-72.