Catalases in Plants: Gene Structure, Properties, Regulation, and Expression

John G. Scandalios, Lingqiang Guan,
and Alexios N. Polidoros
Department of Genetics
North Carolina State University
Raleigh, North Carolina 27695-7614

Catalase action in plant and animal tissues was first observed in 1818 by Thenard, who noted that such tissues readily degraded hydrogen peroxide, a substance he had also discovered some years earlier (Aebi and Sutter 1971). Loew (1901) first established that the degradation of H_20_2 in tissues was due to the effect of an individual, separable enzyme, which he named "catalase." Warburg (1923) suggested that catalase is an iron-containing enzyme, because it is inhibited by cyanide. Evidence for its hematin prosthetic group was presented by Zeile and Hellstrom (1930). Catalase was first purified and crystallized from beef liver, and its identity was made clear by Sumner and Dounce (1937). The earliest genetic studies on catalase were reported by the Russian biologist Koltzoff (1927), who demonstrated that blood catalase levels in several animal species are inherited and segregate according to Mendelian rules.

Catalase has been found in all plants examined, and has been most thoroughly studied biochemically, genetically, and molecularly in the agronomically important species *Zea mays* L. (Scandalios 1990). That catalases can exist in multiple molecular forms or isozymes encoded by multiple genes, in any organism, was first demonstrated by Scandalios *(1965,* 1968) with the maize catalases and has since been found to be the rule rather than the exception, as originally perceived.

OXYGEN AND REACTIVE OXYGEN SPECIES

During respiration, molecular oxygen accepts four electrons to produce two molecules of H_20. However, because of spin restrictions, 0_2 cannot accept four electrons at once but accepts them one at a time (Halliwell and Outteridge 1984). Thus, during the one-electron *(univalent)* reduction of 0_2, stable intermediates are formed in a stepwise fashion (Fig.1).

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$$^{1}O_2$$

$$O_2 \xrightarrow{+e^-} \cdot O_2^- \xrightarrow[2H^+]{+e^-} H_2O_2 \xrightarrow[H^+]{+e^-} \cdot OH \xrightarrow[H^+]{+e^-} H_2O$$

Overall reaction : $O_2 + 4e^- + 4H^+ \longrightarrow 2H_2O$

Figure 1 Pathways in the univalent reduction of oxygen to water leading to the formation of various intermediate reactive oxygen species.

Each of the intermediate reactive oxygen species (ROS) can react with a variety of biomolecules, altering or blocking their biological activity. The combined biological effect of these toxic oxygen species on organisms is termed "oxidative stress." To minimize the damaging effects of activated oxygen, organisms have evolved various enzymatic and nonenzymatic mechanisms that can reduce oxidative stress by detoxifying harmful oxygen species. Among these defenses is the antioxidant enzyme catalase, which converts H_20_2 to oxygen and water.

Unlike the oxygen radicals, H_20_2 can readily diffuse across biological membranes; hence, it can cause oxidative stress far from the site of formation. It will accept an electron and proton to form a molecule of water and the hydroxyl radical. The hydroxyl radical (~OH) is the most reactive of the oxygen species. It is an extremely potent oxidant and reacts with organic molecules at nearly diffusion rates.

Although H_20_2 itself is a relatively stable oxidant and not highly reactive, some biomolecules are directly sensitive to it. For example, many proteins are known to be sensitive to physiologically relevant levels of H_20_2 (about 100 nM). Metalloproteins as a class seem to be sensitive to H_20_2. These include heme proteins (Puppo and Halliwell 1988) and Cu/Zn superoxide dismutase (Hodgson and Fridovich *1975;* Beyer and Fridovich 1987). H_20_2 reacts with these proteins, causing them to release the metal ion and lose their biological activity. Several Calvin cycle enzymes also appear to be especially sensitive to H_20_2. These include fructose biphosphatase, sedoheptulose biphosphatase, glyceraldehyde-3-phosphate dehydrogenase, and phosphoribulokinase (Ellyard and Gibbs 1969; Charles and Halliwell 1981; Asada and Takahashi 1987). H_20_2 appears to act here by oxidizing critical thiols in the enzymes, thereby inactivating them; e.g., H_20_2 has its primary cytotoxic effects through the formation of OH (Fridovich 1975; Imlay et al. 1988). In the Fenton reaction, H_20_2 can form OH in the presence of a suitable transition metal (Fenton 1894; Haber and Weiss 1934).

INTRACELLULAR PRODUCTION OF HYDROGEN PEROXIDE

H_20_2 is produced in significant quantities in various subcellular organelles (see Scandalios, this volume). Each organelle also has potential targets for H_20_2 oxidative stress as well as mechanisms for eliminating H_20_2. Furthermore, H_20_2 can readily diffuse through intra- and intercellular membranes, allowing the interaction of organelles or even cell types.

Peroxisomes

In eukaryotic cells, much of the H_20_2 produced is concentrated in specialized organelles called peroxisomes. They are widely distributed throughout eukaryotes, and in higher eukaryotes they are found in every tissue examined. In peroxisomes, H_20_2 is produced by oxidases, which are involved in the catabolic oxidation of a variety of biomolecules. Oxidases remove two electrons from substrates by way of a flavodoxin intermediate, ultimately transferring the electrons to 02 to form H_20_2.

Peroxisomes contain oxidases for a variety of general metabolic pathways (Tolbert 1981). The enzyme content of peroxisomes varies significantly with the metabolic needs of the specific cell type and developmental stage. In plants, peroxisomes associated with specific metabolic functions have been termed "specialized peroxisomes." These include the glyoxysomes found in tissues of oil-storing seeds during germination (Beevers 1982). They are associated with the glyoxylate cycle and the utilization of fat reserves in oil-storing seeds. During germination, fatty acids are converted directly into carbohydrates in the glyoxylate cycle. Thus, seedlings are provided with a carbon source until the plant is photosynthetically competent. The fatty acids metabolized in the glyoxysomes are converted into succinate. An early enzyme in the glyoxylate pathway is acetyl CoA oxidase, which generates H_20_2. Ultimately, two H_20_2 molecules are produced for every succinate molecule generated. The succinate can then be used as carbon source or metabolized by mitochondria into cellular energy. In addition to all the enzymatic generators of H_20_2 described above, all peroxisomes contain the H_20_2-consuming enzyme catalase.

Chloroplasts

Illuminated chloroplasts generate copious amounts of H_20_2. It is estimated that approximately 10-20% of the electrons that flow through photosystem I reduce 02 to H_20_2 (Asada and Takahashi 1987). As elec

trons flow through the electron chains of photosystem I, they readily "leak" and produce the superoxide radical (O2~~). The O2~ is then reduced to form H_20_2. Herbicides (e.g., atrazine) that block electron transport or act as alternative electron acceptors and increase the flow of electrons to oxygen (i.e., paraquat) significantly increase the production of O2~~ and thereby H_20_2 (Asada and Takahashi 1987; Halliwell 1987). It is hypothesized that the generation of activated oxygen species contributes to the toxic effects of these herbicides.

In the chloroplast, O2~~ is rapidly converted to H_20_2 through the action of superoxide dismutase (SOD) (Baum and Scandalios 1979;
Hayakawa et al. 1984). The chloroplast isozyme of SOD converts O2~
into H_2O_2 and water.

There are also a number of nonenzymatic mechanisms that convert O2~ into H_2O_2 (Asada and Takahashi 1987). For example, O2~ can react with two NAD(P)H molecules to produce H_2O_2 (Nadezhdin and Dunford 1979). Several electron donors can act in this manner, including ascorbate (Cabelli and Bielski 1983) and glutathione (Anderson et al. 1983). Additionally, electrons directly from the electron transport chains of the photosystems can reduce O2~~ to H_2O_2 (Allen 1975).

Chloroplasts scavenge and eliminate H_20_2 via the ascorbateglutathione cycle (Asada and Nakano 1980; Halliwell 1987). In this cycle, ascorbate peroxidase reduces H_2O_2 to form water and dehydroascorbate. Ascorbate reductase then utilizes glutathione to reduce the dehydroascorbate and thereby regenerate the ascorbate. The glutathione is then regenerated by glutathione reductase utilizing reducing equivalents from NAD(P)H. NAD(P)H is not a limiting metabolite in chloroplasts under conditions where excess H_2O_2 is generated, namely light. There is no catalase associated with chloroplasts.

Chloroplasts may have excess capacity to degrade H_20_2 and may play a role in reducing H_20_2 generated in other subcellular compartments. Evidence of this capacity comes from studies with isolated chloroplasts, which can rapidly eliminate H_2O_2 from a 5 mM H_20_2 solution within a few minutes (Asada and Nakano 1980). At this rate, illuminated chloroplasts might be able to scavenge all the H_2O_2 synthesized in the cell.

Mitochondria

In mitochondria the production of H_20_2 is associated with respiration. The rate of peroxide generation depends directly on the metabolic state

of the mitochondria (Loschen et al. 1971). The greater the flow of electrons through the electron chain, the greater the H_2O_2 production. Under physiological conditions, it is estimated that 2% of the O2 utilized by mitochondria is generated into H_2O_2 (Chance et al. 1973). Inhibitors of respiration that block electron flow through the electron transport chain can increase H_2O_2 production (Boveris and Cadenas 1975). Inhibitors that block entry of electrons onto the transport chain decrease H_2O_2 production (Boveris and Chance 1973).

O2~ is believed to be the precursor to H_2O_2 in the mitochondria, as it is in the chloroplast (Forman and Boveris 1982). The electron-rich form of ubiquinone, ubisemiquinone, reduces O2 to form O2~~ (Cadenas and Boveris 1980). 02~, being charged, is not membrane-permeable and accumulates in the mitochondria. A mitochondrial-specific isozyme of SOD degrades O2~ into H_2O_2 and O2 (Fridovich 1975; Baum and Scandalios 1979).

In mammalian systems some of the internally generated H_2O_2 may be partially metabolized by glutathione peroxidase (Nichols 1972; Starke and Farber 1985). There is some evidence that a significant portion of mitochondrially generated H_2O_2 diffuses out of the mitochondria and is scavenged by other systems (Oshino et al. 1975); however, heart mitochondria contain catalase, which directly inactivates H_2O_2 (Radi et al.
1994).

As discussed below, in maize the CAT-.3 catalase isozyme is associated with the mitochondria (Scandalios et al. 1980a). To the extent that CAT-3 instead of glutathione peroxidase is degrading mitochondrially produced H_2O_2, reducing equivalents are conserved, thus allowing for more efficient respiration.

Other Cellular Components

The cytosol, the endoplasmic reticulum, and the nucleus have all been shown to evolve small quantities of H_2O_2 in vitro (Chance et al. 1979). Presently, little is known about the actual quantities generated in vivo or the metabolic processes that generate it. It is generally accepted that catalase is found in the cytosol and could effectively remove cytosolic H_2O_2. Additionally, H_2O_2 generated in these subcellular components may diffuse into other organelles with an excess capacity to degrade H_2O_2 (e.g., peroxisomes). In addition to the above, H_2O_2 has some important functions in plant cells, including roles in signal transduction, cell wall lignification, and defenses against pathogen attack (see Doke, this volume).

CATALASE: CHARACTERIZATION

Catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6; CAT) is a tetrameric heme-containing enzyme that is found in all aerobic organisms and serves to rapidly degrade H_2O_2. Catalase is one of the most active catalysts produced by nature. It decomposes H_2O_2 at an extremely rapid rate, corresponding to a catalytic center activity of about $i0^7$ min-^{1}. Depending on the concentration of H_2O_2, it exerts a dual function (Deisseroth and Dounce 1970). At low concentrations (<10-6 M) of H_2O_2, it acts "peroxidatically," where a variety of hydrogen donors (e.g., ethanol, ascorbic acid) can be oxidized in the following manner.

$$RH_2 + H_2O_2 \quad R + 2H_2O$$

At high concentrations of substrate, catalase decomposes toxic H_2O_2 at an extremely rapid rate using the "catalatic" reaction in which H_2O_2 acts as both acceptor and donor of hydrogen molecules.

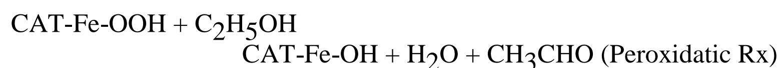
$$2H_2O_2 \quad 2H_2O + O2$$

Spectrophotometric and kinetic evidence suggests that catalase uses a two-step mechanism in both the peroxidatic and catalatic reactions (Deisseroth and Dounce 1970; Dounce 1983). In the first step, the heme iron of catalase interacts with H_2O_2 to form an oxygen-rich iron peroxide.

$$\text{CAT-Fe-OH} + H_2O_2 \quad \text{CAT-Fe-OOH} + H_2O$$

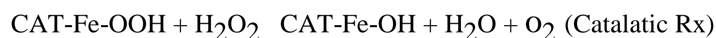
This intermediate iron peroxide (CAT-Fe-OOH) is referred to as compound I, which can be detected in vitro and in vivo, because it alters the spectrophotometric properties of the catalase heme. In fact, because of special kinetic properties of catalase, compound I is utilized as an indicator of H_2O_2 concentrations in vivo (Oshino et al. 1975). At low H_20_2 concentrations, compound I can be reduced by hydrogen donors (e.g., ethanol) peroxidatically.

$$\text{CAT-Fe-OOH} + C_2H_5OH \quad \text{CAT-Fe-OH} + H_2O + CH_3CHO \text{ (Peroxidatic Rx)}$$



At high concentrations of H_20_2, compound I reacts with a second H_2O_2 to produce water and molecular o_2.

$$\text{CAT-Fe-OOH} + H_2O_2 \quad \text{CAT-Fe-OH} + H_2O + o_2 \text{ (Catalatic Rx)}$$



Catalase is unique among H_20_2-degrading enzymes in that it can degrade H_20_2 without consuming cellular reducing equivalents. Hence, catalase provides the cell with a very energy-efficient mechanism to remove H_20_2. Therefore, when cells are stressed for energy and are rapidly generating H_20_2 through "emergency" catabolic processes, H_20_2 is degraded by catalase in an energy-efficient manner. This should result in a net gain of reducing equivalents and, therefore, cellular energy. It has been proposed that catalase may be uniquely suited to regulate the homeostasis of H_20_2 in the cell (Asada and Takahashi 1987). In the catalatic mode, catalase has a very high apparent Michaelis constant and, therefore, is not easily saturated with substrate. Thus, the enzyme activity increases linearly over a wide range of H_20_2 concentrations and, thereby, maintains a controlled intracellular H_20_2 concentration. There is circumstantial evidence that catalase may function in this manner in mammalian systems. Organs with high concentrations of catalase (i.e., liver and kidney) have low levels of endogenous H_20_2, and organs with low concentrations of catalase (i.e., lung and heart) have high endogenous levels of H_20_2 (Thayer 1977). Furthermore, if catalase activity is inhibited, H_20_2 concentrations rise in the liver (Chance et al. 1979).

PLANT CATALASES OCCUR IN MULTIPLE MOLECULAR FORMS

Plants, unlike animals, have multiple enzymatic forms (or isozymes) of catalase. Over 30 years ago, when Scandalios (1965, 1968) first demonstrated that the multiple isozymes of catalase in maize were the products of distinct, unlinked genes, it was considered by many to be an isolated case. However, as recent investigations of catalases in other plant species were undertaken by numerous laboratories, catalase multiplicity proved to be the rule rather than the exception. Among the various plant species examined and found to have multiple isozymes of catalase are *Nicotiana tobacco* (Havir and McHale 1987), cotton (Ni et al. 1990), *Nicotiana plumbaginlfolia* (Willekens et al. 1994b), *Arabidopsis thaliana* (Zhong et al. 1994), *Pinus taeda* (Mullen and Gifford 1993), sunflower (Eising et al. 1989), pumpkin (Yamaguchi et al. 1986), and tomato (Gianinetti et al. 1993). In some plant species (e.g., castor bean), single forms of catalase were initially reported (Gonzalez 1991), but as these were examined in more detail, multiple forms were identified (Suzuki et al. 1994). Some species may indeed prove to possess only one form, but those that have been examined in some detail (e.g., tobacco and *Arabidopsis)* have been shown to have three genetically distinct catalases similar to those in maize. The presence of multiple catalase isozymes

suggested multiple functions for catalases in a variety of plant tissues at various developmental stages and under constantly changing environments from which plants cannot readily escape.

In recent years, interest in plant catalases has gained significant momentum as their role in plant metabolism, defense, and signal perception has been indicated. The cDNAs for a number of plant catalases have been isolated (Table 1), but only six plant catalase genes have been isolated and characterized (Table 2). Such information is useful in establish-

Table 1 Cat cDNA isolated and characterized from plants

Species	Common name	cDNA or protein	Accession number	References
**Zea mays*	maize	*Cat1*	X12538	Redinbaugh et al. (1988)
**Zea mays*	maize	*Cat2*	X54819	Guan et al. (1991)
**Zea mays*	maize	*Cat3*	X12539	Redinbaugh et al. (1988)
Hordeum vulgare	barley	*Cat1*	U20777	Skadsen et al. (1995)
**Hordeum vulgare*	barley	*Cat2*	U20778	Skadsen et al. (1995)
**Oryze sativa*	rice	*CatA*	X61626	Mori et al. (1992)
**Oryze sativa*	rice	*CatB*	D26484	Morita et al. (1994)
Triticum aestivum	wheat	*CatA*	X94352	Z. Song et al. (unpubl.)
Secale cereale	rye	*Cat*	Z54143	M. Schmidt (unpubl.)
**Gossypium hirsutum*	cotton	*Cat1*	X52135	Ni et al. (1990)
**Gossypium hirsutum*	cotton	*Cat2*	X56675	W. Ni. and R.N. Trelease (unpubl.)
**Pisum sativum*	pea	*Cat*	X60169	Isin and Allen (1991)
**Glycine max*	soybean	*Cat*	Z12021	S.H. Isin and R.D. Allen (unpubl.)
**Ipomoea batatas*	sweet potato	*Cat*	X05549	Sakajo et al. (1987)
Solanum tuberosum	potato	*Cat1*	U27082	Wu and Shah (1995)
Solanum tuberosum	potato	*Cat2*	Z37106	A. Niebet et al. (unpubl.)
Solanum melongena	eggplant	*Cat*	X71653	T. Toguri (unpubl.)
**Lycopersicon esculentum*	tomato	*Cat*	M93719	A. Drory and W. Woodson (unpubl.)

mg the evolutionary relationships among catalases from various plant species and all other aerobic organisms (Guan and Scandalios 1996).

Among eukaryotic catalases, the most complex and most thoroughly investigated are those of *Zea mays* L., or maize. A detailed discussion of the maize catalase gene-enzyme system, as a paradigm of the current state of knowledge relative to plant catalases, follows.

THE MAIZE CATALASE GENE-ENZYME SYSTEM

In maize, the three unlinked nuclear structural genes, *Cat1, Cat2,* and *Cat3,* encode three biochemically distinct isozymes, *CAT-1*, CAT-2, and

Table 1 (continued)

Species	Common name	cDNA or protein	Accession number	References
**Nicotiana plumbaginifolia*	*N. plumbaginifolia*	*Cat1*	Z36975	Willekens et al. (1994b)
**Nicotiana plumbaginifolia*	*N. plumbaginifolia*	*Cat2*	Z36976	Willekens et al. (1994b)
**Nicotiana plumbaginifolia*	*N. plumbaginifolia*	*Cat3*	Z36977	Willekens et al. (1994b)
Nicotiana sylvestris	wood tobacco	*Cat1*	U07626	Schultes et al. (1994)
Nicotiana tabacum	tobacco	*Cat1*	U07627	Schultes et al. (1994)
Nicotiana tabacum SR1	tobacco	*Cat1*	U03473	Chen et al. (1993)
Cucurbita pepo	pumpkin	*Cat1*	D55645	M. Esaka et al. (unpubl.)
Cucurbita pepo	pumpkin	*Cat2*	D55646	M. Esaka et al. (unpubl.)
Cucurbita pepo	pumpkin	*Cat3*	D55647	M. Esaka et al. (unpubl.)
Arabidopsis thaliana	Arabidopsis	*Cat1*	U43340	J. Frugoli et al. (unpubl.)
Arabidopsis thaliana	Arabidopsis	*Cat2*	X64271	Chevalier et al. (1993)
Arabidopsis thaliana	Arabidopsis	*Cat3*	U43147	Newman et al. (1994)
Vigna radiata	mung bean	*Cat1*	D13557	Mori and Imaseki (1993)
Helianthus annuus	sunflower	*Cat*	L28740	Kleff et al. (1994)

*Indicates cDNAs used in construction of phylogenetic tree (Fig. 21).

Table 2 *Cat* genes isolated and characterized from plants

Species	Common name	Genomic DNA	Accession number	References
Zea mays	maize	*Cat1*	X60135	Guan and Scandalios (1993)
Zea mays	maize	*Cat2*	X54358	Guan et al. (1996)
Zea mays	maize	*Cat3*	L05934	Abler and Scandalios (1993)
Glycine max	soybean	*Cat*	Z12021	S.H. Isin and R.D. Allen (unpubl.)
Ricinus communis	castor bean	*Cat1*	D21161	Suzuki et al. (1994)
Ricinus communis	castor bean	*Cat2*	D21162	Suzuki et al. (1994)

CAT-3, respectively (Scandalios 1965, 1968, 1979; Scandalios et al. 1980a). Each of the *Cat* genes exhibits temporal and spatial specificity in its expression (Scandalios et al. 1984), and each responds variably to different environmental signals (Matters and Scandalios 1986a,b; Scandalios 1987; Skadsen and Scandalios 1987). In addition, the catalase isozymes exhibit cell (Tsaftaris et al. 1983) and organelle (Scandalios 1974, 1990) specificities. Both overexpression and null mutants have been identified and characterized (Scandalios et al. 1980b; Chandlee and Scandalios 1984a; Bethards and Scandalios 1988; Wadsworth and Scandalios 1990).

Each of the maize catalase isozymes is composed of four approximately 60-kD subunits and is structurally similar to catalases found in other organisms (Chandlee et al. 1983). The differential spatial and temporal expression of these genes has been characterized, and two temporal regulatory loci, *Carl* and *Car2,* have been genetically defined (Scandalios et al. 1980b; Chandlee and Scandalios 1984a,b). *CAT-1* is the only catalase isozyme expressed in mature pollen, the milky endosperm, aleurone, and the scutellum during early kernel development (Scandalios 1983; Wadsworth and Scandalios 1989; Acevedo and Scandalios 1990). During early sporophytic development, levels of CAT-1 in the scutellum decline, while levels of CAT-2 increase, with the CAT-2 developmental profile paralleling that of the glyoxysomes (peroxisomes), the primary intracellular location of these two isozymes (Scandalios 1974). CAT-1 and CAT-3 are the only catalase isozymes present in etiolated leaves and in the coleoptile of the germinating maize seedling.

Upon exposure to light there is rapid accumulation of CAT-2 in leaves due to both increased transcript accumulation (Redinbaugh et al. I 990b) and increased translation of the *Cat2* message (Skadsen and Scandalios 1987). In mature green leaves of maize, CAT-2 is localized in the peroxisomes of bundle-sheath cells, and CAT-1 and CAT-3 are found in mesophyll cells (Tsaftaris et al. 1983). The CAT-3 isozyme is quite different from the other catalases biochemically. In maize, tobacco, and barley, the CAT-3 (i.e., the isozyme specifically immunoreactive with the maize anti-CAT-3 monospecific antibody) isozyme has enhanced peroxidatic activity (70-, 30-, and 28-fold over "typical" catalase, i.e., CAT-2).

Catalase can either catalyze the direct dismutation of H_20_2 into H_20 and 02 (catalatic mode) or use H_20_2 to oxidize substrates such as methanol, ethanol, formaldehyde, formate, or nitrite (peroxidatic mode). The ratio of these is usually calculated as Rpic = (mU peroxidatic/U catalatic) x 10 (Chandlee et al. 1983; Havir and McHale 1989). In maize bundle-sheath, the peroxisomal catalase (CAT-2) has high catalatic but low peroxidatic activity (Rpic = 0.25). In contrast, CAT-3, which is expressed in leaf mesophyll and is not peroxisomal (coisolates with mitochondria), has high peroxidatic but low catalatic activity (Rpic =
17.6). Finally, each catalase isozyme exhibits varying degrees of sensitivity to inhibitors such as cyanide (KCN), azide (NaN_3), and aminotriazol (AT), and to the endogenous maize proteinaceous catalase inhibitor. CAT-3 proved the least sensitive of the three maize catalases to all inhibitors tested (Scandalios 1990, 1994). This again suggests that CAT-3 might have evolved to function under specific conditions or in a specific metabolic role distinct from that of the other catalases. For example, because CAT-3 is cyanide-insensitive, one might hypothesize a role or function under conditions favoring cyanide-resistant respiration (i.e., alternative oxidase respiration) (Elthon and McIntosh 1987).

Although biochemically distinct, the three catalase isozymes are capable of interacting in vitro to form heterotetramers (Scandalios 1965, 1979). The CAT-1 and CAT-2 isozymes also form the expected heterotetramers when expressed together in vivo (e.g., in the scutellum of germinating embryos) (Fig. 2). However, the CAT-3 isozyme is found only as a homotetramer in vivo, even where its temporal and tissue specificity of expression coincide with those of the CAT-1 and CAT-2 isozymes (Scandalios et al. 1980a; Chandlee and Scandalios 1984a). This further implies that CAT-3 is compartmentalized separately from the other catalases in the cell. Because all three catalases are encoded by nuclear genes, this suggests specific targeting of the CAT-3 protein. The

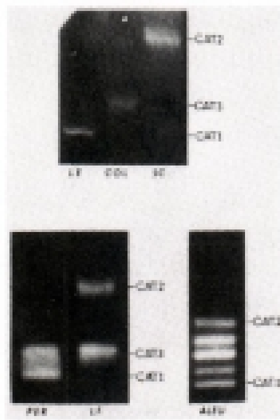


Figure 2 *(Top)* Zymogram showing tissue-specific expression of the three catalase isozymes in maize. CAT-1 in immature milky endosperm (LE), CAT-2 in scutellum (SC) at day 10 postgermination, and CAT-3 in coleoptile (COL). *(Bottom)* Lack of subunit interaction between CAT-3 when coexpressed in the same tissue with CAT-1 (pericarp, PER) or with CAT-2 (green leaf, LF). *(Lower right)* Subunit interaction between CAT-1 and CAT-2 to form intergenic heterotetramers when both are coexpressed in the same tissue (aleurone, ALEU).

CAT-1 and CAT- 2 isozymes have been shown to be cytosolic or, where glyoxysomes/peroxisomes are present, peroxisomal. In contrast, CAT-3 is not found in these cell fractions in leaf but coisolates exclusively with the mitochondrial cell fraction (Scandalios et al. 1980a). In addition, as discussed below, recent findings concerning the novel light regulation of *Cat3* transcription further reinforce the inference of a special metabolic role for CAT-3.

DEVELOPMENTAL EXPRESSION

Each of the maize catalases exhibits a unique and complex pattern of developmental expression throughout the maize life cycle (Scandalios et al. 1984). CAT-3 is detected in the pericarp (a maternal tissue) during ovule development, and during very early postpollination kernel development, but declines rapidly shortly after pollination. CAT-1 is also detected in the pericarp, but at extremely low levels. By days 12-15 postpollination, CAT-1 becomes the predominant form, and is in fact the only catalase detected in the kernel at this time. CAT-1 is the only catalase detected in the milky endosperm of developing kernels. The aleurone, a specialized kernel tissue derived from endosperm cells, expresses CAT-1 and CAT-2 during the latter stages of kernel development. At days 9-20 postpollination,

the scutellum expresses only the CAT-1 isozyme. However, at later stages, as the seed matures, the *Cat2* gene is induced to moderate levels until the seed desiccates and enters developmental arrest. In the scutellum isolated from dry "dormant" seed, CAT-1 appears to be the only isozyme expressed. Upon imbibition and germination, CAT-1 is initially the only form expressed in the scutellum, but as the embryo grows, *Cat2* is rapidly induced, and by day 5 postimbibition, CAT-2 becomes the predominant isozyme. By day 10 postimbibition, catalase expression has shifted almost exclusively to CAT-2. In the coleoptile, CAT-3 is the predominant isozyme detected on zymograms. CAT-1 and CAT-3 are detected in dark-grown leaf tissue. CAT-2 is only expressed with greening of the leaf tissue in the light. The root has very low levels of catalase and is difficult to analyze. However, in young roots, CAT-1 and CAT-3 can be detected. As roots mature and grow, catalase activity decreases and rapidly drops below assay sensitivity. Only CAT-3 is detected in the stem of the mature plant (Acevedo and Scandalios 1991), and only CAT-1 is detected in the pollen after anthesis (Acevedo and Scandalios 1990).

The temporal and spatial distribution of maize catalase activity and isozyme proteins is also reflected in the distribution and accumulation of the *Cat* transcripts. The steady-state levels of *Cat1, Cat2,* and *Cat3* mRNAs generally mirror the level of their respective catalase activities and isozyme patterns (Redinbaugh et al. 1990b; Acevedo and Scandalios 1992) during seed development (Fig. 3), and during early sporophytic development and differentiation (Fig. 4). Whereas these observations are consistent with transcriptional regulation of tissue-specific catalase expression, there is substantial evidence that expression of the maize *Cat* genes is also regulated posttranscriptionally (Skadsen and Scandalios 1987). This multilevel regulation, coupled with the availability of both constitutive *(Cat1)* and tissue-specific *(Cat2* and *Cat3)* gene expression, renders the maize catalase gene-enzyme system a good choice for the study of molecular factors that influence tissue-specific gene expression in plants. The overall developmental program of catalase gene expression presented herein has been corroborated by zymogram analyses, activity profiles, immunochemical measurements, and transcript levels for each of the isozymes.

TEMPORALLY PROGRAMMED TISSUE-SPECIFIC GENE EXPRESSION

The basic developmental program was initially established in the scutellum, since this tissue persists from early kernel development to the time

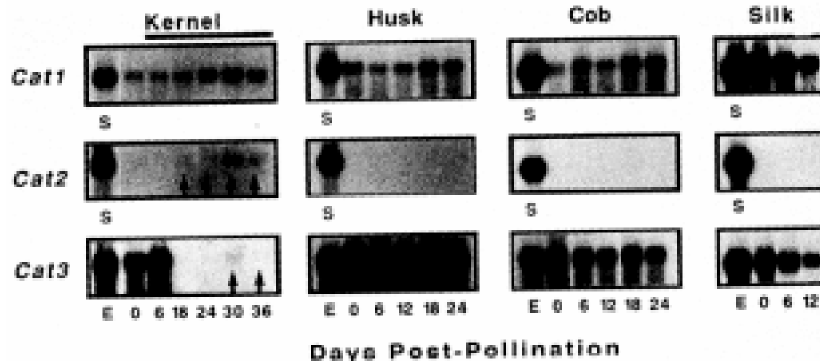


Figure 3 RNA blots of *Cat1, Cat2,* and *Cat3* RNA levels in the maize ear shoot tissues. Each lane contained 10 ~g total RNA from the tissue indicated. RNA isolated from scutellum (S) and epicotyl (E) at 4 dpi was used as positive control. Gene-specific sequences for each gene were used to probe the RNAcontaining filters. Arrows indicate barely detectable amounts of transcript accumulation. Horizontal bar (under kernel) indicates kernel development (6-36 dpp). (0) Unpollinated pistillate flowers.

when the young plant is fully differentiated after germination, whereas the endosperm quickly degenerates following germination, being utilized as a nutrient source. Furthermore, the scutellum is a stable, virtually non-dividing, nondifferentiating, diploid tissue.

Catalase activity in the scutellum, standardized by examining the complete developmental program in a significant number of highly inbred lines, increases rapidly after germination, peaking at 4 days post-germination and thereafter declining to moderate levels. A time-course zymogram analysis reflects the temporal shift in expression from *Cat1* to *Cat2* during this time period (Scandalios 1987). This shift in expression of the two *Cat* genes was shown to be largely due to changes in the rates of synthesis and degradation of the CAT-1 and CAT-2 isozymes during this period (Quail and Scandalios 1971). Interestingly, the *Cat2* mRNA profile increases and decreases in parallel with the CAT-2 protein, whereas the accumulation of steady-state *Cat1* mRNA increases as the

Figure 4 Steady-state levels of *Cat1, Cat2,* and *Cat3* mRNA in the tissues indicated of the developing maize seedling were determined by Si-nuclease protection analysis. The signal generated by 10 or 100 pg in~vitro-synthesized RNA treated in the same manner is shown beside the individual autoradiograms to indicate that different exposures of the autoradiograms are presented.

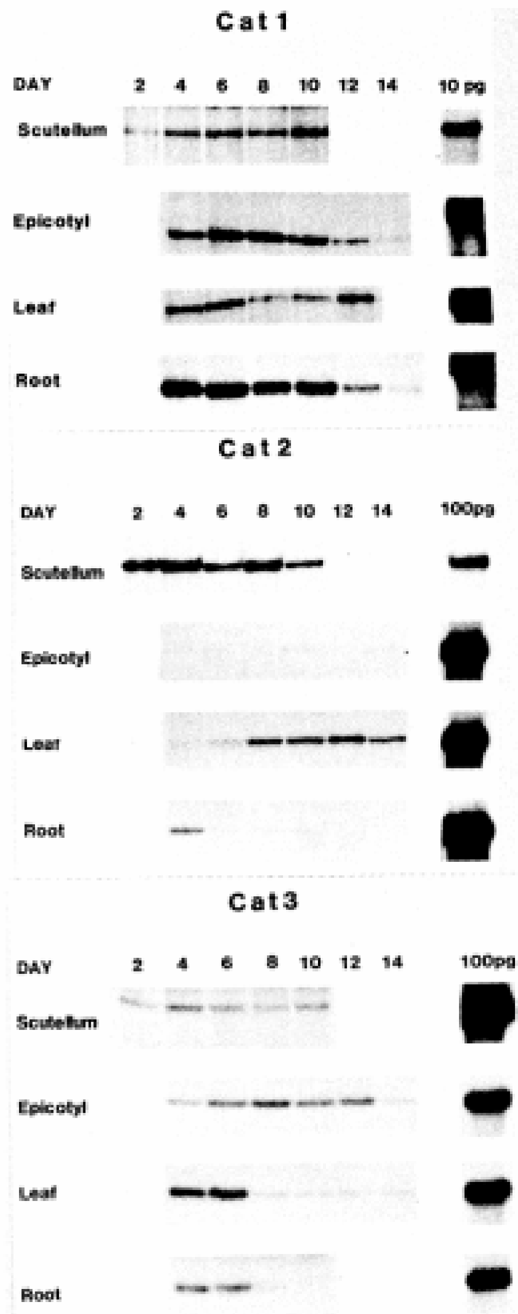


Figure 4 (See facing page for legend.)

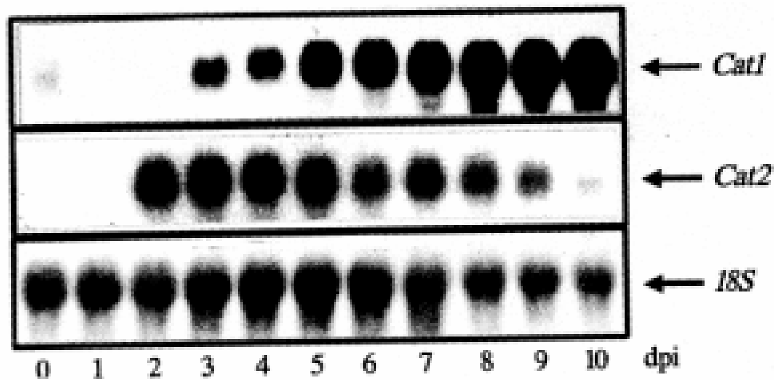


Figure 5 Accumulation of *Cat1* and *Cat2* transcripts in scutella of germinating maize embryos. Embryos were excised from W64A germinated kernels at 0 to 10 dpi; total RNA was isolated, and accumulation of *Cat1* and *Cat2* transcripts in each sample was determined by Northern blot analysis. A DNA fragment containing an 18S ribosomal sequence was used to ensure similar loading and transfer.

CAT-1 protein decreases during the same scutellar developmental period (Fig. 5). These data clearly indicate that the differential expression of the two genes in this tissue involves both transcriptional and posttranscriptional regulation.

The shift in expression from *Cat1* to *Cat2* in the scutellum is under genetic control, as verified by use of several maize lines shown to exhibit altered patterns of catalase expression during the same developmental period (Scandalios et al. 1980b; Chandlee and Scandalios i984a). For example, line R6-67 exhibits a continuous increase in catalase activity after 4 days postimbibition compared to the usual decline seen in most lines (e.g., W64A). The continuous increase in catalase activity is concurrent with an increase in CAT-2 protein synthesis and accumulation. By genetic analysis, a genetic regulatory element, *Carl,* was identified and shown to be responsible for the overexpression of the *Cat2* gene in R6-67 (Scandalios et al. i980b). *Carl* maps 37 map units from the *Cat2* structural gene on the short arm of chromosome 1. The product of *Carl* and the mechanism by which it increases *Cat2* expression are unclear. However, R6-67 has significantly more *Cat2* mRNA than do the normal lines, and it is suggested that *Carl* may encode a trans-acting factor affecting the transcription of *Cat2* (Kopczynski and Scandalios i986).

A second regulatory element, *Car2,* was identified and shown to function in decreasing the rate of CAT-1 synthesis in the scutellum during early sporophytic development (Chandlee and Scandalios i984a).

Car2 acts independently of *Carl* and is either closely linked or contiguous with the *Cat1* structural gene. A number of CAT developmental time-course mutants have been identified and are being analyzed at the molecular level in an effort to decipher the underlying mechanisms involved.

INTERTISSUE SIGNALING AND CAT EXPRESSION IN THE SCUTELLUM

Expression of catalase in the scutellum is apparently regulated by "signals" from tissues that spatially interact with the scutellum. The post-germination induction of *Cat2* occurs as a consequence of a temporal and spatial gradient. Using immunofluorescence assays, CAT-2 is first detected in cells that interact with the aleurone. As development proceeds, CAT-2 accumulates in a spatial gradient, occurring first in cells nearest the aleurone, then in cells farther and farther away from the aleurone, until all scutellar cells express the CAT-2 protein (Tsaftaris and Scandalios 1986). This suggests that the aleurone may be releasing a diffusible molecular signal triggering the induction of *Cat2* in the scutellum. Furthermore, the embryonic axis exerts a specific effect on the accumulation of glyoxysomal proteins, including catalase (Skadsen and Scandalios 1989). Upon excising the embryonic axis from the scutellum prior to imbibition, the developmental accumulation of all glyoxysomal proteins is drastically reduced. In contrast, the developmental patterns of nonglyoxysomal proteins are unaffected; this suggests that an axis-specific factor modulates the level of expression of the glyoxysomal proteins, including catalase (Skadsen and Scandalios 1989). The exact nature of this "factor(s)" is as yet unclear. However, it is apparent that possibly two molecular signals may be involved in regulating the expression of *Cat2* in the scutellum during early sporophytic development; one emanating from the aleurone may act to activate the gene, and another emanating from the embryonic axis may modulate the level of expression of *Cat2*. The nature of the signals is currently under investigation.

CATALASE DEFICIENCIES

Mutants deficient in catalase activity have been identified in barley and maize (Scandalios 1994). The barley mutant (Rpr 79/4) was reported to be unable to survive under photorespiratory conditions (Kendall et al. i983). More recent investigations with this barley mutant (Acevedo et al. i996) indicated that it is able to grow to maturity in normal air, but sustains chlorosis and significant head sterility. Detailed analyses

showed that this mutant lacks one of at least two distinct catalase isozymes; CAT-1 (EP-CAT), analogous to the maize CAT-1 catalase, and CAT-2 (T-CAT), analogous to the CAT-3 of maize, which have been identified in all barley strains examined (Skadsen et al.1995). The mutant barley Rpr 79/4 was found not to express the *Cat* gene encoding the T-CAT isozyme, but retained expression of the *Cat1* isozyme gene (Acevedo et al. 1996). Thus, even though the Rpr 79/4 barley mutant is not completely lacking catalase, the loss of its peroxisomal T-CAT catalase isozyme is evidently harmful, underscoring the protective role of catalase in plants. To date, the molecular basis for the absence of CAT-2 in barley is unresolved.

In maize, a number of null mutations for *Cat2* and *Cat3* were discovered following a large mutant screening program; no such mutants were recovered from similar screens for the *Cat1* gene (Scandalios 1994). Unlike the barley mutant, maize mutants deficient in CAT-2, CAT-3, or both CAT-2 and CAT-3 showed no discernible physiological effects or altered gross phenotypic characteristics when grown under normal conditions. Since the maize catalases appear to have, in addition to scavenging H_20_2, differential responses to a variety of effectors, it is conceivable that the null mutants will respond differently to such effectors. In fact, we have preliminary data indicating that there are compensating or overlapping functions among the three catalases under adverse conditions (e.g., extreme temperature). The various mutants are currently being grown under stress conditions in order to compare their overall phenotypic and physiological responses to the "wild type" grown under similar conditions. It is likely that under highly stressful conditions, the null mutants may be less adaptable.

MOLECULAR CHARACTERIZATION OF THE *Ca~* AND *Cat3* NULL MUTATIONS IN MAIZE

Because the null mutants would be invaluable in deciphering the distinct physiological roles of each catalase isozyme, it was deemed essential to determine their molecular bases. Such a task was undertaken utilizing the available gene-specific cDNA probes for each of the three maize catalases.

RNA blot analyses of 5-day-old, scutellar poly(A)+ RNA from the *Cat2* null line A340 showed that the *Cat2* transcript was about 1400 nucleotides, compared to the 1850-nucleotide *Cat2* transcript in normal maize lines (Bethards and Scandalios 1988).

The molecular basis for the shorter *Cat2* transcript in maize lines null

for the CAT-2 catalase isozyme was further investigated using cDNA libraries and genomic DNA blots. Sequence comparison of partial *Cat2* cDNAs obtained from two CAT-2 null lines and a wild-type CAT-2 encoding cDNA showed that the *Cat2* null transcripts diverged from the wild-type transcript but remained homologous to each other. Genomic DNA blots indicated that the missing portion of the transcript is present in the genomes of lines null for the CAT-2 isozyme. Differences in the hybridization patterns of normal and null lines were revealed when genomic DNA blots were probed with the full-length *Cat2* cDNA, a *Cat2* gene-specific probe, and a "null sequence" probe. Together, the DNA blotting results suggested that a rearrangement of the *Cat2* gene had occurred in the CAT-2 null lines. The available data suggest that this CAT-2 null mutation in maize is due to a DNA insertion into the *Cat2* gene (Abler and Scandalios 1991).

Two maize inbred lines have also been identified as being homozygous for a *Cat3* null allele. CAT-3 monospecific polyclonal antibodies could not detect CAT-3 protein in extracts of any tissues (which in any normal maize line possess CAT-3) of the CAT-3 null lines. RNA blot analysis indicated that *Cat3* transcript does not accumulate in any tissues. Additionally, zymogram and RNA blot analyses showed that the CAT-3 null mutation did not affect the expression of the *Cat1* or *Cat2* genes at the mRNA or protein level. Genomic DNA blots revealed significant structural alterations in the *Cat3* gene of CAT-3 nulls. The results suggest that the molecular basis for the CAT3 null phenotype is most likely due to a deletion in the S 'end of the *Cat3* gene (Wadsworth and Scandalios 1990). To investigate whether the lack of *Cat3* transcript accumulation is due to a lack of transcription of the *Cat3* gene, transcription run-on assays were performed. Results indicated a strong hybridization signal to the ribosomal DNA (a positive control), indicating that the nuclei of both the normal and *Cat3* null mutant lines exhibit high levels of ribosomal RNA transcription in vitro. The *Cat3* cDNA probe hybridized with the transcript from the W64A ("wild type") nuclei. However, the nuclei from CAT-3 nulls did not synthesize a transcript homologous to *Cat3*.

Although there are no major discernible physiological effects of the CAT2 and CAT3 null mutations, such mutants will aid in deciphering the role each isozyme plays in the plant's metabolism. Perhaps the lack of observable major phenotypic effects of these mutants is due to the fact that CAT-1 is still expressed and may compensate for the defect. By breeding experiments, we have created a double null line lacking both CAT-2 and CAT-3. Preliminary data suggest that this line may, in fact,

exhibit a higher germination rate than the normal lines, but it still expresses CAT-1. Eliminating CAT-1 chemically or by antisense mutations might be more telling as to the role of catalases in the life of the plant. It is also likely that each of the catalases may play a distinctly important physiological role under environmental conditions, not yet tested, which increase oxidative stress. Such experiments are currently in progress, utilizing these null mutations in genetically similar backgrounds. By recurrent selection experiments, we have succeeded in putting the null genotype in the W64A background.

CATALASE GENE EXPRESSION IN RESPONSE TO ENVIRONMENTAL STRESS

The expression of catalase genes is not only influenced by genetic and developmental signals, but is also significantly influenced and responsive to various exogenous environmental signals, both physical and chemical. Among the many environmental conditions/substances known to cause oxidative stress and to trigger antioxidant defense responses are temperature extremes, pathogenesis, radiation, drought, anoxia and hyperoxia, wounding, herbicides, hormones, H_20_2, light, and numerous xenobiotics. The response of catalases to some of these effectors is discussed in some detail below.

Fungal Toxin

Many fungi of the genus *Cercospora* produce a light-induced, photoactivated polyketide toxin (cercosporin). In the presence of light and a suitable reducing substrate, an excited form of the cercosporin molecule (triplet state) reacts with 02 to form the O2~ radical (see Scandalios, this volume).

Total leaf protein extracts (7 to 10 days postgermination) showed an approximately 25% increase in total CAT activity in plus-cercosporin versus minus-cercosporin-treated leaves (Fig. 6A). Rocket immunoelectrophoresis, however, showed approximately 20-30% decrease in CAT-2 protein levels. This decrease is apparently at least partially offset by a corresponding increase in the more abundant CAT-3 isozyme (Fig. 6B). Nuclease S-i analyses of leaf total RNA showed a comparable increase in steady-state levels of *Cat3* RNA in the presence of cercosporin, compared to a slight decrease in *Cat2* RNA accumulation (Fig. 6C). Little or no change was observed in total CAT activity in 3-day postimbibition (dpi) embryo axes (not shown). In scutella (embryo minus axis) from

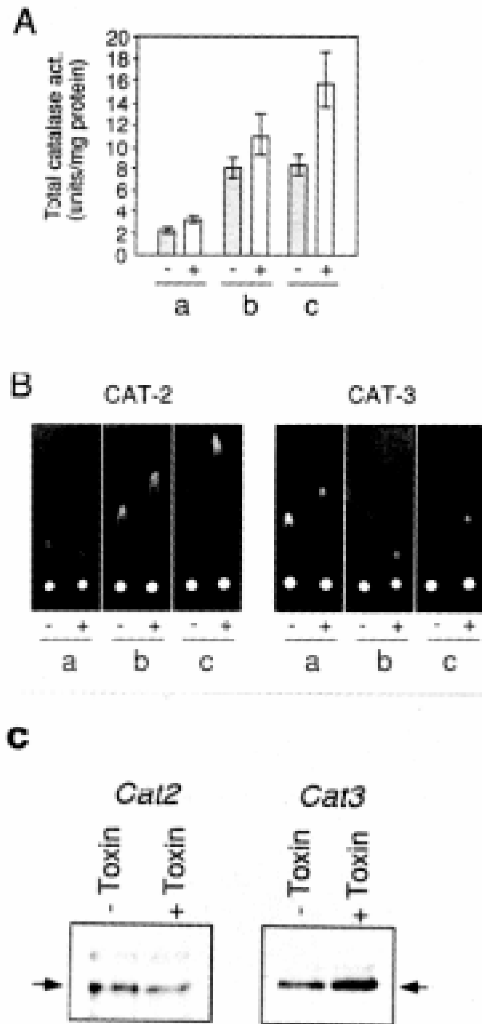


Figure 6 Changes in CAT activity *(A),* isozyme level *(B),* and transcript *(C)* in leaves and scutella of maize treated with *Cercospora* culture filtrate. Sterile, light-grown culture filtrate (+ cercosporin) or dark-grown culture filtrate (- cercosporin) was applied to the surface of 7- to 10-day-old maize leaves *(a* columns). Plants were then grown 24 hr in constant light. Whole kernels *(b* columns) were similarly treated by imbibition, as were excised developing embryos *(c* columns) on organ culture medium, infiltrated with dark- or lightgrown *Cercospora* culture filtrates. After treatment, the respective exposed maize tissues were assayed for changes in total CAT activity *(A).* Accumulation of CAT-2 and CAT-3 isozyme proteins by rocket immunoelectrophoresis *(B),* and *Cat2* and *Cat3* transcript accumulation by Si-nuclease protection analysis *(C).*

3-dpi seed, however, an approximately 50-60% increase in total CAT activity was observed. Rocket immunoelectrophoresis of corresponding scutellar protein extracts showed an increase in CAT-2 and CAT-3 isozyme levels of sufficient magnitude to account for the majority of this increased activity (Williamson and Scandalios 1992a).

Maize embryos incubated in the presence of increasing levels of purified cercosporin showed a dose-dependent increase in total catalase activity, individual isozyme protein levels, and corresponding transcript accumulation. *Cat3* transcript levels increased with increasing doses of purified cercosporin, and *Cat2* transcript accumulation showed a similar increase, followed by a decrease. *Cat1* transcript accumulation showed a distinct decrease followed by an increase (Fig. 7). In contrast to the response of SOD (see Scandalios, this volume), the changes in steady-state levels of the various CAT isozymes were generally consistent with the observed changes in steady-state levels of the corresponding transcripts.

Whereas a number of models may be consistent with these observations, perhaps the simplest is that H_20_2, the dismutation product of O2~~ by SOD, is, by itself, less immediately damaging to the cell than ~ Thus, the time required for the induction of the appropriate *Cat* gene(s) and subsequent message translation might be adequate for defense against this less toxic oxygen species. The observed response of the

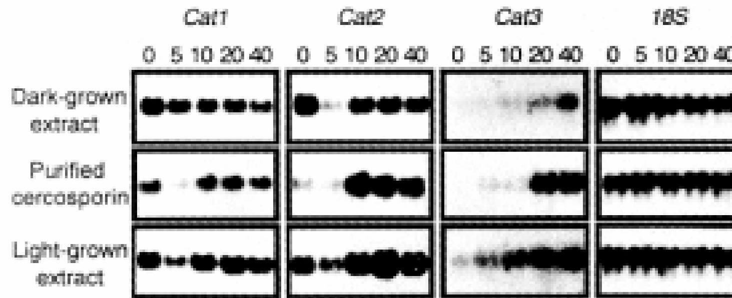


Figure 7 Differential *Cat* transcript accumulation in the presence of *Cercospora* extracts and purified cercosporin toxin. Excised 2 dpi W64A embryos were incubated on culture media supplemented with increasing doses of extract from either dark-grown (-cercosporin) or light-grown *Cercospora (+* cercosporin), or purified cercosporin. All plates contained equivalent amounts of solvent, with 0 cercosporin samples containing solvent only. All samples were exposed to light for 24 hr. Following treatments, accumulation of the various *Cat* transcripts in each sample was analyzed by RNA blots. Results shown are representative of three independent replicates.

catalase gene products to increasing doses of purified cercosporin, in fact, closely mimics the response of cultured maize embryos to increasing doses of H_2O_2 described previously (Scandalios et al. 1984).

A variety of other compounds produced by fungi are also known to affect plant gene expression (Dixon and Harrison 1990). We therefore compared the effects of extracts from dark-grown (lacking cercosporin) and light-grown *Cercospora kikuchii* mycelia with those of purified cercosporin (Williamson and Scandalios 1993). The results from these experiments showed that the *Cat* transcripts also respond to compounds other than cercosporin present in the extracts from both light- and dark-grown fungi (Fig. 7). The complex response of *Cat2* (a single-copy gene in maize), which shows a decrease followed by an increase at S ~M while *Cat3* shows a simple increase to increasing doses of extract from darkgrown mycelia, suggests the presence of one or more differentially active compounds in the non-cercosporin-containing extracts. Although our results do not specifically reveal the type or number of active components present in extracts of dark-grown *Cercospora,* a number of fungal compounds other than cercosporin have been identified that elicit altered plant gene expression (Dixon and Harrison 1990), including the nonphotosensitizing (non-cercosporin) phytotoxic B-toxin in *Cercospora spp.*

Our results also demonstrated that the response of the maize catalases to toxin-containing *Cercospora* extracts is different at two separate developmental time points in maize scutella (Williamson and Scandalios 1993). These two time points represent distinct and rather well-defined alternate pathways in developmental programming, embryogenesis, and germination. Different responses to the same treatment at these two stages is, therefore, a significant observation. These results suggest a number of possibilities to explore. For instance, a specific trans-acting factor involved in catalase gene expression might be present only at a particular developmental stage. Thus, the response of a single gene to the same environmental signal could be quite different at different developmental stages. Such complex control of expression might reflect the specific functions of these genes in cellular responses to oxidative stress.

Hydrogen Peroxide

The response of catalase to exogenously applied H_2O_2 varied between the normal catalase lines (e.g., W64A) and the Cat2-null mutants (e.g., A338F) during postpollination kernel development. Total catalase activity

increased in W64A but decreased in A338F. In the normal catalase lines, CAT-1 isozyme activity increased slightly at low concentrations (1 mM H_20_2), then decreased to very low levels with increasing [H_20_2] of up to 50 mM. In contrast, the activity of CAT-2 increased significantly above 10 mM H_20_2. This differential response of the CAT-1 and CAT-2 isozymes to H_20_2 during kernel development leads to a pattern shift (Fig. 8) from CAT-1 to CAT-2 reminiscent of the catalase developmental program observed in scutella during postgerminative development (Quail and Scandalios 1971). In W64A, the response of catalase to H_20_2 during postpollination kernel development is similar to that observed during postimbibition scutellar development (Scandalios et al. 1984). In the *Cat2* null mutant, the activity of the CAT-1 and CAT-3 isozymes increased slightly at the lower concentrations [1-10 mM H_20_2], but decreased significantly at higher concentrations, leading to the complete disappearance of CAT-1 at >30 mM H_20_2 (C. Auh and J.G. Scandalios, unpubl.).

It is apparent from the above experiments that the three maize catalases respond differentially to exogenously applied H_20_2 at different developmental stages. We have preliminary data indicating that endogenous H_20_2 levels measured over extended developmental time periods in maize tissues show an inverse correlation between CAT activity and H_20_2 (C. Auh and J.G. Scandalios, unpubl.), suggesting that fluctuations in H_20_2 levels may play a significant signaling role in effecting the tissue-specific and temporal expression of the different *Cat* genes in maize.

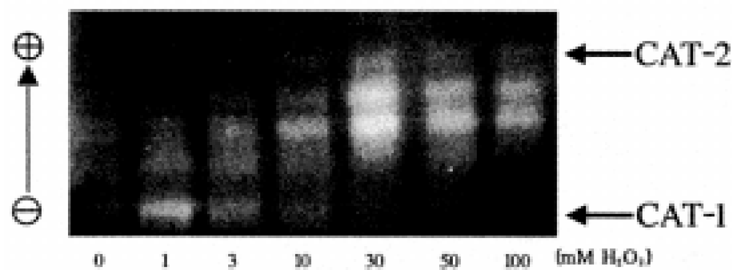


Figure 8 Zymogram analysis of maize catalase from W64A scutella extracted 28 days postpollination following treatment with various doses of H_20_2. The samples were homogenized in 25 mM glycylglycine buffer (pH 7.4). Equal amounts of total protein were electrophoresed. The final concentration of H_20_2 in the medium is indicated below each lane. CAT-1 and CAT-2 homotetramers are indicated by arrows.

Temperature Extremes

Organisms have evolved a variety of ways to adapt to fluctuations in temperature. The most dramatic and readily discernible response to thermal stress in most organisms is the "heat shock" response, during which a number of genes are induced to produce the so-called "heat shock proteins " (hsps) that serve to protect cells from thermal damage. Less is known about responses to extreme cold, but what is known is based largely on recent work with plants (Tomashaw 1990). The most dramatic manifestation of cold acclimation is the increased freezing tolerance that occurs in many plant species. It is known that extreme environmental conditions often create oxidative stress conditions, which in turn might affect the antioxidant defense system.

The developmental profile of catalase activity in the high catalase activity mutant (R6-67) and the "wild type" activity profile line W64A are differentially affected by chronic exposure to high temperature (40^{0}C) (Matters and Scandalios 1986a). At 25^{0}C, R6-67 has significantly higher CAT activity after day 3 postgermination compared to W64A; until about day 3, the rate of increase and level of catalase activity between the two lines are about the same. Following about day 3-4 at 40^{0}C, however, the level of CAT activity in line R6-67 drops precipitously to the level of W64A, whereas the basic activity profile in W64A changes only slightly. The decline in R6-67 is associated with a decrease in CAT-2 protein and mRNA, whereas CAT-1 remains relatively constant (Scandalios 1994). When both CAT isozymes and transcripts were examined in day 1 post-germination W64A scutella following incubation at 35^{0}C and 40^{0}C for 24 hours, both CAT-1 and CAT-2 proteins and their respective transcripts increased at 35^{0}C and remained so, although CAT-1 protein and mRNA appeared more stable than CAT-2 at 40^{0}C (Fig. 9). In *N. plumbaginifolia, Cat1* and *Cat2* transcripts decreased during a 5-hour exposure to 37^{0}C but rapidly recovered following a return to 22^{0}C, whereas *Cat3* mRNA levels remained constant at both temperatures (Willekens et al. i994c). Thus, like the maize catalases, the tobacco catalases exhibit differential responses to thermal stress.

Low temperature (4^{0}C and i4^{0}C) treatments led to significant increases in total catalase activity and resulted in isozyme pattern shifts in germinating maize embryos. A more dramatic increase in total CAT activity was observed in the embryonic axes than in scutella. In the latter, both CAT-1 and CAT-2 isozymes increased in response to the low temperatures, but there was a significant shift toward CAT-1 (Fig. bA). The CAT-1 homotetramer appeared only after the cold treatment, the CAT-1/CAT-2 heterotetramers also increased, and the CAT-2 homo

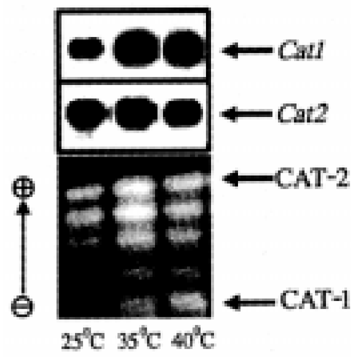


Figure 9 Changes in catalase transcripts and isozyme proteins in response to high temperature. W64A seeds were imbibed in water for 24 hr and incubated in germination trays at 25^0C, 35^0C, and 40^0C for 24 hr. Scutella were isolated from treated seeds, and equal amounts of protein were examined by zymogram. The CAT-1 and CAT-2 homotetramers are indicated. Transcripts were detected by Northern blot hybridization with *Cat1* and *Cat2* gene-specific probes. Representative results from duplicate blots are shown.

tetramer did not change. In the embryonic axes, CAT-2 increased, CAT-1 was newly induced by low temperature, and CAT-3 (normally expressed in this tissue) decreased (Fig. lOB).

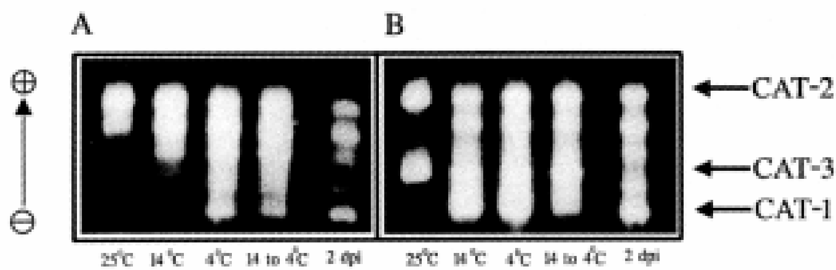


Figure 10 Changes in catalase isozyme protein in response to cold treatment. W64A embryos were isolated from 2 dpi germinating seeds and incubated in MS basic medium at 25^0C, 14^0C, or 4^0C for 24 hr, or at 14^0C for 12 hr, then transferred to 4^0C for 12 hr. Scutella *(A)* and embryo axis *(B)* were isolated, and equal amounts of protein were examined by zymogram analysis for catalase isozyme activity. The 2 dpi scutella were used as a catalase standard control. CAT-1, CAT-2, and CAT-3 are indicated by arrows; intermediate bands are CAT-1/CAT-2 heterotetramers. CAT-3 does not form heterotetramers, in vivo, with either CAT-1 or CAT-2.

Eftects of Plant Hormones

Salicylic Acid

Salicylic acid (SA) is found in the leaves and reproductive structures of many plant species (Raskin et al. 1990) and modulates many biological processes in plant cells (Raskin 1992). Recent evidence has indicated that SA may serve as an endogenous signal molecule in the induction of systemic acquired resistance (SAR) in tobacco (Gaffney et al. 1993). SAR is the phenomenon whereby plants, upon infection, develop enhanced systemic resistance to subsequent microbial attack (Madamanchi and Kuc 1991). A cellular SA-binding protein that shares high sequence identity to plant catalase was recently identified in tobacco (Chen et al. 1993). It was proposed that SA can bind to catalase and inhibit its activity, leading to an increase in cellular H_20_2 levels, which then serve to transduce the signal that mediates a response to pathogen attack by triggering the plants' defense mechanisms such as SAR (Chen et al. 1993). Although tobacco is known to possess three distinct catalases (Havir and McHale 1987), no distinction was made by Chen et al. (1993) as to whether all three serve as SA-binding proteins. These findings are somewhat controversial, because other laboratories have reported different results with the same system and have presented alternative hypotheses (Bi et al. 1995; Neuenschwander et al. 1995).

The response of the maize catalase *(Cat)* genes to SA was examined at two distinct developmental stages: embryogenesis and germination. A unique, germination-related differential response of each maize catalase gene to various doses of SA was observed (Guan and Scandalios 1995). During late stages of embryogenesis, total CAT activity in scutella increased dramatically with 1 mM SA treatment. The accumulation of *Cat2* transcript and CAT-2 isozyme protein is the major contributor to the observed increase in total CAT activity (Fig. 1 lA). This event was paralleled by the induction of germination of embryos at that stage. In a CAT-2 null mutant line, a full compensation of total CAT activity by the CAT-1 isozyme was observed in the presence of SA. Thus, in a maize line missing one catalase isozyme (i.e., CAT-2 null), another catalase isozyme (CAT-1) is dramatically increased to compensate for the loss of CAT activity due to the missing isozyme. This suggests that catalase is important in maintaining normal cellular processes under stress conditions. SA (1 mM), which induces germination of immature embryos, appears to inhibit seed germination at 1 dpi. Furthermore, *Cat2* transcript accumulation was inhibited at this stage, whereas the *Cat1* transcript was increased with 1.5 mM SA treatment (Fig. liB). During the later stages of germination, CAT activity again increased dramatically with 1 mM SA

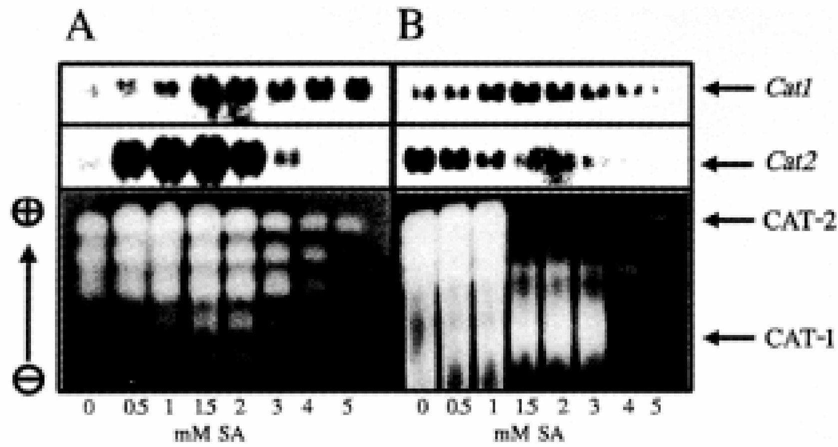


Figure 11 Changes in catalase transcripts and isozyme proteins of maize embryos in the presence of SA. Embryos were isolated from 28 dpp kernels *(A)* and 1 dpi seeds *(B)* and treated with 0-5 mM SA for 24 hr. Scutella were isolated from treated embryos, and equal amounts of protein were examined by zymogram. The CAT-1 and CAT-2 homotetramers are indicated by arrows; intermediate bands are CAT-1/CAT-2 heterotetramers. Transcripts were detected by Northern blot hybridization with *Cat1* and *Cat2* gene-specific probes. Representative results from duplicate blots are shown.

treatment likely due to the activation of the CAT-1 protein (L. Guan and J.G. Scandalios, unpubl.). We conducted SA-binding assays with total protein extracted from leaves and scutella, as well as with purified CAT-2 protein. Our results indicated only slight inhibition of catalase activity with 1 mM SA for 1 hour. These observations imply that the maize catalases, unlike the tobacco catalase, may not serve as SA-binding proteins, and the observed differential responses to SA may be indicative of unique metabolic roles for each of the three maize catalases, in addition to their role in scavenging H_20_2. Our results suggest that the mechanism of action for SA, with respect to catalases and H_20_2, may be different between monocots and dicots.

Abscisic Acid

The effects of the phytohormone abscisic acid (ABA) on the expression of maize catalases have been studied in several developmental stages in the scutella during embryogenesis and germination. Embryos from developing maize seed at days 18-21 were isolated and incubated on

growth medium supplemented with ABA. Zymogram analyses of total extracted proteins of these embryos showed that CAT-1 protein accumulated in embryos incubated in the presence of ABA. In contrast, excised embryos incubated on growth media alone accumulated increased levels of the CAT-2 isozyme, whereas CAT-1 levels appeared to decrease. *Cat1* transcript levels increased substantially in the presence of ABA but remained unchanged in the absence of ABA. Conversely, *Cat2* increased in the absence of ABA but failed to accumulate in the presence of ABA in scutella of developing embryos (Williamson and Scandalios 1992b).

The effects of ABA on the expression of catalase have also been studied in mature embryos. A similar catalase expression pattern was observed in scutella of 1 dpi embryos after ABA treatment. The CAT-1 isozyme level increased in response to ABA, whereas CAT-2 did not respond to ABA (L. Guan and J.G. Scandalios, unpubl.). When ABA was applied to 5 dpi embryos, the opposite effect of ABA on catalase expression was observed. The CAT-1 isozyme protein increased upon ABA treatment while *Cat1* transcript remained unchanged (Williamson and Scandalios 1994). On the other hand, both CAT-2 protein (Fig. 12) and *Cat2* transcript (Guan et al. 1996) increased substantially after ABA treatment. These data suggest that the *Cat1* and *Cat2* of maize respond differentially to ABA, and the response of each *Cat* gene to ABA is developmental-stage-dependent.

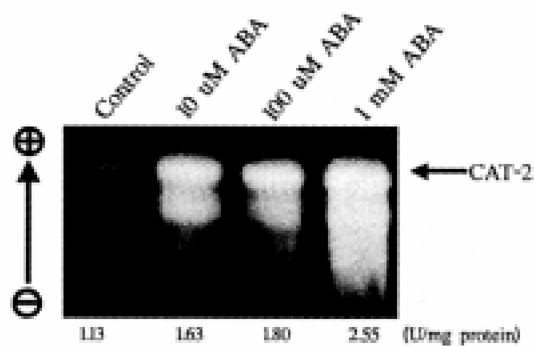


Figure 12 CAT response to ABA in *6* dpi scutella. Changes in catalase isozymes and activity in the presence of ABA. Embryos were isolated from *5* dpi W64A seedlings and treated with increasing doses of ABA for 24 hr in the dark. Scutella were isolated from treated embryos, and equal amounts of protein were examined by zymogram analysis for catalase activity. The CAT-2 homotetramer is indicated by an arrow. The number below each lane represents total catalase activity (unit per mg protein) after ABA treatment.

Addition of ABA in culture medium leads to a large increase in *Cat1* RNA accumulation in excised immature embryos but not in the germinating mature embryos. The *Cat1* transcript, however, also accumulates to high amounts in scutella of germinating embryos, where ABA content is low and decreasing, indicating that ABA up-regulates *Cat1* transcript accumulation in scutella during embryogenesis but not during germination (Williamson and Scandalios 1994). Thus, factors other than ABA control *Cat1* expression during germination.

The *Cat1*promoter has been sequenced, revealing several regulatory elements, and has been used for the construction of promoter-reporter gene fusions that can drive the expression of the reporter gene *Gus* in transgenic tobacco. A minimal 800-bp promoter fragment is sufficient for the regulation of *Gus* expression with a pattern similar to the *Cat1* expression observed in maize (Guan and Scandalios 1993). An ABA-responsive element (the G-box-consensus sequence *Em1a)* (Guiltinan et al. 1990) was located at -110 relative to the transcriptional start site and displays differential binding of nuclear proteins isolated from germinated and developing embryos (Fig. 13A) and from embryos grown in the presence and absence of ABA (Fig. 13B). Southwestern analyses show that one protein (CAT1BP-20) accumulated in the absence of ABA in immature embryos and in germinating mature embryos (Fig. 13C), suggesting that a repressor-mediated mechanism accounts for at least a portion of the ABA regulation of *Cat1*.

Indoleacetic Acid

Auxin (indoleacetic acid; IAA) was the first plant hormone to be identified. It regulates many aspects of plant growth and development including cell elongation, tissue differentiation, maintenance of tissue polarity, and leaf expansion. IAA is the principal naturally occurring auxin in most plants. The effects of IAA on the expression of maize catalase gene expression were examined at several developmental stages during seed maturation and germination. Results showed that IAA enhances the expression of catalase isozymes at every developmental stage examined, even at 12 dpi when ABA has no effect on CAT expression. Total catalase activity increased upon IAA treatment, with CAT-2 being the major isozyme contributing to the total catalase activity increase (Fig. 14). CAT-1 isozyme activity also increased with IAA treatment, but to a lesser extent. IAA treatment also enhanced the germination process of maize embryos during the late stages of seed maturation and early stages

of germination. It has been reported that the endogenous content of IAA in germinating maize seeds increases up to threefold after 36 hours postimbibition and then drops to low levels after 72 hours postimbibition (Tillberg 1977). It is interesting to note that the developmental profile of catalase activity in scutella matches that of IAA content in the germinating seeds. All this implies that IAA may play a role in controlling the developmental pattern of catalase expression in scutella during seed germination. The action of IAA on maize scutella may promote the initial increase and retention of catalase activity during the early stages of seed germination.

Photoregulation and Photooxidative Stress

The three maize catalase genes respond differentially to light signals. The expression of the *Cat1* gene is light-independent throughout development (Scandalios 1994). The control of *Cat2* expression in maize leaves in response to light (Skadsen and Scandalios 1987) appears to involve a unique form of translational inhibition in leaves grown in constant dark (DD), preventing translation of isolated *Cat2* mRNA. The mRNA is rendered translatable only after the leaves are exposed to white light, leading to a dramatic induction of the CAT-2 isozyme in leaves when DD-grown seedlings are exposed to light (Skadsen and Scandalios 1987; Scandalios 1994). *Cat3* exhibits a transcriptionally regulated circadian rhythm (Redinbaugh et al.1990a; Acevedo et al. 1991; Abler and Scandalios 1993, 1994; Boldt and Scandalios 1995). This differential light response of the three maize catalases is also reflected in the expression pattern during photooxidative stress caused by UV irradiation.

We have recently shown (Boldt and Scandalios 1997) that UV light pulses in the range of 240-400 nm could entrain the *Cat3* circadian expression (see Circadian Regulation of Plant Catalases below) and cause a strong transient induction of *Cat2*. The most distinct response was obtained with UV light in the range of 290-400 nm (containing UV-B and UV-A). This effect was reduced by about 60% when the UV-B portion was removed using a polyester filter (240-310 nm cutoff). This reduction was reversed by applying UV light plus visible light. The reversibility of the UV-light effect indicates DNA damage that can be repaired via photoreactivation. However, UV-light pulses in the range of UV-B and in the range of UV-AIblue light applied with visible light caused a similar induction of *Cat2* mRNA accumulation. Therefore, it is suggested that the induction of *Cat2* may be also mediated by a UV-B and a UV-AIblue light photoreceptor. The transient character of the induction

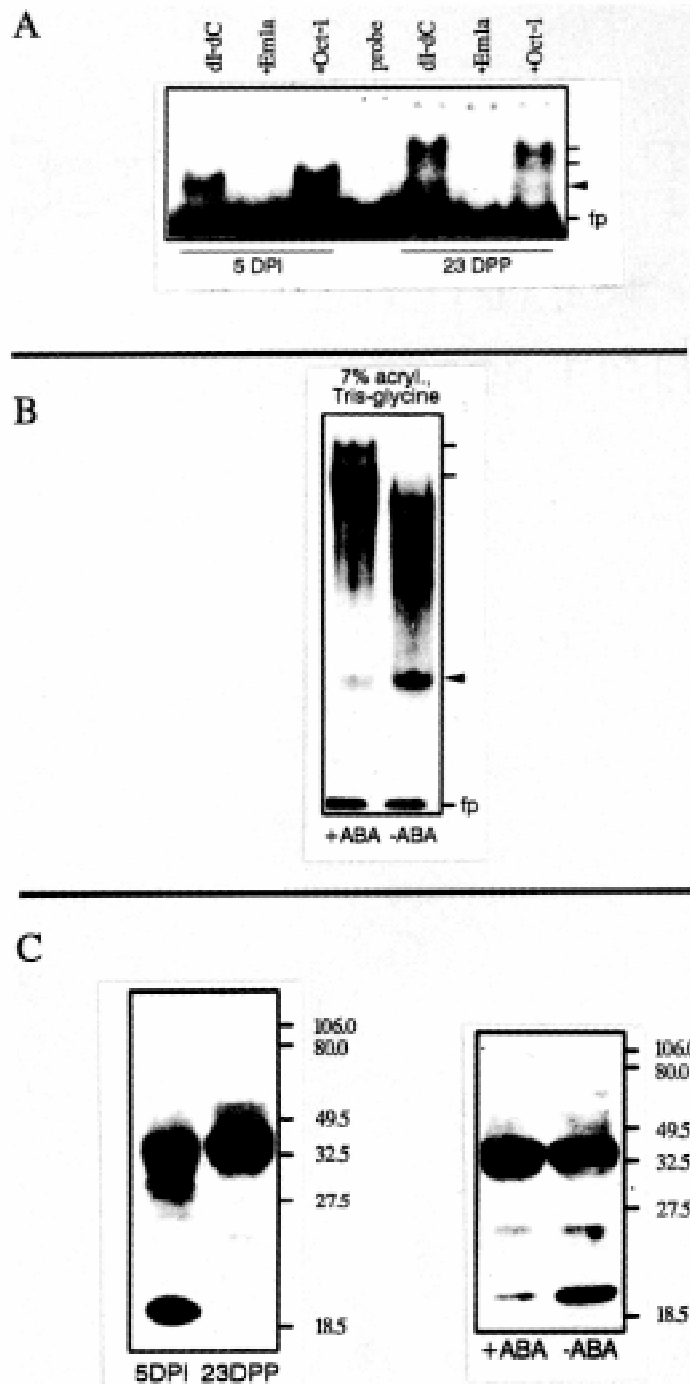


Figure 13 (See facing page for legend.)

of *Cat2,* and its repair by photoreactivation, imply an early response to UV-light stress.

A slight induction of *Cat2* was also observed after long-term exposure to UV light. However, the level of *Cat2* transcript accumulation was lower than that obtained with UV-light pulses, even though the transcript level remained slightly enhanced over the standard controls. Considering the stable circadian expression of *Cat3* under UV-light stress, *Cat2* may ensure a basic level of H_20_2 scavenging, particularly at the period when *Cat3* reaches a minimum during its circadian oscillation.

Figure 13 Band-shift and Southwestern blot analysis of DNA-binding proteins from scutella of immature and germinated embryos, and from scutella of +ABA and -ABA-treated embryos. Embryos were excised from germinated kernels of the maize inbred line W64A at 5 dpi and from developing kernels at 23 dpp. Embryos were also excised from developing kernels 23 dpp and incubated for 14 hr in the dark on GM alone (-) or GM supplemented with $i0^{-4}$ M *RIS* racemic ABA (+). Embryo axes were removed, and total nuclear proteins were isolated from the scutella. *(A)* Band-shift analysis of nuclear protein extracts from scutella of immature and germinated embryos. Binding reaction containing end-labeled 188-bp *Cat1* promoter (containing ABRE Emla) alone or probe incubated in the presence of nuclear extracts *(5* ijg) from either 5 dpi or 23 dpp scutella were separated on 7% polyacrylamide Tris-acetate EDTA gels. Reactions also contained 30-fold excess poly(dI-dC) alone(dI-dC) or in combination with unlabeled 28-bp Emla oligonucleotide (+Emla) or unlabeled Oct-i DNA consensus (+Oct-1) as competitor DNA. Migration of free probe (fp) and major retarded bands are indicated (-). The major band enhanced in the absence of ABA is indicated by an arrowhead. *(B)* Band-shift analysis of nuclear protein extracts from scutella of +ABA- and -ABA-treated developing embryos. Probes used in these experiments were prepared by end-labeling a 28-hp synthetic oligonucleotide spanning the G-box consensus sequence *Emla.* Binding reactions containing the end-labeled 28-bp *Emla* oligonucleotide incubated in the presence of nuclear extracts from either +ABA- or -ABA-treated scutella from 21 dpp immature embryos (10 and 5 ~g, respectively) were separated on 7% polyacrylamide Tris-Gly gels. Migrations of free probe (fp) and major low-mobility complexes are indicated (-). The major band enhanced in the absence of ABA is indicated by an arrowhead. *(C)* Southwestern blot analysis of DNAbinding proteins present in nuclear extracts of scutella of immature and germinated embryos or in nuclear extracts of scutella from +ABA- and -ABAtreated embryos. Nuclear extracts were separated on 12% SDS-PAGE gels, renatured, and blotted to nitrocellulose. Blotted proteins were probed with endlabeled 28-bp oligonucleotide containing Emla sequence. Blots were rinsed free of excess probe, and protein-binding labeled promoter DNA was visualized by autoradiography. Migration of molecular mass standards is indicated.

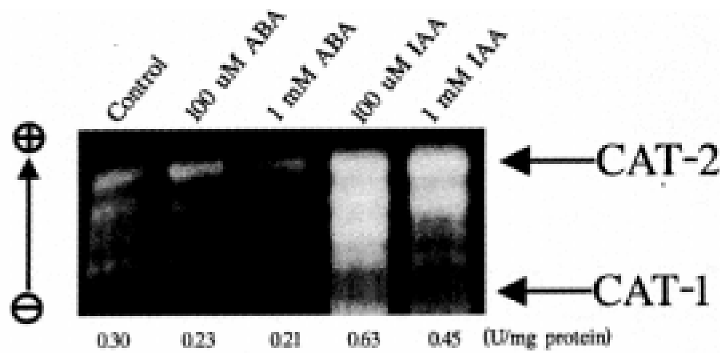


Figure 14 Changes in catalase isozymes and activity in the presence of ABA and IAA in 12 dpi scutella. Embryos were isolated from 12 dpi W64A seedlings and treated with different doses of ABA or IAA for 24 hr in the dark. Scutella were isolated from treated embryos, and equal amounts of protein were examined by zymogram analysis for catalase activity. Positions of the CAT-2 and CAT-1 homotetramers are indicated by arrows; intermediate bands are CAT1/CAT-2 heterotetramers. The number below each lane represents total catalase activity (unit per mg protein) after ABA or IAA treatment.

In *N. plumbaginifolia* leaves, *Cat1* and *Cat2* (not identical to the maize *Cat1* and *Cat2* genes) transcript levels changed dramatically in response to UV-B stress; *Cat1* was repressed, whereas *Cat2* was induced fourfold after UV-B exposure. In addition, tobacco *Cat3* (also not identical to the maize *Cat3* gene) was induced by UV-B (Willekens et al. 1994a). These data suggest a distinct role of catalases in response to UVB-stressed *N. plumbaginifolia* leaves.

Circadian Regulation of Plant Catalases

The *Cat3* gene of maize (a monocot C_4 plant) was the first catalase gene reported to exhibit a transcriptionally regulated circadian rhythm (Redinbaugh et al. 1990a). Since then, three additional plant catalases (in dicot C_3 plants), the *Arabidopsis CAT2* (Zhong et al. 1994) and *CAT3* (previously called *CA Ti;* Zhong and McClung 1996), and the *Cat1* gene of *N. plumbaginifolia* (Willekens et al. 1994c) have been shown to be transcriptionally regulated by a circadian clock. Among them, two different patterns of circadian regulation are observed. The maize *Cat3* and the *Arabidopsis CAT3* show maximal expression late in the light period, approximately 10 hours after the light onset (CT-b, circadian time) and minimal transcript accumulation late in the dark period at CT-22. The *Arabidopsis CAT2* and the *N. plumbaginifolia Cat1* exhibit a circadian

rhythm similar to the typical rhythm observed for the photosynthetic genes *cab* and *rbcS* with maximal transcript accumulation early in the light, and minimal at the start of the dark period. Considering that different patterns of circadian expression of catalase genes are present within the same *(Arabidopsis)* as well as between different plant species *(Arabidopsis, Z. mays, N. plumbaginifolia),* it is reasonable to assume that each gene fulfills different metabolic roles. In C_3 plants like *Arabidopsis* and *N. plumbaginlfolia,* the phase of accumulation of catalase transcripts early in the light period coincides with that of photosynthetic genes and is in good agreement with a potent role of catalase in decomposing H_20_2 generated during the photorespiratory oxidation of glycolate in the peroxisome (Ogren 1984).

Photorespiration in maize, a C_4 plant, occurs to a lesser degree as compared to C_3 plants, and the phasing of *Cat3* in maize with maximal expression late in the light period and early in the dark period suggests that the role of this gene is likely not associated with photorespiration. The existence of a C_3 plant catalase *(Arabidopsis CA T3)* exhibiting a similar circadian expression phase as *Cat3* of maize (C_4) implies a common metabolic necessity in C_3 and C_4 plants for catalase during the dark period. The nature of such a metabolic requirement remains obscure. However, there is substantial evidence that the maize CAT-3 protein is associated with the maize mesophyll mitochondria.

In C_4 plants, malate oxidation in mesophyll mitochondria is sensitive to cyanide and shows strong respiratory control, as in mitochondria from leaves of C_3 species and etiolated tissues. In bundle-sheath mitochondria, however, malate oxidation is largely insensitive to cyanide and shows no respiratory control. On the other hand, this oxidation is strongly inhibited by salicylhydroxamic acid (SHAM), showing that the alternative oxidase is involved (Douce 1985). Localization of CAT-3 in mesophyll mitochondria is in good agreement with ROS production during cyanide-sensitive respiration. The need for the circadian oscillation of *Cat3* may be explained by two different hypotheses that converge to a fluctuation of ROS produced during the dark-light cycles. First, mitochondrial respiration can be higher late in the light period and in the early dark period when metabolites from photosynthesis are amply supplied. Later, during the dark period, the rate of respiration becomes lower as metabolites are depleted. That could result in a fluctuation of ROS produced at a rate similar to that of respiration. Second, the engagement of the alternative oxidase in the maize mesophyll cells could be light-dependent. Actually, differential engagement of the alternative oxidase during the darklight cycle has been reported in plants with crassulacean acid metabolism

(CAM) (Robinson et al. 1992). Light-dependent induction of the alternative oxidase pathway (AOP) has been reported in etiolated soybean cotyledons exposed to light (Robinson et al. 1995). The physiological role of the AOP is still uncertain (with the exception of a few cases, as in *Arum* lilies wherein the AOP is apparently directly related to thermogenic metabolism). Several lines of evidence lead to the suggestion that the AOP may be related to prevention of oxidative stress. An apparent induction of alternative oxidase by superoxide in the yeast *Hansenula anomala* has been shown (Minagawa et al. 1992). It has also been suggested that oxidation of ubisemiquinones by the alternative oxidase would prevent them from reacting with molecular oxygen to generate superoxide (Purvis and Shewfelt 1993). Apparently, diversion of the electron flow from the cytochrome pathway where the sites of free radical production are present could result in lower ROS production. If that is true, then inhibition of the AOP could result in induction of other protective mechanisms for ROS scavenging.

Our working hypothesis (currently under investigation in our laboratory) is that a diurnal fluctuation of ROS produced by mesophyll mitochondria, dependent on the metabolite accumulation-consumption rhythm and/or differential alternative oxidase engagement during the dark-light cycle, could account for the necessity of the circadian rhythm of *Cat3,* whose product is associated with mitochondria.

There are many additional differences between the oppositely cycling catalases *CAT2* of *Arabidopsis* and *Cat3* of maize. The *Arabidopsis CAT2* mRNA reaches a peak of abundance 1 dpi in continuous dark (DD) or continuous light (LL)-grown seedlings. A second peak of mRNA abundance appears 6 dpi only in light-grown seedlings and may be associated with the development of photosynthetic competence and induction of photorespiration. This second peak is regulated by light and is not seen in etiolated seedlings. *CAT2* mRNA accumulation is induced by exposure to high-fluence blue or far-red light but not by red light. In addition, light induction is unaffected by mutations that block blue-light-mediated inhibition of hypocotyl elongation *(bluel, blue2, blue3, hy4),* suggesting phytochrome involvement. Upon transfer of etiolated seedlings to constant white light, *CAT2* mRNA rapidly accumulates, and its abundance undergoes robust oscillations with 24-hour periodicity, indicating control by an endogenous circadian clock. The rhythm is also present in plants growing under dark-light cycles and persists for at least S circadian cycles when plants are transferred either to constant dark or to constant light, indicating the robustness of the circadian rhythm (Zhong et al. 1994). In contrast, the maize *Cat3* mRNA is present at high

levels in leaves of either DD- or LL-growing seedlings (Fig. 15) without an apparent oscillation (Acevedo et al. 1991). The same is observed with the *Arabidopsis CA T3,* as its expression is similar in seedlings germinated and growing either in continuous dark or in continuous light (Zhong and McClung 1996).

The circadian expression of the maize *Cat3* gene requires the entrainmg signal of a light-dark cycle. This is a marked difference from what is observed with the *Arabidopsis CA T2,* or photosynthetic genes like *cab* (Nagy et al. 1988), which oscillate under continuous light.

An effort was undertaken in our laboratory to define the necessary conditions for the entrainment of the maize *Cat3* circadian rhythm (Boldt and Scandalios 1995). Our results demonstrated that the circadian rhythm of *Cat3* is inducible by transferring DD- or LL-growing plants to a cycling dark-light regime. A rhythm is induced by the first D to L or L to D transition, but seems to be more stable and synchronized after a second transition to the initial condition. The photoperiod (12h D/12h L or 8h LI 16h D) does not affect the appearance of the maxima and minima in *Cat3* expression which is set at CT-10 and CT-22, respectively. However, a different pattern of circadian expression concerning the phase of the rhythm and related to the initial light conditions is observed when LL- or DD-growing plants are transferred to the opposite continuous regime. When DD-growing plants are transferred to LL (Fig. 16), the induction of the rhythm starts with an increase in mRNA abundance that reaches a maximum 10 hours, and a minimum 22 hours, after the transition, which represents the normal phase of an established rhythm in 12h D/12h Lgrowing plants. The entrainment of the rhythm in plants transferred from LL to DD begins with a rapid decrease of *Cat3* mRNA accumulation, reaching a minimum 10 hours, and a maximum 22 hours, after the transition (Fig. 16). In that case, although the phase of the rhythm is the opposite of what was observed in the DD to LL transition, it still represents the normal phase of an established rhythm in plants growing under a 12h D/12h L regime. Thus, the phase in the entrainment of the circadian expression of *Cat3* depends on the nature of the transition and is in accordance with the phase of a normal rhythm that exhibits 12h D/12h Lgrowing plants. In a very interesting analysis of the UV-light effects on the induction of the *Cat3* circadian expression, Boldt and Scandalios (1997) demonstrated that single transitions of LL- or DD-growing plants to constant full-spectrum (240-400 nm) UV light strongly induce the circadian expression of the *Cat3* gene, and significantly, in a manner similar to that observed in DD to LL or LL to DD transitions. Surprisingly, not only did the DD to UV transition ("dark" to "light") induce the

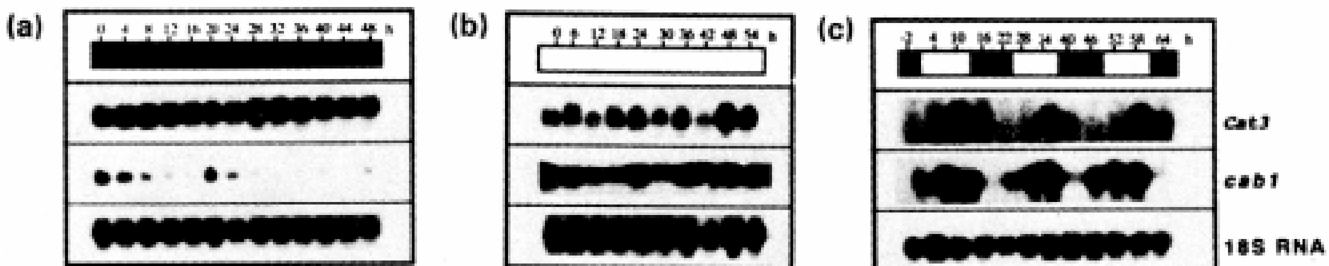


Figure 15 Accumulation of *Cat3* mRNA in leaves of maize seedlings grown under (*a*) continuous dark, (*b*) continuous light, and (*c*) in a 12 hr L/12 hr D period for 8 days. Leaf material was collected every 4 hr over a period of 48 hr for the constant dark and constant light growing plants, and every 6 hr over a period of 72 hr for the 12 hr L/12 hr D growing plants. Total RNA was extracted, and Northern blots were performed using a *Cat3* gene-specific probe. Filters were reprobed with a 18S ribosomal DNA probe as loading control, and a maize *cab1* probe as control of a typical photosynthetic gene circadian rhythm. The light and dark periods as well as the time points are indicated by bars and time in hours above the Northern blots.

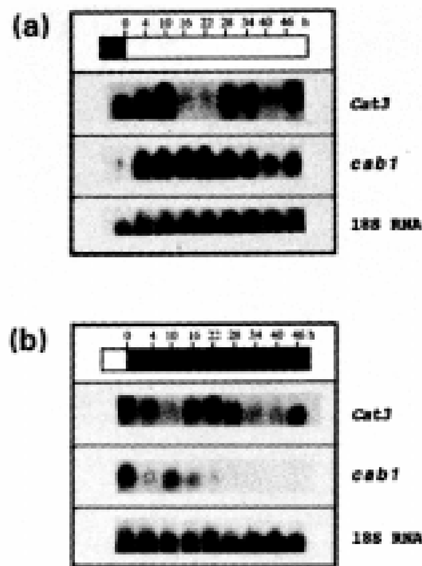


Figure 16 Entrainment of the *Cat3* circadian rhythm after single transitions from constant conditions. Accumulation of *Cat3* mRNA in leaves of maize seedlings transferred from *(a)* constant dark to constant light or *(b)* constant light to constant dark. Leaf material was collected every 6 hr for 48 hr. Total RNA was extracted, and Northern blots were performed using a *Cat3* gene-specific probe. Filters were reprobed with a 18S ribosomal DNA probe as loading control and a maize *cabi* probe as control of a photosynthetic gene circadian rhythm. The light and dark periods, as well as the time points, are indicated by bars and time in hours above the Northern blots.

circadian *Cat3* expression, but in addition, the LL to UV transition ("light" to "light") also induced a circadian rhythm similar to the one observed in the transition from constant light to constant dark. It is therefore tempting to speculate that the internal "regulator" of the rhythm reacts not only to light-on and light-off signals, but also to signals related to the quality of the light (wavelength), indicating the complex and sensitive nature of the oscillator.

In addition to dark-light transitions, short 15-minute light pulses of different wavelength monochromatic or polychromatic light given to 8-day-old etiolated seedlings can induce the circadian *Cat3* expression in a markedly uniform way, regardless of the wavelength of the light (Fig. 17). In doing so, light pulses of white, red, far-red, red followed by farred, UV (290-400 nm), or UV from which different portions of the spectrum (UV-C, UV-C and UV-B, or UV-C and UV-B and UV-A) have

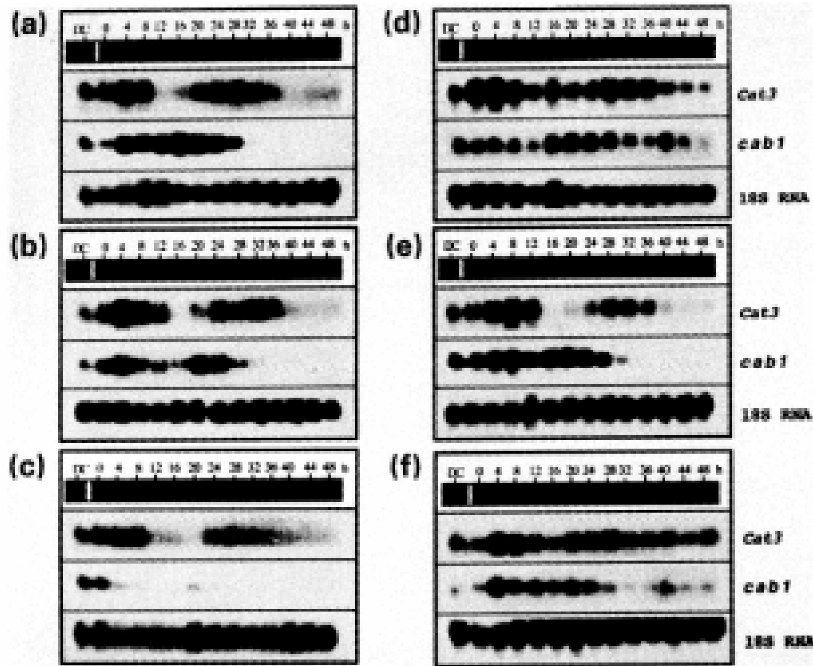


Figure 17 Influence of polychromatic and monochromatic light pulses in the induction of the *Cat3* circadian rhythm. Accumulation of *Cat3* mRNA in leaves of maize seedlings grown under constant dark for 8 days and then subjected to 15-mm light pulses of *(a)* white light, *(b)* red light, *(c)* far-red light, ***(d)*** red followed by far-red light, *(e)* blue light, and (1) UV light in the range of 290-400 nm. After the light treatments the plants were placed back into constant dark, and leaf material was collected before the light pulse (DC dark control), immediately after the light treatments and then every 4 hr for 48 hr. Total RNA was extracted, and Northern blots were performed using a *Cat3* gene-specific probe. Filters were reprobed with a 18S ribosomal DNA probe as loading control and a maize *cabi* probe as control of a photosynthetic gene circadian rhythm. The light and dark periods, as well as the time points, are indicated by bars and time in hours above the Northern blots.

been removed, using appropriate filters, can rapidly induce *Cat3* mRNA accumulation that reaches a maximum 4-8 hours after the pulse. The *Cat3* mRNA decreases thereafter, reaches a minimum 12-16 hours after the pulse, and then peaks again 24-28 hours after the pulse. These results indicate that the *Cat3* circadian expression is regulated by a very low fluence phytochrome response and/or a blue-light/UV-A and a UV-B photoreceptor (Boldt and Scandalios 1995, 1997).

The *Cat3* mRNA damps to constant expression levels when 12h D/12h L-growing plants are transferred to continuous light or darkness. Damping of the rhythm occurs rapidly, and slight oscillations are detected 3 days after transfer to constant conditions (Abler and Scandalios 1994), in contrast to the observed robust oscillations of the *Arabidopsis CAT2* gene, which persist for 5 days after transfer to constant conditions (Zhong et al. 1994). Because the *Cat3* gene is continually transcribed in the absence of a cycling light regime, the normally observed diurnal variation of *Cat3* expression has been proposed to be the result of a circadian-regulated transcriptional repressor (Acevedo et al.
1991).

Studies with other plant genes have fortified the assumption that light-responsive and circadian-responsive sequences are located in the 5 '-flanking regions of structural genes (Nagy et al. 1988; HerreraEstrella and Simpson 1990; Millar and Kay 1991; Borello et al. 1993; Anderson and Kay 1995; Carre and Kay 1995; Teakle and Kay 1995). The complete genomic sequence of the maize *Cat3* gene has been isolated and characterized (Abler and Scandalios 1993). Recent reports and knowledge on light and circadian regulation of more plant genes has prompted us to reexamine the *Cat3* ***5*** 'upstream region for the presence of regulatory motifs. Several transcription factor-binding sites were recognized and, surprisingly enough, the presence of a transposable element in the *Cat3* promoter was identified. The transposable element located at -171 belongs to a large family of small inverted repeat elements designated *"Tourist"* (Bureau and Wessler 1992), first described in the wx-B2 mutation of maize. Members of this family of elements are highly repetitive in genomes of some and perhaps all members of the grasses, are associated with insertion mutations, and are also found in the introns and flanking sequences of wild-type genes (Bureau and Wessler 1994). Of all *Tourist* elements reported in S '-flanking sequences of wild-type genes to date, only the one found in the *Cat3* 5'-flanking region is proximal to the promoter. Moreover, the sequence of this element, termed *Zml3,* revealed very interesting motifs that are not present in other members of the *Tourist* family. The terminal inverted repeats of the element are two oppositely oriented core motifs of the 5V40 enhancer (Hatzopoulos et al. 1988), located in a perfect palindrome with sequence CAATCCAT|ATGGATTG (the vertical axis denotes the symmetry center). The two CAAT boxes of each palindrome are two of the three CCAAT motifs located on the *Cat3* promoter. The third CCAAT motif is also present within the transposable element overlapping a GATA repeat present at -196. This region shares striking similarities with the -111 to

-38 region sequence of the *Arabidopsis CAB2* promoter that has been shown to be sufficient for induction of a luciferase *(luc)* reporter gene by red light in etiolated tobacco plants, as well as for rhythmic expression in green tissue (Anderson et al. 1994). The GATA repeat of the *Arabidopsis CAB2* promoter lies between -55 and -74 and serves as a binding site for the GATA-binding protein CGF-1 that is closely related to the GT-1 transcriptional factor and can play a role in light regulation (Teakle and Kay 1995). In vivo analysis of *CAB2::luc* fusion constructs in transgenic *Arabidopsis* demonstrated that a circadian-regulated element lies within a 36-bp sequence immediately upstream of the GATA repeat that overlaps a conserved CCAAT box and contains binding sites for three putative transcription factors (Carre and Kay 1995). On the basis of the evidence provided from the described analyses of the *Arabidopsis CAB2* gene (probably the most well characterized circadian-regulated plant gene at the molecular level), it is likely that the *Zml3* transposable element present in the *Cat3* promoter is a perfect candidate regulatory sequence directing the light-inducible and circadian expression of the gene. If that is true, we may be able to identify maize genotypes lacking the transposable element in the promoter of the *Cat3* gene and, thus, not exhibiting a circadian rhythm. Alternatively, in genotypes exhibiting circadian regulation of the *Cat3,* we shou~d be able to identify the *Zml3* element in the *Cat3* promoter. Actually, limited data (obtained after analysis of three different genotypes) indicated that genetic background and allelic variation do not directly affect the temporal pattern of *Cat3* accumulation in leaves (Acevedo et al. 1991). It would be very interesting to examine these genotypes for presence of the *Zml3* element in the promoter of the *Cat3* gene. Certainly the most efficient way to provide evidence for the regulatory role of the element is to use transcriptional fusions with a reporter gene and to examine the pattern of the reporter's expression in transient assays and/or transgenic plants. Such approaches are currently pursued in our laboratory to obtain thorough insights into the molecular basis of the maize *Cat3* circadian regulation of expression.

STRUCTURE AND CHARACTERIZATION OF THE THREE MAIZE CATALASE GENES

An essential element in any effort to understand the underlying mechanisms by which the *Cat* genes perceive signals and effect a response is the understanding of the architecture of each gene and its regulatory components. To these ends, the available cDNAs (Scandalios 1992) were effectively used to isolate and characterize the maize *Cat* genes.

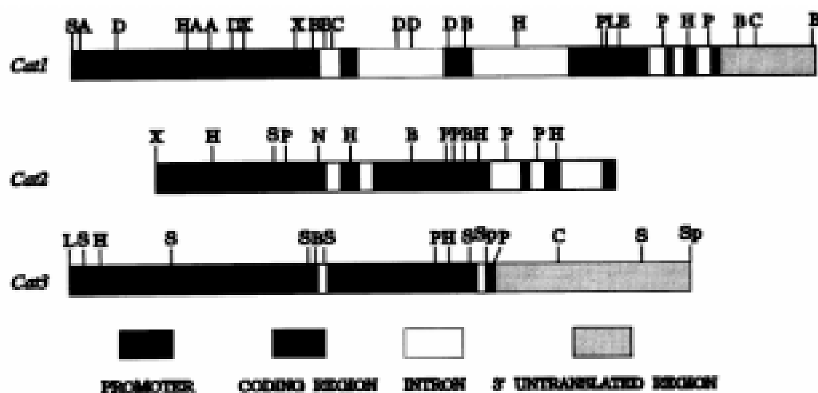


Figure 18 Restriction map of the maize *Cat1, Cat2,* and *Cat3* genes. The map was constructed from clones isolated from a maize W64A genomic library. The black box indicates the DNA fragments that hybridized to the full-length *Cat1, CatZ* or *Cat3* cDNA probe, respectively. The introns, coding region, and 5'-and 3'-flanking regions are indicated. Restriction sites are: (A) *Aval,* (B) BamHI, (C) *Clal,* (D) HindIll, (E) *EcoRI,* (H) HindIl, (N) *NcoI,* (P) *PstI, (S) Sad,* (Sp) *SphI,* (X) *XbaI.*

The *Cat1, Cat2,* and *Cat3* genes of maize have been isolated from W64A and fully characterized (Abler and Scandalios 1993; Guan and Scandalios 1993; Guan et al. 1996). The structural map of each of the three maize catalase genes shows differences and similarities in their structural components (Fig. 18). Each gene has been sequenced, and the coding region is almost identical to its respective cDNA, except the variable introns in each gene. The *Cat1, Cat2,* and *Cat3* genes are interrupted by six, five, and two introns, respectively. Some of the introns in each gene share identical positions (Fig. 18). The third intron of the *Cat1* gene is missing in its relative position in the maize *Cat2* gene and only the first (#1) and the last (#6) introns of the *Cat1* gene are found in the same position of the maize *Cat3* gene. The relevant introns among the three catalase genes share no sequence identity and vary in size. All intron/exon junctions obey the GT/AG rules described in other eukaryotic genes (Shapiro and Senapathy 1987).

The start of transcription for each of the three maize catalase genes was determined by primer extension. The *Cat1* and *Cat3* genes contain promoter sequences of approximately 2.6 kb, whereas the promoter region of the maize *Cat2* gene is only about 1.6 kb. There is no sequence homology among the three maize catalase promoters. The TATA-like sequence is only found in the promoter of the maize *Cat2* gene, but no

CAAT-like consensus sequence was found in the *Cat2* promoter. In con**trast,** CAAT consensus sequences were found in the *Cat1* and *Cat3* gene promoters. No typical TATA-like sequences are present at the usual locations of either the *Cat1* or *Cat3* gene.

Computer searches revealed that the promoter of each *Cat* gene contains a unique set of putative cis-acting elements (Fig. 19), which are important for eukaryotic gene regulation. In the promoter region of the *Cat1* gene, two GC-rich sequences with one nucleotide mismatch relative to the core sequence of the GC box (CCGCCG, GGGCTG) (Dynan and Tjian 1985) were identified. Such sequences serve as binding sites for the protein Spi and have been found in promoters of many viral and cellular genes (Kadonaga et al. 1986). Two 8-bp sequences (CACGTACG, CACGTGGA) which are similar to an ABA response element (ABRE, CACGTGGC) (Guiltinan et al. 1990) were located at -110, -220 relative to the start of transcription (+1) of the *Cat1* gene. An 1 1-bp element (5 '-AGTGACATTGG-3'), located at -470 was identified which is homologous to the antioxidant responsive element (ARE, 5' -puGTGACNNNGC-3') of the rat glutathione S-transferase Ya subunit and rat NAD(P)H:quinone reductase genes (Favreau and Pickett 1991; Rushmore et al. 1991). The ARE might represent a cis-acting element that activates genes that protect eukaryotic cells against oxidative stress. One 1 10-bp fragment from -622 to -512 of 5'-flanking region of the *Cat1* gene is 80% homologous to the first 100 bp of the coding region of the U2 snRNA gene of maize and potato (Brown and Waugh 1989; Waugh et al. 1991). A 26-bp element within this fragment is identical to part of the U2 snRNA gene from both organisms. The major function reported for U2 products is to form the ribonucleoprotein complex for intron splicing (Steitz et al. 1988). The potential function of this fragment in the *Cat1* 5 'region needs to be investigated further. Two direct repeats were identified further 5 'upstream. Two adjacent 32-bp perfect repeat sequences were located between -902 and -871 relative to the +1 site. Another 51-bp direct repeat with a 6-bp mismatch was identified between -530 and -1426.

The possible regulatory roles of ARE, as well as that of other regions or elements of the *Cat1* promoter, for the expression of the gene in the scutellum during germination were examined. Interactions of nuclear proteins isolated from 10 dpi scutella with promoter regions of *Cat1* have been examined by electrophoretic mobility shift assays. Results indicate that a 158-bp fragment containing the ARE core sequence, as well as a 90-bp fragment located 5' and in continuous sequence with the 158-bp fragment containing part of the U2 snRNA sequence, interacts strong-

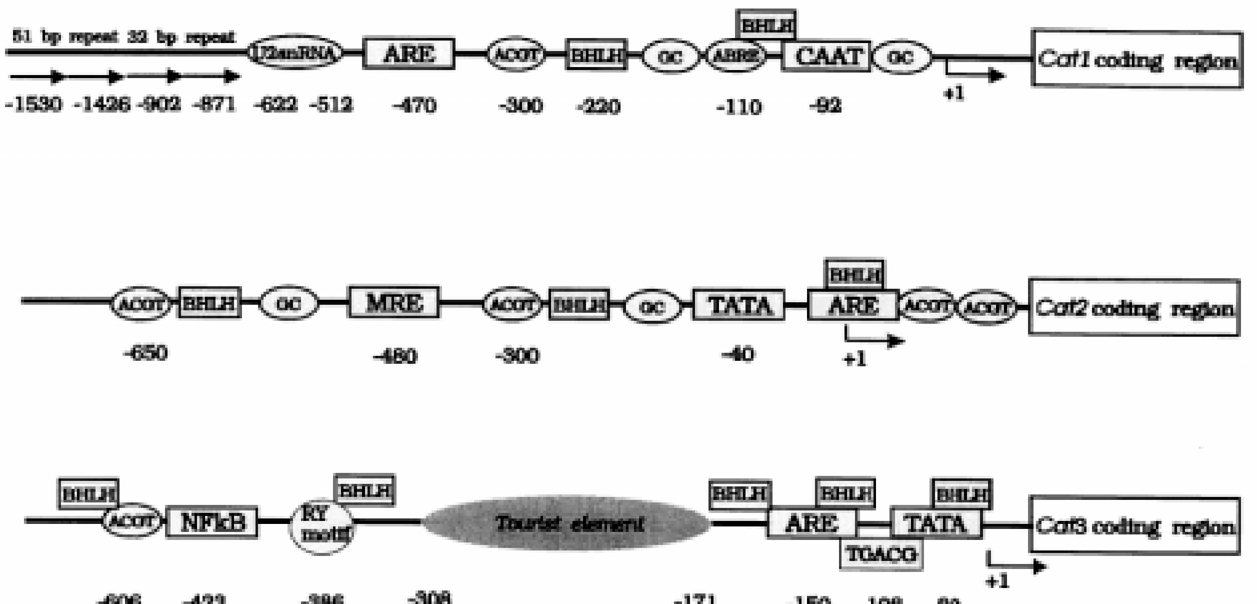


Figure 19 Schematic representation of the motifs located in the promoter region of the maize *Cat1*, *Cat2*, and *Cat3* genes. The location of each motif is relative to the start of transcription (+1 site) of each catalase gene. (BHLH) Basic helix-loop-helix protein binding site; (ARE) antioxidant responsive element; (ACGT) ACGT core or leucine zipper protein-binding site; (GC) GC box or Sp1-binding site; (MRE) metal-responsive element. Refer to text for details.

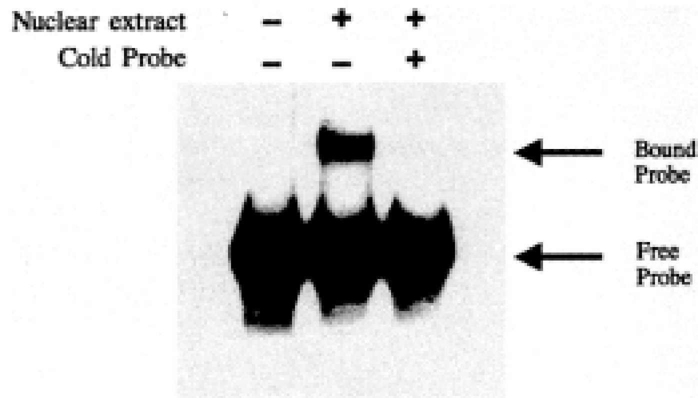


Figure 20 Electrophoretic mobility shift assay probed with a 90-bp *XbaI-Sa~A Cat1* promoter fragment containing the U2 snRNA homologous region. The 90-bp probe (1 ng labeled DNA) in binding buffer containing 100 ng poly(dI-dC) nonspecific competitor was incubated with (lane *1)* no nuclear extract and no specific competitor (-,-) (lane *2)* 0.1 mg nuclear extract and no specific competitor (+,-); and (lane 3) 0.1 mg nuclear extract and 30x cold probe-specific competitor (+,+). A shifted band was detected when the probe was incubated for 20 mm at room temperature with nuclear proteins and disappeared after addition of 30x cold probe in the binding mixture, indicating the specificity of the binding. The Free Probe and Bound Probe bands are indicated by arrows.

ly with nuclear extracts (Fig. 20). Experiments are in progress to examine analogous interactions in early germination and to define their importance in the regulation of *Cat1* expression during germination.

In the promoter region of the *Cat2* gene, two GC box-like sequences (GCGCGG, GGGCAG) were found around nucleotides -114 and -570. Two 8-bp sequences (CACGTCGC, CACGTAAC), which are similar to an ABRE, were located at -308, -648 relative to the +1 site. The core sequence of the ABRE (ACGT) is the binding site for basic leucine zipper transcriptional factors or common plant regulatory factors (CPRFs) (Armstrong et al. 1992). Promoter elements with an ACGT core are recognized by both homo- and heterodimers of leucine zipper transcriptional factors. An 1 1-bp element (GGTGACCTTGC), which is identical to the ARE, PuGTGACNNNGC) of the rat glutathione S-transferase Ya subunit, was identified at a position close to the +1 site. Two direct ACGT repeat sequences, which is the core sequence for leucine zipper protein-binding sites, were located at about 15 bp downstream of the +1 site. A DNA motif, CAGGTG, which is identical to the core sequence for a class of transcriptional factors bHLH (CANNTG, basic helix-loop-

helix proteins), was located near the +1 site and overlapping with the 5' portion of the ARE motif. This transcriptional factor shares similarities with the basic leucine zipper family and can form homo- and heterodimers to exert regulatory functions (Pabo 1992). Two additional bHLHbinding motifs (CATCTG, CATTTG) were also found at -245 and -625 relative to the +1 site. Both of them are located downstream from the two leucine zipper core sequences ACGT. A DNA sequence (CGTCCCGGAACG) was located at -480 with 2-bp mismatch to the 12-bp metal-responsive element (MRE, CGNCCCGGNCNC) core sequence (Stuart et al. 1984). This motif can be found in the promoter region of metal-lothionein proteins that bind heavy metal ions and has been functionally implicated in heavy metal detoxification (Westin and Schaffner 1988).

The promoter of the *Cat3* gene has a unique feature among the three maize catalases. At position -171, a 137-bp *Tourist* transposon element is located (Bureau and Wessler 1994). This element harbors putative *cis*acting motifs. Their significance in the regulation of the *Cat3* gene was discussed above (see Circadian Regulation of Plant Catalases). Putative regulatory motifs have been identified in the *Cat3* S 'upstream region. Among them are motifs identified in the two other catalases as the ACGT core at position -606, the bHLH motif at -16, -136, -180, -236,
-328, -649, -920, and the ARE motif at -148 (with 1-bp mismatch) and
-2404. Several additional putative cis-acting elements were identified on the *Cat3* 5 '-flanking sequence. Two motifs serving as binding sites for light-regulated transcriptional factors, the GT-1 consensus binding motif (G/T)(A[[)GTGPu(A/T)AA(A/T)Pu(A/T) (Green et al. 1988) and the 1-box or GATAA motif (Terzaghi and Cashmore 1995), were found repeatedly in the *Cat3* promoter. GT-1-binding sites are located at -174,
-332, -556, -796, and GATAA motifs at -428, -721, and -842. The binding site GGGPuNNPyPyCC of the mammalian factor NF-KB (Meyer et al. 1993; Poellinger 1995), which is related with oxidative-stress responses, was identified at -423. A TGACG motif that exists in many plant promoters and is related with transcriptional activation of several genes in response to auxin and/or salycilic acid (Terzaghi and Cashmore 1995) is located at -108.

Two motifs with the sequences (C[F)AAC(G/T)G (Myb-like) and ACCTAC(A/C) (H-box) serve as binding sites for the mammalian Myblike transcriptional activators. Myb-like regulatory proteins exist in plants like the P and Cl proteins of maize (Paz-Ares et al. 1987) that control the expression of anthocyanin biosynthetic genes. Myb-like proteins modulate transcription of several genes of the phenylpropanoid pathway in maize, as well as in some other plant species. The binding

sites for Myb-like proteins are present in the *Cat3* S 'upstream region at the positions -992, -1509, -1527, -2424, -2584, and the H-box at
-1988.

The RY motif CATGCATG is present in the 5 'upstream region of a large number of plant genes mostly expressed during seed development (Baumlein et al. 1992). It is also present as the *SphI* element in the 5V40 enhancer (Zenke et al. 1986). Destruction of this motif by short internal deletions nearly abolishes the function of the *Cl* maize gene (Hattori et al. 1992) and the legumin *B4* gene of *Vicia faba* (Baumlein et al. 1992). Strong positive effects have also been reported for glycinin (Lelievre et al. 1992) and b-conglycinin (Chamberland et al. 1992) expression in soybean. In contrast, in *V. faba,* the RY motif of the *usp* gene promoter exerts a silencing effect, since its destruction increases transcription (Fiedler et al. 1993). It has been hypothesized that positive or negative effects of the RY motif in transcription are controlled by the presence of enhancers, or silencer-like elements, upstream of the motif (Baumlein et al. 1992; Fiedler et al. 1993). In addition, the hypothesis is supported by a number of reported unsuccessful attempts to demonstrate binding of nuclear proteins to the RY motif (Riggs et al. 1989; Meaklm and Gatehouse 1991). In *Cat3,* the RY motif is present at -386. In a region about 20 nucleotides upstream of the RY motif, the NF-icB-binding site is located, and about 30 bp downstream, we found a perfect GT-1 box. The functional significance of the above-described putative regulatory sequences in the maize catalase gene promoters are currently under investigation in our laboratory.

MOLECULAR EVOLUTION OF MAIZE CATALASES AND THEIR RELATIONSHIP TO OTHER PLANT CATALASES

Comparison of nucleotide and deduced amino acid sequences indicated that the three maize catalases are not very highly conserved. The maize CAT-1 and CAT-3 proteins are more closely related to other plant catalases than to each other. To better understand the relationship between maize catalases and other plant catalases, available nucleotide and protein sequences were used to examine any apparent evolutionary correlations. We analyzed 16 plant catalase protein sequences. Phylogeny was constructed using the unrooted parsimony method (Felsenstein 1993). We also compared the codon usage of the 16 plant catalases and the gene structures of known plant catalases, especially the intron position within each gene. Structure-function (where available) relationships have also been considered.

Molecular Phylogenies of Plant Catalase Proteins

We utilized the parsimony method to establish the degree of phylogenetic and evolutionary links among the various plant catalase proteins (Guan and Scandalios 1996). The parsimony tree (Fig. 21) required a total of 899 steps. In general, plant catalases can be divided into three groups. The major group includes the monocot maize CAT-1 and most of the dicot catalases. The second group includes the *N. plumbaginifolia* CAT-3 and tomato catalase, and the third group includes the monocotspecific catalases maize CAT-3, rice CAT-A, and barley CAT-2. The monocot-specific catalases (group III) form a single group supported by the bootstrap values (Felsenstein 1985) of 100%. *N. plumbaginifolia* CAT-2 and tomato catalases seem to derive from the same ancestor and are supported by 100% bootstrap values. Within group I, catalases can be separated into two major clades: the monocot and the dicot clades. The soybean and pea catalases appear more closely related to monocot CAT

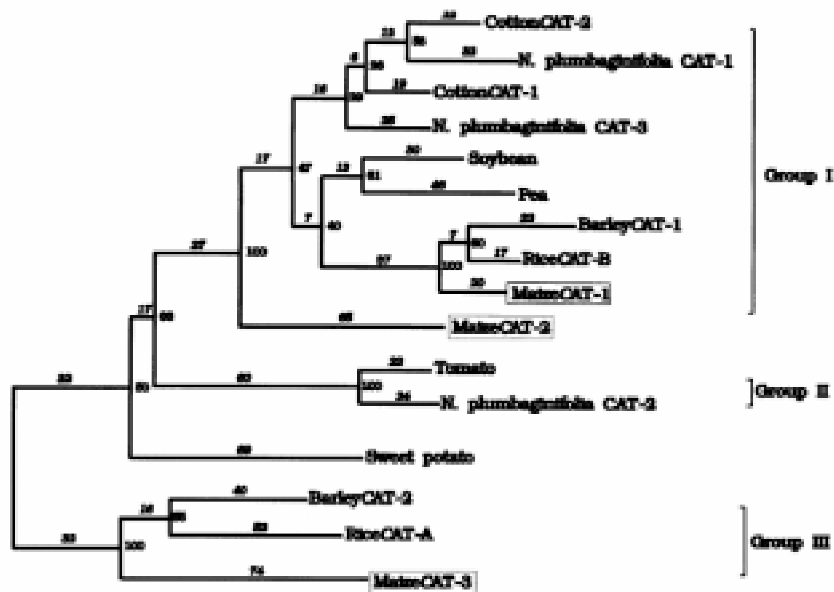


Figure 21 Unrooted phylogenetic tree based on the amino acid sequences of 16 plant catalases constructed by the parsimony method. The maximally parsimonious tree required a total of 899 steps. The numbers at the nodes are the bootstrap values from 100 replicate samples. The three maize catalases are indicated in boxes.

1-like clades than the dicots. The relationship among dicot catalases in group I is not clear because of the low bootstrap value. The cotton CAT-2 and *N. plumbaginifolia* CAT-1 form one dade in both trees, but with low bootstrap values of 53%. The maize CAT-2 is most related to the group I, supported by 100% bootstrap values in the parsimony analysis.

These results clearly indicate that most of the dicot catalases and some monocot catalases are very closely related to each other (group I). Monocot-specific catalases (group III) are closely related, but have low sequence identities with other plant catalases. The sequences which are more like the maize CAT-3 are found only in monocot plants and may derive from either the maize CAT-2 or CAT-1-like sequence after the monocot/dicot separation. The nucleotide sequence alignment among plant catalase cDNAs generated similar sets of results, except that the monocot-specific catalase sequences are mostly related to the *Cat2* of maize rather than the *N. plumbaginifolia Cat1* and the cotton *Cat2,* as determined by the amino acid sequence comparison. This implies that the maize CAT-3-like sequences in monocots may derive from maize CAT-2-like sequences after the separation of monocots and dicots, and the *Cat3* gene maintained the high G + C at the third codon position. These two sets of genes might have been under different evolutionary pressures, causing the divergence in amino acid similarity. To date, the *Cat2*-like sequence has been found only in maize. It is not clear whether this sequence has been lost in other monocot plant genomes, or whether it may just not yet have been identified in other species.

Codon Usage at the Third Nucleotide Position of 16 Plant Catalases

The three maize catalase genes have different nucleotide preferences at the third codon position (Bethards et al. 1987; Redinbaugh et al. 1988). *Cat1* has no codon bias at the third position with a G + C content of about 51%; however, *Cat2* and *Cat3* have extremely high codon biases at the third position with a G + C content of about 95%. Therefore, we examined the G + C content at the third position of all 16 plant catalases (Table 3). Surprisingly, all dicot catalases have less than 50% G + C at the third codon except the sweet potato catalase (65%). Catalases in one monocot dade (including maize *Cat1,* rice *CatB,* and barley *Cat1)* which are close to the dicot catalases have G + C contents ranging from 51% to 59%. The monocot-specific catalases show extremely high G + C content at the third positions (94-96%). These observations correlate well with the results of the phylogenetic trees. Finally, the *Cat2* of maize and sweet

Table 3 The percentage of G+C content at the third codon positions of 16 plant catalase genes

Cat gene	% G+C content	*Cat* gene	% G+C content
Barley2	96	Maizel	51
RiceA	95	Cotton1	48
Maize2	95	Cotton2	45
Maize 3	94	*N. plumbaginifolial*	44
Sweet potato	65	*N. plumbaginifolia2*	44
Barleyl	59	*N. plumbaginifolia3*	47
Soybean	54	Tomato	43
RiceB	52	Pea	43

potato catalase have low sequence identity to other plant catalases and, therefore, were classified separately from the above groupings. These two catalases also show a high codon bias at the third position. These observations are not limited to catalase genes; in fact, higher GC contents at the third codon position are very common in other monocot genes, but not common in dicot genes (Murray et al. 1989; Campbell and Gown 1990). Exactly when and how this event occurred in monocot genes is not known. In unicellular organisms, codon bias reflects the tRNA population and the level of specific gene expression (Ikemura 1985). In rabbit, the muscle-specific glycogen phosphorylase cDNA has a high nucleotide bias for G or C in the third codon position (86%), whereas the human and the rabbit liver-specific transcript does not (60%) (Newgard et al. 1986). Other organisms such as thermophilic bacteria and the protozoan *Leishmania,* which are exposed to such environmental stresses as high temperature and low pH, respectively, have high G + C content in their coding sequence (Bibb et al. 1984; Kagawa et al. 1984). This is presumably due to the stability of the GC base pairs that stabilizes the processes of gene replication, transcription, and translation. Thus, the codon bias of the maize *Cat2* and *Cat3-like* sequences may play a role in the regulation and expression of these genes. In fact, the expression of the three catalases in maize is highly regulated. The *Cat1* of maize is expressed, at low levels, in almost all tissues examined, whereas *Cat2* and *Cat3* are expressed in specific tissues and developmental stages and respond more dramatically to environmental signals. This suggests that CAT-1 may predominantly function as the base-level scavenger of H_20_2 in plant cells. The CAT-2 and CAT-3 of maize are highly regulated and may serve additional and divergent functions (Scandalios 1994).

Intron Position and Evolution

Sequence data indicate that for each of the three maize *Cat* genes the genomic coding region corresponds to the respective cDNA sequence except for the regions interrupted by introns. The 5, promoter regions of the three maize *Cat* genes share no sequence identity, possibly explaining the differential regulation observed for each of these genes. The *Cat1* gene coding sequence is interrupted by six introns, *Cat2* contains five introns, and *Cat3* contains only two introns. The intron positions are a good indicator for analysis of the evolution of these genes. We compared the intron positions among plant catalase genes and the relationship with other known non-plant catalase genes from human (Quan et al. 1986), *Drosophila* (fly), and fungi (Fowler et al. 1993). The position of all plant catalase introns was found to be conserved (Fig. 22). Because of the limited data, we can only speculate that the majority of plant catalase genes may contain six introns. The vast majority of introns in plant genes are found in different positions from the introns in animal genes; however, we found one exception in a plant catalase gene. The castor bean *Cat2* contains an extra intron that shares the same position with the human intron 11. The position of intron 3 of soybean catalase does not match any other plant catalase introns; however, it locates two amino acids downstream from the human intron 9, possibly due to intron sliding (Gilbert and Glynias 1993). The castor bean *Cat1* gene shares the identical intron number and location with the *Cat1* gene of maize. It is also tightly linked to *Cat2* of castor bean, indicating a direct duplication event and subsequent loss of one intron. These data imply that the ancestor of plant catalase genes contained seven or more introns with some sharing similar positions to animal introns. If the same intron location of plant (i.e., castor bean) and animal (i.e., human) is a true evolutionary event, this may provide evidence that plant and animal catalases have derived from a common ancestral form. The fungal catalases share no similar intron positions with any other catalase genes. Together with the observation that most of the plant and animal catalases share no similar intron positions, this may imply that the fungi and most of the plant and animal introns were inserted at later stages following the divergence. The catalase genes tend to lose introns following gene duplication. The *Cat2* of maize lost one intron and *Cat3* of maize lost four introns following several duplication events. The data presented herein support the evolutionary deletion of introns in the *Cat* genes in plants. Recently, three catalase genes from rice were also isolated and characterized (Higo and Higo 1995 and pers. comm.). *Ca~,* the maize *Cat3-like* gene, possesses three introns with identical positions to most of the plant catalases. The

Figure 22 Comparison of intron positions of nine catalase genes from different organisms. The intron positions are located after comparing with the cDNA sequence of the same genes. The nine catalase genes are from maize *(Cat1, Cat2,* and *Cat3),* castor bean *(Cat1* and *Cat2),* soybean, human, *Drosophila* (fly), and fungi *(CatR).* The arrows indicate the positions of the plant introns that are located at or near the same position as the human *Cat* introns.

rice *CatB* and *CatC* have high sequence identity to the maize *Cat1* and possess similar introns and position with the *Cat1* gene of maize. This further supports our observation that in plants, especially in monocots, there is a general trend in the evolution of intron number reduction.

Structural and Functional Relationships among Plant Catalases

The relationship among plant catalases has been established from sequence alignment and phylogenetic analysis. We found that the functions of some plant catalases are also conserved between monocots and dicots. The expression of maize catalases has been extensively studied. *Cat1* is

expressed during the early stages of seed germination and *Cat2* becomes the predominant form of catalase after 3 dpi (Wadsworth and Scandalios 1989; Redinbaugh et al. 1990b; Scandalios 1994). The two cotton catalases have been reported to have similar patterns of expression (Ni and Trelease 1991). *N. plumbaginifolia Cat3* also has a similar pattern of expression to the cotton *Cat1* and the maize catalases (Willekens et al. 1994c). The expression in leaves of the maize *Cat2* gene is light-dependent, and a similar expression was observed for the cotton *Cat2* and *Cat1* of *N. plumbaginifolia,* implying their roles in photorespiration. The maize *Cat3* gene ha~ some unique characteristics. It has enhanced peroxidatic activity, and it copurifies with mitochondria (Scandalios et al. 1980a). *Cat3* is expressed preferentially in dark-grown maize leaves and in stem (Acevedo and Scandalios 1991) and is under circadian regulation (Redinbaugh et al. 1990a; Boldt and Scandalios 1995), as is the CAT-3 of *Arabidopsis* (Zhong and McClung 1996). CAT-2 of barley has a high amino acid identity to CAT-3 of maize and is also expressed in stem (Skadsen et al. 1995).

CONCLUDING REMARKS

Oxygen presents living organisms with a variety of physiological challenges collectively termed "oxidative stress." These challenges are more severe in plants than in other eukaryotes, because plants both consume 02 during respiration and generate 02 during photosynthesis. Catalase, which is present in all aerobic organisms and has efficient enzyme kinetics, plays a crucial role in modulating and/or eliminating H_20_2 from cells.

Unlike animals, all plants examined to date possess multiple enzymatic forms (isozymes) of catalase, as initially demonstrated for maize. Among higher plants, the maize catalases discussed herein have been purified and most extensively characterized genetically, biochemically, and at the molecular level.

The differential regulation and expression of the genetically distinct catalase isozymes during maize development and in response to various exogenous chemical and physical signals provide a clear indication that in addition to scavenging H_20_2, catalases play other important roles. For example, data from several laboratories have indicated that H_20_2 may play an important role in signal transduction; therefore, its modulation by the various catalases within specific cell types or organelles at different developmental stages is likely a critical role of catalases. The eveningspecific expression of CAT-3 in maize and in *Arabidopsis* suggests a

critical role for this catalase in regulating H_2O_2 levels during metabolic activity at night. The modulation of catalase and/or H_2O_2 levels affects a variety of biological processes ranging from responses to various environmental insults, defenses against pathogens, and specific gene expression (see, e.g., Fig. 8) during development.

The isolation and characterization of the three *Cat* genes in maize clearly indicate distinct roles for each of the three catalases, in addition to their common role as H_2O_2 scavengers. As more plant *Cat* genes are similarly isolated and characterized, more meaningful data will be derived as to structure/function relationships among a greater diversity of plant species. On the basis of such comparisons to date, plant catalases can be grouped phylogenetically into three distinct classes (Guan and Scandalios 1996).

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