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Coniferyl Alcohol Dehydrogenase, a Multifunctional Isozyme-Gene-System in Norway Spruce, Affects the Armillaria Resistance of Young Trees

By F. BERGMANN, B. VORNAM and B. HOSIUS

Abteilung für Forstgenetik und Forstpflanzenzüchtung, Georg-August-Universität, Büsgenweg 2, D-37077 Göttingen, Germany

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Summary

Coniferyl alcohol dehydrogenase (CADH) is a key enzyme in lignin biosynthesis of conifers. Because it functions differently in different ontogenetic stages, tissues and organs of trees, a more in-depth investigation of this isozyme-gene-system in Norway spruce (*Picea abies* L.) appeared worthwhile.

As a result of CADH analysis in somatic and gametic (megagametophytes of single trees) tissues of various spruce trees, one isozyme zone was detected in dormant buds, megagametophytes and embryos, which is controlled by one gene locus (CADH-A). Comparisons between a plus-tree and a random tree collection from the same Bavarian Norway spruce provenance showed a higher degree of heterozygosity at this gene locus among the plus-trees.

A study of isozymes from buds of young trees which suffer from *Armillaria* infections showed a reduced CADH activity in zymograms as compared to healthy trees of the same stand. PCR-based amplification experiments of the DNA encoding CADH were performed with needles from the same spruce material. Using a specific primer pair designed from a cDNA of this gene, only a single band of 520 bp could be detected in agarose gels. Whereas this band was strong in healthy trees, needles of infected trees showed only a very faint DNA band at this position in agarose gels. Since the DNA of other chromosomal regions in infected trees did not indicate similar reductions of the amplification process, it may be postulated that considerable mutations or modifications of the CADH open reading frame are correlated with the susceptibility of trees to *Armillaria* infections.

Key words: Picea abies, coniferyl alcohol dehydrogenase, isozymes, DNA amplification, *Armillaria* infections, susceptibility.

FDC: 165.3; 165.53; 443; 174.7 *Picea abies.*

Introduction

The maintenance of isozyme major polymorphisms in tree populations is a process not yet satisfactorily understood, although a number of experimental and theoretical studies have dealt with this problem (for review and literature compilation, see MITTON, 1995). Based on a reanalysis of numerous isozyme data from 6 different conifer and broad-leaved species, it was recently suggested that major polymorphisms are the result of heterozygote advantage due to ontogenetic differences in the metabolic efficiency of the 2 allozymes (GREGORIUS and BERGMANN, 1995). A more convincing confirmation of this suggestion would be the finding of major polymorphisms for which the 2 frequent allozymes even exhibit different functions in different ontogenetic stages and/or different organs of a heterozygous tree.

One isozyme-gene-system that appears to have multifunctional properties during the ontogenetic development of trees is coniferyl alcohol dehydrogenase (CADH, also referred to as cinnamyl alcohol dehydrogenase, CAD, E.C. 1.1.1.195). This enzyme is primarily involved in lignin biosynthesis of conifers, where it catalyses the reduction of coniferyl aldehyde to the corresponding alcohol, which is the main lignin precursor in the xylem tissue of all conifers (GRISEBACH, 1981).

Surprisingly, the same CADH isozyme can be found not only in the differentiated xylem but also in megagametophytes of loblolly pine seeds, where it could be involved in other functions or pathways (O'MALLEY et al., 1992). It has also recently been established that CADH biosynthesis can be induced in spruce in response to ozone fumigation and fungal infections where it may be involved in the induction of pathogen defense response (GALLIANO et al., 1993).

In the course of a search for new isozyme systems of conifer species which can be used in population and ecological genetic studies, we recently found CADH isozymes to show typical major polymorphisms in Norway spruce and silver fir, with the proportion of heterozygous genotypes exceeding the panmictic expectations (BERGMANN, in prep.). Therefore, the objectives of this study are to (1) demonstrate the occurrence and genetic control of CADH isozymes in Norway spruce (*Picea abies*), (2) present preliminary data on the CADH heterozygosity in plus-tree as compared to random tree collections, and (3) show a correlation between DNA coding for CADH and the susceptibility of young trees to *Armillaria mellea* infections.

Material and Methods

The test material for isozyme analyses consisted of buds and seeds from single spruce trees. The plus-tree collection originated from a seed orchard located near Landshut in Bavaria, and the random tree samples were harvested in 2 stands in the Bavarian Forest. Both collections were described in an earlier study (BERGMANN and RUETZ, 1991).

The material for isozyme and DNA analyses consisted of needles of 10-year-old Norway spruce trees from a plantation located near Prüm in the Eifel region. The altitude of the stand is 450 m above sea-level, the annual average temperature is 7 °C and the annual precipitation is about 850 mm. About 20% of the trees were infected by the *Armillaria mellea* fungus. This is a common problem for Norway spruce plantations that follow broad-leaved tree stands or farm-lands. The infection by *Armillaria mellea* can be unambiguously diagnosed by the presence of mycelia underneath the bark. Twenty pairs of trees, each pair consisting of a healthy and an infected individual, were chosen for the isozyme and DNA analyses (see GREGORIUS, 1989); a typical pair of such trees is shown in *figure 1.*

Isozyme analysis

The CADH isozymes were resolved by means of horizontal starch-gel zone electrophoresis using the Ashton buffer system pH 8.1. Following electrophoretic separation, the CADH activity zones in the gel slabs were stained with a mixture composed of 100 ml 0.02 M TRIS-HCl pH 9.0, 100 mg coniferyl alcohol, 20 mg NADP, 20 mg MTT, 2 mg PMS, and several drops of 10% MgCl. Since cinnamyl alcohol as substrate yields only a very faint activity zone in zymograms, we prefer the name "coniferyl" alcohol dehydrogenase.

Figure 1. – A pair of trees consisting of a healthy and a strongly infected individual from a plantation near Prüm.

DNA-extraction

Genomic DNA was extracted from needles according to the procedure described by ZIEGENHAGEN et al. (1993). DNA concentration was estimated by analytical agarose gel electrophoresis, with 50 ng λ-DNA serving as the standard.

Amplification with Primers

Amplification of DNA was performed by means of a primer pair designed from the cDNA sequence CADH from *Picea abies* L. (EMBL/GenBank Accession X 72675). The sequences are as follows: primer 1 (bp No. 495) 5'TGGTTCGAATCCCGGA GAATCTAC 3' (518 bp), primer 2 (bp No. 916) 5' GAGCCGTTG-CACTTTGTTACTCCT 3' (893 bp) (SPERISEN et al., in prep.).

PCR-conditions

For PCR, the mixtures had a total volume of 50 μl containing 20 ng of template DNA, 0.2 μmole of the 2 primers, all 4 dNTP's (each at 200 mM), 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 2.5 mM $MgCl_2$, and 1 unit Taq polymerase (Boehringer, Mannheim, Germany). The reaction mixtures were overlayered with parafin oil (Merck, Darmstadt, Germany) before being placed in a Biometra thermal cycler with the following profile (*i*) 95 °C for 3 min for 1 cycle; (*ii*) 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min for 40 cycles; and (*iii*) 72 °C for 10 min for the last cycle.

Visualization of PCR products

The PCR amplification products were precipitated by the addition of 0.1 volume 3 M NaAc and 2.5 volume of 96% EtOH, washed in 70% EtOH, dried, and redissolved in TE buffer (10 mmol/l, pH 8.0, 1 mmol/l EDTA, pH 8.0). DNA fragments were separated by length on 1.8% agarose gels with 1 X TAE (40 mmol/l Tris acetate, pH 7.8, 1 mmol/l EDTA) as the running buffer. DNA fragments were stained with ethidium bromide and gels were photographed with a MP4-Polaroid camera under UV light (302 nm). The plasmid pBR 328 digested with *Bgl*I and *Hinf*I (Boehringer DNA molecular weight marker VI) served as a standard for fragment size determination.

Results and Discussion

Occurrence and inheritance of CADH isozymes

One CADH activity zone could be observed in zymograms after electrophoretic resolution of crude extracts of dormant buds, megagametophytes and embryos of germinated seeds. Dry seed extracts yielded no visible enzyme activity in zymograms, which is in contrast to many other isozyme systems.

In the limited tree sample investigated so far, only 2 CADH variants were detected in this zone. These appear as single bands in the haploid megagametophytes and as single or triple bands in the diploid bud tissues (*Figure 2*). These isozyme patterns are assumed to represent allozymes encoded by 2 alleles at 1 controlling gene locus (CADH-A). To confirm this assumption, megagametophyte samples from putative heterozygous trees were scored for segregation ratios. In all cases the CADH variants behaved as simple allelic products, since they occurred alternatively and showed no significant deviation from 1-to-1 proportion among the megagametophytes of single

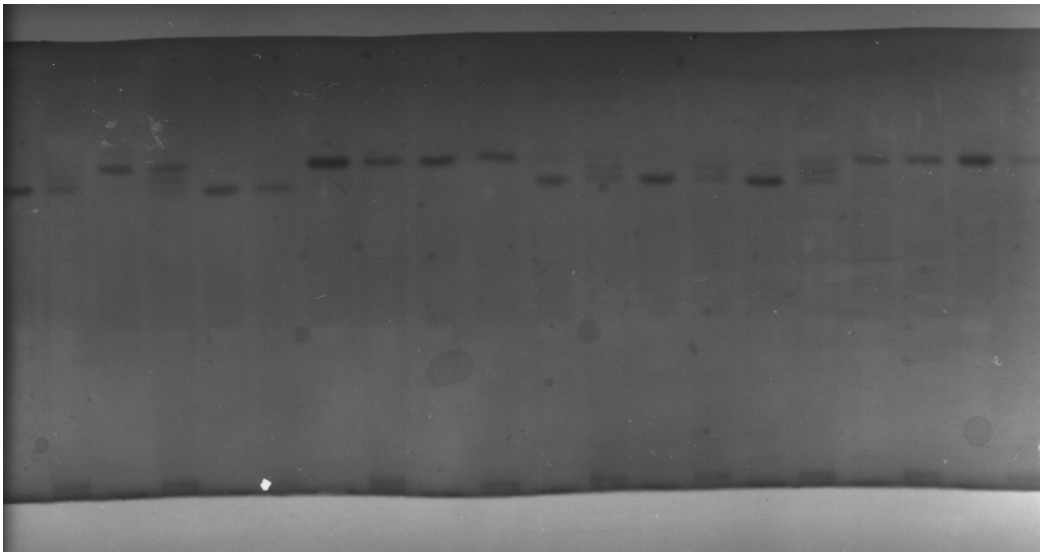

Figure 2. – Electrophoretic CADH patterns of megagametophytes and the corresponding embryos (side by side) from one heterozygous tree (Landshut Nr. 19).

trees (e.g. Oberhof No. 5 showed 18 A1 and 23 A2; Landshut No. 19: 24 A1 and 20 A2). The zymograms of megagametophytes and the corresponding embryos of the tree Landshut No. 19 are shown in *figure 2*. Since the CADH patterns of several embryos consist of triple-banded phenotypes indicating a heterozygous state, it is suggested that this enzyme has a dimeric quaternary structure.

Multiple forms of coniferyl alcohol dehydrogenase isolated from cambial regions have already been described for many plant species including several conifers (MANSELL et al., 1976). In Norway spruce an increase of CAD(H) mRNA in response to ozone exposure and fungal elicitors demonstrates the involvement of this enzyme-gene-system in defense reactions of conifers (GALLIANO et al., 1993). The same authors showed that DNA Southern blot analysis reveals only one CAD gene in cell cultures, which is in accordance with our results inferred from isozyme analyses. Only one CAD gene locus was also identified in loblolly pine (*Pinus taeda*), where the allozyme patterns strongly suggest a dimeric enzyme structure (O'MALLEY et al., 1992; MACKAY et al., 1995). In close correspondence to the determined enzyme activity, CAD(H) mRNA could be detected in xylem, needle and megagametophyte tissues, but not in embryos (MACKAY et al., 1995). The latter result does not agree with our findings in Norway spruce, because single bands (homodimers) or triple bands (heterodimers and homodimers) appear in embryo zymograms (see *Figure 2*). An explanation for this discrepancy may relate to a possible disruption between mRNA synthesis and enzyme presence.

CADH heterozygosity in plus-tree and random tree collections from the same provenance

In an earlier study it was shown that a plus-tree collection from a seed orchard possesses a higher degree of heterozygosity for several isozyme gene loci than a random tree collection chosen from the same Bavarian provenances, from which the orchard plus-trees were selected years ago (BERGMANN and RUETZ, 1991). A part of this material is still available and, therefore, the distribution of heterozygosity at the CADH-A locus was assessed in the 2 collections.

The resulting data are compiled in *table 1*, where in accordance with the small sample sizes only 2 classes, homozygotes (irrespective of the genotype) and heterozygotes, were compared between the 2 collections. The frequency of the CADH heterozygotes is clearly higher among the plus-trees than among the randomly chosen trees, although the deviation measured by the G-test of homogeneity only approaches the 0.05 level. This result suggests an advantage of the heterozygous state at CADH-A for selection as plus-trees. Similarly, the relatively high level of CAD(H) variation and heterozygosity found in loblolly pine (MACKAY et al., 1995) may be associated with some variation in lignin biosynthesis and wood characteristics assumed to be related to physiological or developmental homeostasis (MITTON, 1993).

Table 1. – Frequencies of homozygotes and heterozygotes at CADH-A in a plus-tree and a random tree collection. The homogeneity between the 2 distributions was tested by the log-likelihood ratiotest (G-test).

Tree collection	Frequency of		N	G-Test value
	Homozygotes	Heterozygotes		
Plus-trees	0.33 (10)	0.67 (20)	30	
Random Trees	0.57 (17)	0.43 (13)	30	3.33 n.s.

n.s.: not significant at level of 0.05

Isozyme and DNA analyses in healthy and infected trees

a.) Isozymes

In order to detect possible associations between the CADH polymorphism and fungal infections, healthy and by *Armillaria* infected trees from a spruce plantation (see Material and Methods) were examined for their isozyme patterns. Based on a sample of 40 trees, no correlation between genotype (e.g. heterozygotes) and degree of infection could be established, however, the extracts of all infected trees yielded only a low staining intensity in zymograms, which was primarily attributed to a general destruction of metabolites in diseased trees.

b.) Amplification of the DNA encoding CADH

Total DNA was isolated from the needles of several healthy and infected trees. The quality of the high molecular DNA showed no differences between healthy and infected trees and was appropriate for RFLP and RAPD analyses. Also, no differences in the behaviour of the DNA from the 2 types of trees were detected during the digestion with several restric-

tion enzymes or the amplification with randomly chosen oligonucleotide primers. These comparisons demonstrated that the DNA from the infected trees was in general not impaired.

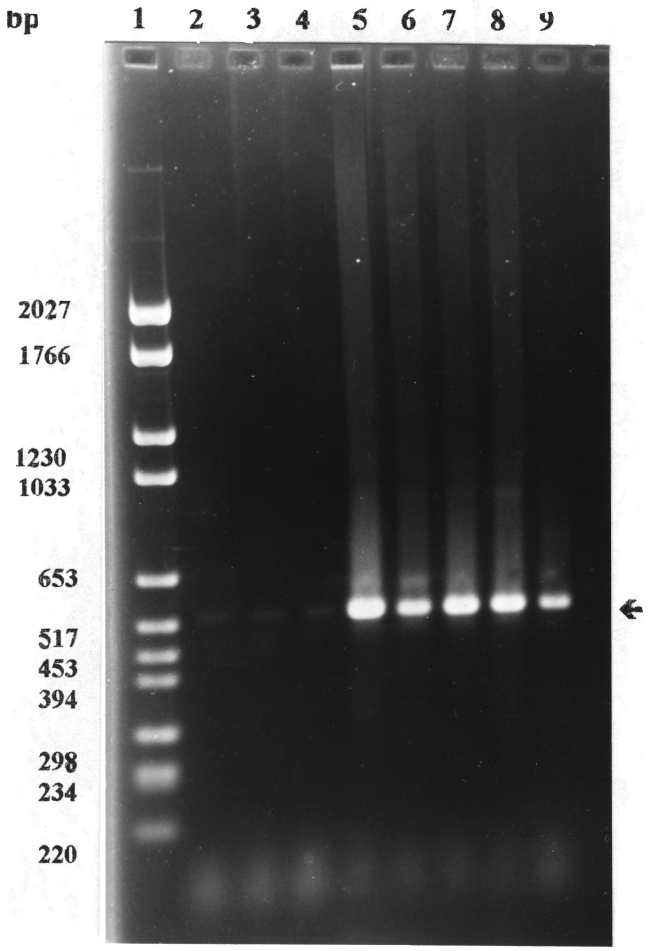

Figure 3. – Electropherogram of DNA from Norway spruce after amplification with a primer pair specific for the CADH gene. Lane 1: size standard (Boehringer DNA molecular weight marker VI); lanes 2 to 4: infected trees; lanes 5 to 9: healthy trees.

The amplification of the DNA encoding CADH was carried out with a specific primer pair designed from a cDNA sequence of this gene. *Figure 3* shows the amplification products of the DNA from 3 infected (lanes 2 to 4) and 5 healthy (lanes 5 to 9) trees. The amplification product, indicated by an arrow, is about 520 bp long for all analysed DNA probes. Analogous to isozyme analysis, this preliminary result of DNA amplification suggests the presence of only one CADH gene in Norway spruce. Although the PCR reactions were carried out with the same amount of template DNA from the different trees, the amplification of the DNA from the healthy trees was more effective than that of the infected trees, as indicated by the intensity of the ethidium bromide staining. Thus the infected trees showed only one faint band of 520 bp compared to the strong band of the healthy trees (*Figure 3*). Therefore we assumed that parts of the DNA encoding CADH were damaged or modified in some trees, making the annealing of the primers to their complementary sequences more difficult. Our assumption was just recently confirmed by preliminary data from DNA sequencing which showed point mutations to occur at primer 1 annealing site in diseased spruce (SCHUBERT, pers. comm.)[1]). Perhaps these mutations lead to a reduced CADH biosynthesis in spruce which explains the low CADH activity in zymograms of all infected trees, thus indicating a direct connection between the DNA coding activity and the amount of enzyme.

Based on our own results and on data elaborated by SCHUBERT, the following hypothesis is put forward:

A certain proportion of younger spruces carries mutations at specific DNA sites of the CADH gene which affect the function of its open reading frame. As a consequence, the transcription rate is reduced so that the CADH enzyme molecules are synthesized only in a low quantity, which can lead to a distortion of the lignification processes in the xylem. Trees affected in this way are then more susceptible to infections by *Armillaria* if they grow at adverse sites.

Earlier investigations also suggested that the stimulation of CADH may play a role in the generation of secondary signals involved in pathogen defense response (WALTER et al., 1988).

Although additional spruce samples from another region (Sauerland) recently showed a similar trend (8 heavily infected trees yielded only a faint DNA band following amplification), further studies with a greater number of healthy and infected tree pairs must show whether this kind of analysis is suitable for predicting the susceptibility of young trees to infections by *Armillaria mellea*.

[1]) Lehrbereich Forstgenetik, LMU München.

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