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Class III Chitinase Expression and Salicylic Acid Accumulation in Chestnut after Challenge with Hypovirulent and Virulent *Cryphonectria parasitica* (Murr.) Barr

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K e y w o r d s : Chestnut, *Castanea sativa*, *Cryphonectria parasitica*, class III chitinase, salicylic acid.

S u m m a r y

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The expression of homologous sequences to tobacco acid and basic class III chitinases (chi-a and chi-b respectively) and the accumulation of endogenous salicylic acid (SA) was investigated in stems of chestnut plants (*Castanea sativa* Mill.) grown in vitro after inoculation with the causal agent of chestnut blight, virulent (v) *Cryphonectria parasitica*, and with a biocontrol strain against chestnut blight, hypovirulent (hv) *Cryphonectria parasitica*. Wounded plants were used as a control.

On day three after treatment, only in stems of wounded plants chi-a transcription was found to be stimulated. Neither chi-b expression nor SA levels were altered significantly three days after fungal treatment. But on day seven after infection, hypovirulent *C. parasitica* treated plants exhibited clearly elevated levels of SA and the expression of chi-a and chi-b was strongly induced by hypovirulent *C. parasitica* compared to plants inoculated with virulent *C. parasitica* or wounded control plants. This shows that hypovirulent *C. parasitica* stimulates defence mechanisms of the host stronger than the virulent strain.

I n t r o d u c t i o n

Chestnut (*Castanea sativa* Mill.) is an ecologically and economically important tree species with a long cultural tradition in many areas of Europe. Two major fungal diseases, ink disease (*Phytophthora* ssp.) and chestnut blight

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(*Cryphonectria parasitica*), are the principal causes of the disappearance of this valuable tree. In Austria, the major pathogen of chestnut causing severe damages is *Cryphonectria parasitica*, the causal agent of chestnut blight. Nearly 90 years ago in North America, and over 50 years ago in Europe, chestnut trees began dying in groves from this canker disease (ANAGNOSTAKIS 1987).

One possibility to combat chestnut blight is biocontrol by application of hypovirulent strains of *C. parasitica* (HEINIGER & RIGLING 1994). Hypovirulent *C. parasitica* contains a double stranded RNA virus of the CHV type, which gives rise to the hypovirulent phenotype by interacting with calcium- and G-protein linked signal transduction pathways of the fungus (CHOI & al. 1995). The CHV virus can be transmitted through hyphal anastomoses from hypovirulent to virulent *Cryphonectria* strains of identical or closely related vegetative compatibility groups (ANAGNOSTAKIS 1987).

The interaction between virulent and hypovirulent *C. parasitica* and chestnut plants grown in vitro was investigated on protein level (SCHAFLEITNER & WILHELM 1997). It was found that extracellular pathogenesis-related (PR) protein activity (ß-1,3-glucanases, partly also chitinases) increased after inoculation with hypovirulent *C. parasitica*. Class III chitinases, which are investigated in this work, have been found to be extracellularPR-proteins expressed during systemic acquired resistance (SAR) in many plant species, e.g. tobacco (LAWTON & al. 1992) and cucumber (MÉTRAUX & al. 1989). During the establishment of SAR, the expression of PR-proteins is highly induced (WARD & al. 1991). This stimulation was found to correlate with an increase of SA in many plants (YALPANI & al. 1993). For some plants the induction of PR-proteins through SA-mediated pathways has been demonstrated (GAFFNEY & al. 1993).

SA appears in plants in two forms: as free salicylic acid and conjugated as ß-O-D-glucoside. The free form is supposed to act as an activator of PR-gene transcription, whereas the glucosidic form most probably is not directly involved in the induction of SAR related defence responses like PR-gene transcription (YALPANI & al. 1993).

In this work, preliminary investigations on the expression of chestnut genes homologous to tobacco chi-a and chi-b genes in connection with the accumulation of SA after infection with virulent or hypovirulent *C. parasitica* three and seven days post treatment is described.

Material and Methods

Plant material, fungal strains, inoculation and harvest were essentially as described in SCHAFLEITNER & WILHELM 1997. Plants were sampled three and seven days after treatment. Defoliated stems of seven replicate plants of each treatment were pooled, necrotic lesions caused by the fungi were discarded.

cDNA probes: cDNA probes of the tobacco PR-genes acid and basic class III chitinase were kindly provided by J. RYALS, Novartis Inc., Triangle Park, NC, USA. The cDNA probes were excised from the vector with appropriate restriction enzymes, purified by size fractionation on a low melting point agarose gel and labelled with ^{32}P-dCTP with a Stratagene Random Prime Kit according to the instructions of the supplier.

RNA extraction and northern blotting: RNA extraction from stems was performed according to LAGRIMINI & al. 1987. mRNA was purified by the mRNA isolation Kit (Qiagene). RNA and mRNA were quantified photometrically as described by SAMBROOK & al. 1989. 3µg mRNA per treatment were size-fractionated by the procedure described by ROSEN & al. 1990, blotted on a Hybond N membrane (Amersham). Hybridisation, washing and detection was performed according to the instructions of the supplier of the membrane.

Salicylic acid measurements: 200 mg stem tissue of wounded, hypovirulent or virulent *C. parasitica* inoculated plants, which did not contain the necrotic lesions caused by the fungi, was powdered in liquid nitrogen and freeze dried. The measurements of the levels of free and bound salicylic acid were performed according to MEUWLY & al. 1995.

Results

Both, genes homologous either to chi-a or chi-b of tobacco were found to be expressed in chestnut plants grown in vitro after fungal infection or wounding. Chi-a was found to be induced after wounding on day three (Fig. 1). The wound-inducible effect seemed to be reduced more by virulent and less by hypovirulent *C. parasitca*.

a) chi-a b) chi-b

Fig. 1. Northern blot; 1) wounded day 3, 2) hv *C. parasitica* day 3, 3) v *C. parasitica* day 3, 4) wounded day 7, 5) hv *C. parasitica* day 7, 6) v *C. parasitica* day 7.

On day seven, wounded control plants exhibited lower levels of chi-a mRNA than on day 3, whereas in plants inoculated with hypovirulent *C. parasitica* the gene was expressed as high as on day three. Virulent *C. parasitica* infection did not seem to alter the transcription of chi-a from day 3 to 7 and showed the lowest expression level from all treatments performed. This shows that besides wound induction on day three, chi-a of chestnut is stronger expressed after inoculation with hypovirulent than with virulent *C. parasitica*.

In contrast to chi-a, chi-b transcription was not induced after wounding on day three (Fig. 1). The expression was alike after all treatments on day three. On day seven, highest transcript accumulation was found in hypovirulent *C. parasitica* inoculated plants. Also in wounded and virulent *C. parasitica* infected plants the transcription level increased compared to day three, however, less than observed in hv. *C. parasitica* treated plants.

Endogenous SA is present in chestnut in free and glucosidic bound form. On day three only little differences of free SA levels appeared between wounded or fungal challenged plants, however, in hypovirulent *C. parasitica* inoculated plants

the quantity of free SA was slightly higher than in wounded and virulent *C. parasitica* treated plants.

Later on during the infection process, on day seven, a strong increase of both, free and glucosidic bound SA was observed in hypovirulent *C. parasitica* inoculated plants. The measurements revealed a 2.6-fold increase of free SA after hypovirulent *C. parasitica* treatment compared to wounded and a 2.9-fold increase compared to virulent *C. parasitica* infected plants, whereas in wounded and virulent *C. parasitica* treated plants the level of free SA remained about the same as on day three. The amount of free SA in hypovirulent *C. parasitica* inoculated plants of day seven was 2.7-fold higher than on day three. In virulent *C. parasitica* infected plants only a moderate 1.3-fold increase of free SA was observed during the same time span (Fig. 2).

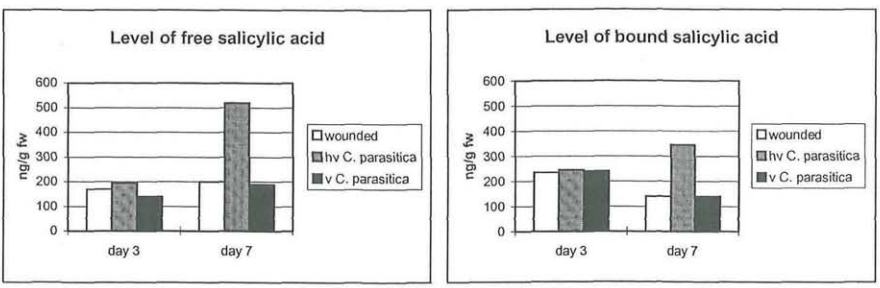

Fig. 2. Hv *C. parasitica* causes an increase of free and bound SA seven days post inoculation compared to wounding and inoculation with v *C. parasitica*.

The amount of glucosidic bound SA was almost exactly the same after all treatments on day 3. In wounded and virulent *C. parasitica* infected plants the level of conjugated SA decreased from day three to day seven, whereas in hypovirulent *C. parasitica* inoculated plants a 1.4-fold increase compared to day three was detected, where the level of bound SA was 2.5-fold higher than in wounded and 2.4-fold higher than in virulent *C. parasitica* inoculated plants (Fig. 2).

Discussion

cDNA probes for tobacco chi-a and chi-b are useful to study PR-gene expression in chestnut. Homologous sequences to these genes are shown to be present and expressed in chestnut. It is anticipated that these genes function as chitinases also in chestnut.

Chitinases exhibit lytic activity on fungal cell walls in vitro and inhibit growth of fungal hyphae (SCHLUMBAUM & al. 1986). High expression of these enzymes in wounded tissue or after fungal infection could lead to the protection of the plant against invading fungi. Also antifungal chitinases of chestnut were characterized previously (ALLONA & al. 1996), these enzymes were isolated from cotyledons and belonged to the class Ib.

The transcription of chi-a was found to be stimulated by wounding on day three after treatment. Wound-inducible chitinases of trees were described previously (CLARKE & al. 1994), but the enzymes described belonged to other chitinase classes, they were found to be expressed in leaves, and have not been investigated in stem tissue.

Induction of chitinases after fungal infection was described (MÉTRAUX & BOLLER 1986). There, during a plant-pathogen interaction, resistant cultivars accumulate more chitinase transcript than susceptible ones. Also in *C. parasitica* tolerant chestnut species (*Castanea molissima)* a higher expression of PR-proteins was shown than in susceptible ones (SHAIN & SPALDING 1995).

However, quantitative measurements in chestnut stems produced only slight variation of chitinase activities between inoculations with hypovirulent and virulent *C. parasitica*, but the activity in intercellular fluid from hypovirulent *C. parasitica* inoculated stems was higher than in stems inoculated with hypovirulent *C. parasitica* or wounded controls (SCHAFLEITNER & WILHELM 1997). There, the total activity of all chitinase isoenzymes present in the intercellular fluid of stems was measured and not the activity of a particular isoenzyme.

On day seven we observed a distinct increase of chi-a and chi-b transcription in hypovirulent *C. parasitica* inoculated plants compared to wounded or virulent *C. parasitica* treated plants, parallel to the rise of free and glucosidic bound SA levels.

The induction of chi-a did not correlate with the SA accumulation: on day three, wounded plants exhibited high levels of acid class III chitinase transcription but low SA levels. Therefore SA levels are not induced by tissue damage in chestnut and the up-regulation of chi-a after wounding seems not to be regulated by a SA dependent pathway. It has been demonstrated that plants possess at least two separate systems of resistance induction, one involving pathogens through SA and another involving wounding, mediated by ethylen and methyl jasmonate. But it has been shown that PR-proteins can be induced by SA and/or wounding (DERCKEL & al. 1996).

However, on day seven, both, chi-a and chi-b were strongest transcribed in plants where the SA level was found to be elevated. The SA levels after hypovirulent *C. parasitica* infection were stimulated up to 2.5-fold compared to wounded control plants.

These preliminary experiments show that SA is present in chestnut, its level rises after inoculation with a hypovirulent fungal pathogen and the rise of SA occurs at least partly in parallel with the stimulation of PR-gene transcription. However, more data are needed to elucidate the role of SA in PR-gene activation in chestnut.

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