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Associations of genes encoding allozymes peroxidase and superoxide dismutase in poplar and spruce species

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Summary. Isozymes of peroxidase (PER) and superoxide dismutase (SOD) were analyzed in vegetative buds or very young leaves of seven species and two interspecific hybrids of *Populus,* in progenies of seven controlled crosses of three *Populus* species, and in needles of five *Picea* species and one putative hybrid. One to three PER, and one or two SOD zones of activity were observed. Electrophoretic mobility (EM) and banding phenotypes of isozymes of one PER locus were identical to those of one SOD locus in vegetative buds of five *Populus* species and hybrid. In leaves of the four *Populus* species and hybrid and progenies of controlled crosses, EM and phenotypes of isozymes of two PER loci were identical to those of two SOD loci. In *Picea* species, EM of isozymes of the only SOD locus was somewhat similar but not identical to that of one PER locus, and isozyme phenotypes of all individuals at the SOD locus were not identical to those at a PER locus. Chi-square tests verified the single-gene Mendelian control of the segregating allozyme variants at each of *Per-L1* and *Sod-1* in the three *Populus* species. The results of joint two-locus segregation tests indicated a very tight linkage and no recombination between *Per-L1* and *Sod-1* in three *Populus* species. Genes coding for isozymes of one or two PER loci are either presumably the same as, or very tightly linked to, the genes coding for isozymes of one or two SOD loci in the *Populus* species.

Key words: *Populus* – *Picea* – Peroxidase – Superoxide dismutase – Allozyme gene associations

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Introduction

Isozymes of peroxidase (PER, E.C. No. 1.11.1.7) and superoxide dismutase (SOD, E.C. No. 1.15.1.1), because of their activity in a wide variety of tissues, their ease of detection, and their biological (physiological) importance, have been commonly used for various biological studies in plants and animals. Recently, great attention has been focussed on understanding the molecular biology of SODs (Touati 1988). In early studies, activity and isozymes of SOD were identified as that of tetrazolium oxidase (TO), and it was demonstrated that TO is SOD (Lippitt and Fridovich 1973). In forest trees, isozymes of PER have been used extensively and those of SOD to a limited extent for population genetic studies (Paule 1990).

In respiring living cells, activities of PER and SOD are linked and provide solutions to the problems of living with oxygen (Halliwell 1974; Fridovich 1975, 1978, 1986; Butt 1980). Although PER and SOD are linked in their activities and their isozymes have been used for various studies, the relationships of genes coding for these enzymes are not known. Early observations in potato (*Solanum tuberosum* L.) and soybean (*Glycine max* L.) indicated that some peroxidase isozymes may act as superoxide dismutases. In soybean, one seed coat peroxidase band was found to have INT-oxidase activity (Larsen and Benson 1970). This band was the same variant as the tetrazolium oxidase variant in seedling tissue of soybean (Gorman and Kiang 1977, 1978). In potato, some but not all isozyme bands of peroxidase were at the same position as tetrazolium oxidase isozyme bands (Oelshlegel and Stahmann 1971).

In genetic studies of North American aspen species (*Populus tremuloides* Michx., and *P. grandidentata* Michx.) and European white poplar (*Populus alba* L.)

Table 1. Poplar (*Populus*) and spruce (*Picea*) species and hybrids studied

Genus/Species/Hybrid	No. of individuals	Origin
Populus L.		
(a) Section *Leuce* Duby		
P. alba L.	22	Bulgaria, Germany, Austria, Hungary, Italy, Yugoslavia, France, and Canada (planted)
P. tremula L.	19	Norway, Finland, USSR, England, France, Italy, Yugoslavia, Austria, and Czechoslovakia
P. × *canescens* Sm.	15	The Netherlands, England, Germany, Poland, Yugoslavia, Czechoslovakia, and Canada (progeny of controlled crosses)
P. grandidentata Michx.	24	Southeast part of Michigan state of USA
P. tremuloides Michx.	30	Edmonton, Canada
(b) Section *Aigeiros* Duby		
P. deltoides Marsh.	14	Ontario and Manitoba provinces of Canada; Indiana, Illinois, Kansas, Mississippi and North Dakota states of USA
P. nigra L.	13	The Netherlands, Czechoslovakia, Hungary, Germany, and France
P. × *canadensis* Moench syn. *P.* × *euramericana* (Dode) Guinier	17	*Cultivars:* 'Baden 431,' 'Blanc du Poitou,' 'Canada Blanc,' 'Dorskamp 925,' 'Eugenei,' 'Gelrica,' 'Grandis,' 'Heidemij,' 'I-55/56,' 'I-132/56,' 'I-214,' 'Jacometti,' 'Ostia,' 'Regenerata,' 'Robusta,' 'Steckby,' and 'Zurich 03/3' *Countries of origin:* France, Italy, The Netherlands, Germany, and Spain
(c) Section *Tacamahaca* Spach.		
P. maximowiczii Henry	8	China and Japan
(d) Controlled crosses		
P. deltoides × *P. deltoides*	20	2 crosses: D17 (Toronto, Canada) × D476 (Toronto); D17 × D477 (Toronto)
P. deltoides × *P. nigra*	30	3 crosses: D17 × N166 (Hungary); D17 × N167 (Hungary); D32 (Ottawa, Canada) × N167
P. deltoides × *P. maximowiczii*	20	2 crosses: D17 × M10 (Japan); D17 × M11 (Japan)
Picea A. Dietr.		
Picea glauca (Moench) Voss	120	Four different locations in Alberta province of Canada
Picea engelmannii Parry ex. Engelm.	150	Five different locations in Alberta province of Canada
Natural putative hybrids of *P. glauca* and *P. engelmannii* named as *P. glauca* var. *albertiana*	130	Five different locations in Alberta province of Canada
Picea pungens Engelm.	8	Planted at the University of Alberta campus, origin unknown
Picea abies (L.) Karst.	2	Planted at the University of Alberta campus, origin unknown
Picea mariana (Mill.) B.S.P.	3	Alberta province of Canada

using isozymes, we observed that the isozyme phenotypes and genotypes of the individuals for one locus coding for PER were identical to those for one locus coding for SOD. These observations led us to expand this study to other *Populus* species representing angiosperms and to spruce (*Picea* A. Dietr.) species representing conifers. We analyzed and compared isozymes of PER and SOD in seven species and two interspecific hybrids of *Populus* and in five species and one putative hybrid of *Picea*. Inheritance and linkage of allozyme genes coding for PER and SOD were studied in progenies of *Populus deltoides* Marsh. controlled crosses with *P. deltoides, P. nigra* L., and *P. maximowiczii* Henry. In this paper, we present results of these investigations and provide evidence that one or two genes encoding PER are very tightly linked with or may be same as one or two genes encoding SOD in poplars.

Materials and methods

Poplar species and individuals

One hundred and thirty individuals of seven *Populus* species, 32 individuals of two interspecific *Populus* hybrids, and 70 progenies of seven controlled crosses of *Populus* species (Table 1) were

studied. The *P. deltoides*-controlled crosses with *P. deltoides, P. nigra,* and *P. maximowiczii* were made in 1983 (Rajora 1986, 1990), and the sampled progenies are located in a field progeny test.

Dormant shoots with vegetative buds of *Populus alba, P. tremula,* and *P.* × *canescens* were collected from the arboreta of Ontario Tree Improvement and Forest Biomass Institute (OTIFBI), (Maple, Ontario), while those of *P. grandidentata* and *P. tremuloides* were from the natural populations. Dormant shoot cuttings of *P. deltoides, P. nigra, P.* × *canadensis,* and *P. maximowiczii* were collected from the arboreta of OTIFBI, and those of progenies of controlled crosses from the field progeny test. These shoot cuttings were rooted in a greenhouse (Rajora 1986). Only 70 individuals of the seven controlled crosses were available for this study.

Spruce species and individuals

Four hundred and thirteen individuals of five spruce species and of natural putative hybrids of two spruce species were sampled (Table 1). The needle samples of these individuals were collected from natural populations or planted trees in Alberta province of Canada (Table 1).

Tissue collection and preparation

Tissues of dormant vegetative buds of *Populus alba, P. tremula, P.* × *canescens, P. grandidentata,* and *P. tremuloides,* and tissues of very young emerging leaves from sprouts of rooted shoot cuttings of *P. deltoides, P. nigra, P.* × *canadensis, P. maximowiczii,* and progenies of controlled crosses (Table 1) were used for enzyme electrophoresis. Tissues of needles were used for enzyme electrophoresis in *Picea* species (Table 1).

The formulation of the extraction buffer for poplar buds and leaves was as follows: 0.1 *M* TRIS, 0.2% ascorbic acid (w/v), 1% tween, 0.2% magnesium chloride (w/v), 0.2% calcium chloride (w/v), 17.1% sucrose (w/v), and 0.03% 2-mercaptoethanol, pH 7.5.

The formulation of extraction buffer for spruce needles was as follows: 0.1 *M* TRIS, 0.1% ascorbic acid (w/v), 0.095% cystein (w/v), 1% tween, 0.2% magnesium chloride (w/v), 0.2% calcium chloride (w/v), and 0.05% 2-mercaptoethanol, pH 8.0.

The crude enzyme extract was prepared by grinding the leaf and needle tissue in the extraction buffer manually with the help of mortar and pestle, and by grinding vegetative bud tissue with the help of power-driven, stirrer-type homogenizer. The filter paper wicks were soaked in the homogenate to absorb the enzyme extract.

Enzyme electrophoresis and detection

PER and SOD were analyzed on 12.5% w/v starch gels by horizontal gel electrophoresis using TRIS-citrate and lithium borate (pH 8.1) buffer (Ridgeway et al. 1970). Zones of PER were detected by immersing the gel slice in the following mixture and incubating at 37°C: 92.5 ml of 0.05 *M* sodium acetate (pH 5.0), 0.5 ml of 3% hydrogen peroxide, 2 ml of 0.1 *M* calcium chloride, 50 mg of 3-amino 9-ethyl carbazole dissolved in 5 ml of dimethyl formamide. Zones of SOD activity were detected by staining the gel slice in a mixture of 10 ml of 0.2 *M* TRIS (pH 8.0), 40 ml of distilled deionized water, 40 mg β-nicotineamide adenine dinucleotide phosphate, 40 mg phenazine methosulphate, 40 mg nitro-blue tetrazolium, and 4 ml of 10% magnesium chloride, incubating for 10 min at 37°C and then placing the staining tray with gel on light. Same SOD zones of activity were also detected on gels stained for dehydrogenases.

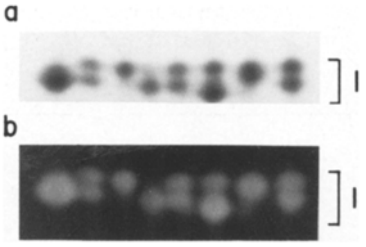

Fig. 1 a, b. Electrophoretic isozyme phenotypes of some individuals of *Populus tremuloides* for PER locus (**a**) and SOD locus (**b**). Each lane represents the isozyme phenotype of a single individual. The loci are numbered on the *right side*

Genes, genotypes, inheritance, and linkage

The genotypes of the individuals were inferred from the banding patterns. The loci were designated in numerals (prefixed by L for only PER loci in *Populus* leaves) and the alleles in letters within an enzyme system, progressively from the anodal to the cathodal direction. Enzyme patterns of PER and SOD were compared for each individual.

Inheritance of two PER and one SOD genes was tested in 70 progenies of seven controlled crosses of poplars by comparing the genotypes of parents and offspring by a Chi-square test. Linkage between one PER and one SOD genes was tested by investigating their joint segregation and independent assortment.

Results and discussion

There was one zone of PER and one zone of SOD activity in vegetative buds of *Populus tremuloides* and *P. grandidentata.* These zones were inferred to be controlled by a single gene each, *Per-1* and *Sod-1,* respectively. The mobility and phenotypes of isozymes of *Per-1* and *Sod-1* were identical in all individuals of *P. tremuloides* and *P. grandidentata* (Fig. 1). Inferred homozygotes at these loci showed single-banded phenotypes, whereas heterozygotes showed double-banded phenotypes (Fig. 1).

In vegetative buds of *Populus alba, P. tremula,* and *P.* × *canescens,* there were three zones of PER activity (*Per-1, Per-2,* and *Per-3*) and one clear zone of SOD activity (*Sod-1*) (Fig. 2). Each of these zones was inferred to be controlled by a single gene. The only SOD locus, *Sod-1,* was identical to *Per-1* for mobility of its isozymes, and isozyme phenotypes and genotypes of the *P. alba, P. tremula,* and *P.* × *canescens* individuals (Fig. 2). Inferred homozygotes at each of *Per-1, Per-2, Per-3,* and *Sod-1* showed single-banded phenotypes and heterozygotes showed double-banded phenotypes. Four alleles were detected at *Per-1* and *Sod-1.*

There were two anodal PER zones of activity (*Per-L1* and *Per-L2*) identical to two anodal SOD zones of activity (*Sod-1* and *Sod-2*) in very young leaves of *Populus deltoides, P. nigra, P.* × *canadensis,* and *P. maximowiczii.* PER also had one cathodal zone of activity. Allozyme phenotypes and genotypes of the individuals at *Per-L1*

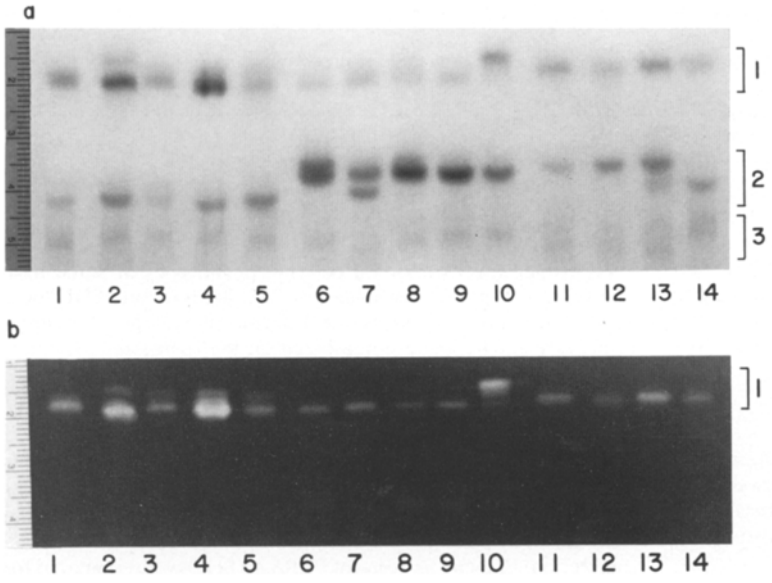

Fig. 2a, b. Electrophoretic isozyme phenotypes of *Populus alba* (1–5), *P. tremula* (6–10), and *P.* × *canescens* (11–14) individuals for PER loci (**a**) and SOD locus (**b**). The loci are numbered on the *right side*

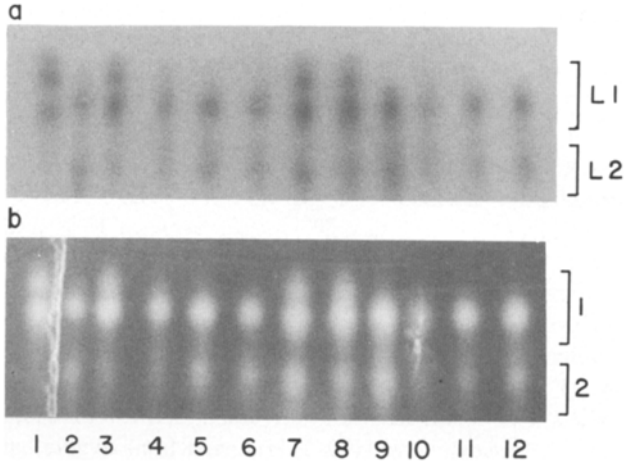

Fig. 3a, b. Electrophoretic isozyme phenotypes of *Populus deltoides* female parent D32 (*lane 1*), *P. nigra* male parent N167 (*lane 2*), and progenies of D32 × N167 (*lanes 3–12*) for PER loci (**a**) and SOD loci (**b**). The loci are numbered on the *right side*

were identical to those at *Sod-1*, and at *Per-L2* they were identical to those at *Sod-2*. Three alleles were detected at *Per-L1* and *Sod-1*. *Per-L2* and *Sod-2* were monomorphic. Homozygotes showed a single-banded phenotype and heterozygotes showed a double-banded phenotype at *Per-L1* and *Sod-1*. Isozymes of each of *Per-L1, Per-L2, Sod-1,* and *Sod-2* conformed to the single-locus Mendelian inheritance. The genotypes of the parents and progeny and isozyme inheritance at *Per-L1* were identical to those at *Sod-1*. At *Per-L1* or *Sod-1*, four parents were inferred to be homozygous and four to be heterozygous as follows: D17 and D476 as AA, N166 as BB, N167 as CC, D32 and D477 as AC, and M10 and M11 as AB. At *Per-L1* and *Sod-1*, all progenies of the crosses between trees homozygous for the same allele were single-banded homozygous, all progenies of the crosses between trees homozygous for different alleles were double-banded heterozygous, and progenies of the crosses between a homozygous parent and a heterozygous parent segregated into two classes, single-banded homozygotes and double-banded heterozygotes (Fig. 3), with no significant deviation from the expected 1:1 ratio ($P > \chi^2 = 0.21-0.53$). Segregation ratios were consistent over the crosses of the same type. All parents and progenies of their crosses were single-banded homozygous monomorphic at *Per-L2* and *Sod-2* (Fig. 3). Joint two-locus segregation patterns of *Per-L1* and *Sod-1* indicated only parental classes and no recombinational classes between these loci (Table 2). Although a smaller number of progenies per cross were sampled, Chi-square analysis indicated a significant deviation from the independent assortment of *Per-L1* and *Sod-1*.

The inheritance results suggest that isozymes of each of *Per-L1, Per-L2, Sod-1,* and *Sod-2* are controlled by a single gene in leaves of *P. deltoides, P. nigra,* and *P. maximowiczii*. The results also indicate that functional PER and SOD are monomeric molecules in leaves of *P. deltoides, P. nigra,* and *P. maximowiczii*. Genetic control of PER isozymes and the monomeric nature of functional PER have been demonstrated in roots of these *Populus* species (Rajora 1986, 1990). The functional PER is generally known to have a monomeric subunit structure in plants and animals. Isozymes of SOD have been reported to show dimeric banding patterns in plants (e.g., Gorman and Kiang 1978; Griffin and Palmer 1989). Most plants and other eukaryotes have copper- and zinc-containing SOD, CuZnSOD, and a few plants also have manganese-containing SOD, MnSOD (Fridovich 1986). In eukaryotes, CuZnSODs have been found to have dimeric or

Table 2. Joint two-locus segregation patterns and Chi-square analysis for the test of linkage of *Per-L1* and *Sod-1*

Cross	Parental genotypes *Per-L1/Sod-1*	No. of offspring	Offspring genotypes Observed – *Per-L1/Sod-1*	Expected ratio	χ^2, $df=3$	$P>\chi^2$
D17 × M10	AA/AA × AB/AB	10	4(AA/AA):6(AB/AB): 0(AA/AB):0(AB/AA)	1:1:1:1	10.8	0.012
D17 × M11	AA/AA × AB/AB	10	6(AA/AA):4(AB/AB): 0(AA/AB):0(AB/AA)	1:1:1:1	10.8	0.012
D17 × D477	AA/AA × AC/AC	10	4(AA/AA):6(AC/AC): 0(AA/AC):0(AC/AA)	1:1:1:1	10.8	0.012
D32 × N167	AC/AC × CC/CC	10	3(AC/AC):7(CC/CC): 0(AC/CC):0(CC/AC)	1:1:1:1	13.2	0.004

tetrameric subunit structure, whereas MnSODs are frequently dimeric (Fridovich 1986). Ours may be the first report of SOD isozymes showing monomeric banding patterns. However, the best evidence of subunit structure of SOD isozymes in the studied *Populus* species can only be obtained by isolating and analyzing SOD proteins.

The results of the joint segregation and independent assortment of *Per-L1* and *Sod-1* suggest that these loci are very tightly linked in *P. deltoides, P. nigra,* and *P. maximowiczii.*

In needles of spruce species, there were three clear zones of PER activity (*Per-1, Per-2,* and *Per-3*) and one clear zone of SOD activity (*Sod-1*). Each of these zones was assumed to be controlled by a single gene. The migration of isozymes of *Per-1* was somewhat similar but not identical to those of *Sod-1.* Isozyme phenotypes and genotypes of all individuals of *Picea* species (Table 1) at *Per-1* were not identical to those at *Sod-1.* However, some (0–50% in different populations) individuals showed similar isozyme phenotypes at *Per-1* and *Sod-1* in *Picea glauca, P. engelmannii,* and their putative hybrids. Isozyme phenotypes of both individuals of *Picea abies* were the same at *Per-1* and *Sod-1,* while those of three *Picea mariana* and eight *Picea pungens* individuals were different at these loci. Both *Per-1* and *Sod-1* showed high allelic variability in *Picea glauca, P. engelmannii,* and their putative hybrids. Due to unavailability of controlled crosses and viable open-pollinated seeds, it was not possible to test the inheritance and linkage of isozymes in spruce species.

The results of our study indicate that the two loci in each of the following pairs *Per-1* and *Sod-1* in vegetative buds of *Populus tremuloides, P. grandidentata, P. alba, P. tremula,* and *P. × canescens,* and *Per-L1* and *Sod-1,* and *Per-L2* and *Sod-2* in leaves of *Populus deltoides, P. nigra, P. maximowiczii,* and *P. × canadensis* are identical for electrophoretic mobility of their isozymes and isozyme phenotypes and genotypes of the individuals. Therefore, isozymes of one PER and one SOD loci in each of these pairs *Per-1* and *Sod-1, Per-L1* and *Sod-1,* and *Per-L2* and *Sod-2* may be controlled by the same gene or very tightly linked genes in the *Populus* species studied. The results of the joint segregation test for *Per-L1* and *Sod-1* in *P. deltoides, P. nigra,* and *P. maximowiczii* suggest a very tight linkage between these genes. As demonstrated by these results, it can be said that *Per-L1* and *Sod-1* are very closely located on the same chromosome. To ascertain whether PER and SOD loci in each of the above pairs represent the same gene, further investigations on the analysis of proteins, m-RNA, and DNA sequences are needed.

A very tight linkage of one or two SOD-encoding genes with one or two PER-encoding genes, as observed in the *Populus* species studied, does not seem to be very surprising if we consider the linked roles of PER and SOD in metabolism of living organisms. SOD, an ubiquitous enzyme, mediates the dismutation of the superoxide radical, $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ (Fridovich 1975). Hydrogen peroxide is removed by catalases, which convert it to water plus oxygen, and by peroxidases, which reduce it to water (Halliwell 1974; Fridovich 1978). The superoxide radical is a product of the biological reduction of molecular oxygen and of many other reactions (Fridovich 1978). The O_2^- radical is an agent of oxygen toxicity; SOD scavenges this radical and provides an important defense against this aspect of oxygen toxicity (Fridovich 1975, 1978, 1986).

In spruce species, the situation seems to be different. In *Picea glauca, P. engelmannii,* and their putative hybrid complex, *Per-1* and *Sod-1* may be loosely linked. However, this has to be tested by further studies on the inheritance and linkage of isozymes. The oxygen metabolism in poplars may be different from that in spruces. Poplars are more demanding for oxygen than conifers. Therefore, associations of allozyme genes coding for PER and SOD may be different in poplars and spruces.

Our study has provided the first information on association of allozyme genes encoding PER and SOD in forest trees. Further studies are needed to understand the relationships of the genes coding for these two metabolically important and linked enzymes in the living organisms.

Independence among loci is an assumption in some applications of allozyme genes in various genetics and breeding studies. Therefore, it would not be advisable to use PER and SOD genes at the same time for such investigations of the *Populus* species studied. In spruce species, caution should be exercised in using PER and SOD loci at the same time.

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