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Engineering stress tolerance in maize

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A wide range of environmental stresses (such as chilling, ozone, high light, drought, and heat) can damage crop plants, with consequent high annual yield losses. A common factor in all these unrelated adverse conditions, called oxidative stress, is the enhanced production of active oxygen species (AOS) within several subcellular compartments of the plant. AOS can react very rapidly with DNA, lipids and proteins, causing severe cellular damage. Under normal growth conditions, AOS are efficiently scavenged by both enzymatic and non-enzymatic detoxification mechanisms. Nevertheless, during prolonged stress conditions such detoxification systems get saturated and damage occurs. The main players within the defence system are superoxide dismutases, ascorbate peroxidase, and catalases. These enzymes directly eliminate the harmful AOS. By enhancing the levels of these proteins in transgenic plants via transformation technology the improvement of tolerance against oxidative stress is being attempted. In our research, we are generating transgenic maize lines that overproduce various antioxidative stress enzymes and we are assessing the performance of these plants during chilling stress.

Why enhance chilling tolerance in maize?

Maize (*Zea mays* L.) is the most important agricultural crop in Europe and North America. Estimated production worldwide of maize (grain) in 1997 was 62 million tons. In 2005, the production will have increased by 35%, giving a total of 87 million tons.[1] The energy value and high nutritional quality makes maize silage and grain a basic element for animal feeding and human consumption (Figure 1). Within the European Union, 24% of the cereal used for animal feeding is maize. In 1995, maize was grown on 7.5×10^6 ha in Europe. Being such an economically important crop, maize has been the subject of extensive research. The main research programmes focus on the improvement of maize (grain quality and nutritional value) and on resistance to pests and diseases. These research programmes mostly make use of conventional breeding programmes and have improved the quality of maize drastically over the past decades.

Because maize originates from subtropical regions, it is not surprising that it is very sensitive to environmental stresses, such as chilling and freezing. Following expansion of maize-growing areas towards northern climates (northern Europe and northern America), acclimation to chilling conditions became a major research target. Optimal growth conditions for maize are 20–30°C. However, in northern Europe, temperatures of 4°C to 15°C

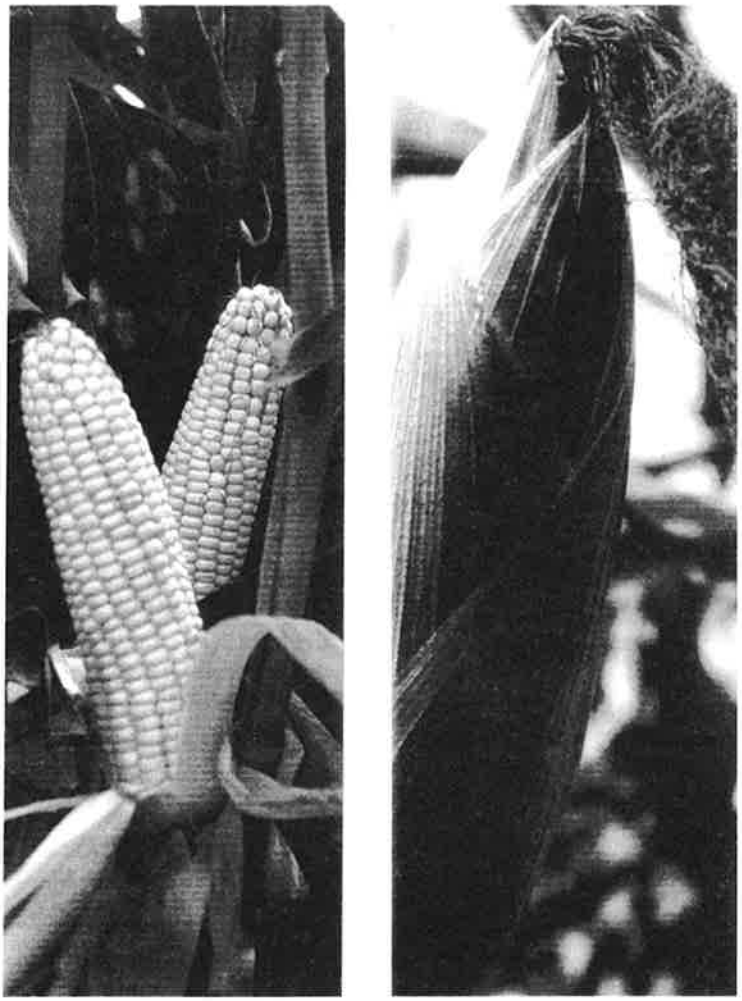

Figure 1. The energetic value and high nutritional quality makes maize silage and grain a basic element for animal feeding and human consumption. Photographs with permission of COOP de PAU.

are not rare in the early growing season. Moreover, the combination of high light intensities and low temperatures, such as those experienced on chilly but sunny mornings in the early growing season, can cause dramatic damage to young maize seedlings.

Low-temperature stress is an important factor for a total area of 1.3×10^6 ha of maize, grown in the northern parts of Europe. The average yearly losses have been estimated to be 18×10^6 ECU. Significant economic losses due to low-temperature stress are encountered in northern France, Belgium, the United Kingdom, Ireland, Germany, Denmark, and The Netherlands. Conventional breeding programmes in conjunction with transformation technologies are now being employed to overcome problems in these areas with sub-optimal growing temperatures.

Four major advantages to the production of chilling-tolerant maize lines are:

(i) improved stress tolerance would allow the production of the same quantity on fewer hectares, thereby reducing production costs;
(ii) maize varieties with a higher tolerance to low-temperature stress might be grown in regions where, until now, no maize or limited areas of maize could be cultivated;
(iii) maize varieties that grow faster in low-temperature conditions are more resistant to parasites, thus considerably reducing chemical treatments; and
(iv) the ability to plant maize in early spring would avoid hot dry summer periods during pollination and fertilization.

As with many agriculturally important traits, chilling resistance is a genetically complex and polygenic system. A limitation of conventional breeding approaches for the improvement of chilling tolerance in commercial hybrids is the use of yield (a genetically complex trait) as the main selection index. In addition, it takes 10 to 15 years for one typical crop improvement cycle.[2]

Genetic engineering can greatly contribute to crop improvement programmes and will accelerate the production of chilling-tolerant lines. The identification of specific genes involved in chilling tolerance, the availability of various methods for the production of transgenic maize, and the development of *in vitro* assays for the rapid measurement of tolerance will save considerable amounts of time on the itinerary towards the production of stress-tolerant maize.

Chilling stress in maize

Domestic plants in cold climates are well adapted to the ambient chilling temperatures. The adaptation to low temperatures in these plant species is directed more to survival than to performance. For crop plants such as maize, which are grown outside their natural environment, performance (harvestable yield) is the only valuable criterion. For maize, a sub-optimal stress could be defined as any reduction in growth or induced metabolic, cellular, or tissue injury that results in limitations to the genetically determined yield potential, caused as a result of exposure to temperatures below the thermal thresholds for optimal biochemical and biophysical activity or morphological development.[3]

Optimal growth of a maize crop occurs in climates with midsummer temperatures between 21°C and 27°C and the optimal temperature for maximum maize grain yields lies around 25°C. Maize plants that are subjected to temperatures below 20°C are believed to undergo physiological and biochemical changes. Of course, the damage will increase according to the duration and severity of the chilling conditions. For example, maize plants grown continuously at 17°C/15°C (day/night temperatures) are seriously retarded in growth. The stressed plants reach the same developmental stage (fully expanded leaf

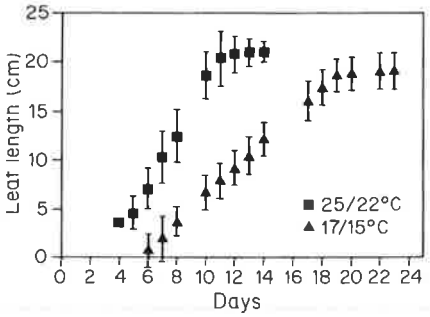

Figure 2. Growth characteristics of maize plants (var. H99) grown at normal and chilling temperatures. Elongation of leaf 2 of maize plants, during continuous growth at normal (25°C day and 22°C night) and chilling (17°C day and 15°C night) temperatures (L. Slooten, personal communication).

2) only ten days later than the non-stressed plants (Figure 2).

Besides the disruption of plasma membranes and decreased activities of metabolic enzymes, photo-oxidative stress is the main cause of damage during chilling stress. In particular, in combination with high light intensities, photo-oxidative stress occurs rapidly in chilling-sensitive species such as maize. This review will mainly focus on oxidative stress and the related plant defence mechanisms during chilling.

Molecular effects of chilling on plants

Chilling-induced alterations in the membrane structures

The plasma membrane is probably the first site of chilling and freezing injury. Phase transition and lateral phase separation of membrane lipids, followed by membrane leakiness, have been proposed as primary molecular events leading to the development of chilling injury symptoms. Most plants react to cold temperatures by changing the relative abundance of unsaturated phospholipids in their membranes. The saturation level of membrane lipids is a determining factor in chilling sensitivity or resistance in higher plants. Low temperatures have an effect on the fluidity of the membranes, and consequently on the activity of membrane-bound enzymes. Higher desaturation levels of membrane lipids compensates for this decreased fluidity. Plants which have higher or lower levels of saturated fatty acids (indigenous or genetically engineered) are more sensitive or resistant to chilling stress, respectively. The changes in membrane composition can be considered as a primary defence (adaptation) mechanism of plants against chilling stress. In transgenic tobacco plants, the chilling sensitivity was significantly enhanced by modulating the saturation levels of membrane fatty acids by overproducing an *Anacystis nidulans* acyl-lipid desaturase.[4]

Another effect on the membranes is the selective peroxidation of unsaturated fatty acids in membrane phospho- and glycolipids. During environmental stress in plants, peroxidation of the membrane fractions is observed. Peroxidation initiates a chain reaction of the production of carbon radicals, which ends in the accumulation of lipid hydroperoxides. Lipid hydroperoxides can react with Fe^{2+} to form hydroxyl radicals. But lipid hydroperoxides can also degrade in aldehydes such as malondialdehyde, and hydrocarbons such as ethane and ethylene. Specifically in plants, chilling-induced oxygen radicals (see below) also provoke the random de-esterification of phospholipids in the cell membrane. This leads to the accumulation and consequent degradation of free saturated and unsaturated fatty acids within the plant cell. Besides the cytotoxic effect, degradation of fatty acids could also serve as a sensing and signalling mechanism to initiate defence responses. The carbon radicals produced, some of the fatty acid degradation products, or even the hydroxyl radicals can serve as signals to switch on a defence response within the cell. In this way membranes can sense a stress situation and quickly produce an amplified signal.[5]

Expression of low-temperature-induced genes

Low temperatures also rapidly induce the expression of nuclear genes. A large array of genes are induced upon growth under suboptimal conditions. In general, they are denominated low-temperature-responsive (LTR) genes.[6] Most of these genes are also induced by other environmental stresses such as drought stress and by the phytohormone abscisic acid (ABA). The LTR genes encode different classes of proteins: anti-freeze proteins, late-embryogenesis-abundant (LEA) proteins, RNA-binding proteins, heat shock proteins, alcohol dehydrogenases, lipid transfer proteins and osmotins. Although the exact function of the LTR genes is not always clear, their function can arbitrarily be divided into two classes: protection of cellular structures from damage caused by dehydration (membranes and proteins) and regulation of water householding. In maize, several genes whose products are involved in the anthocyanin pathway, an alcohol dehydrogenase, a DNA-binding protein, and a calcium-dependent protein kinase, have been identified as LTR genes.[7]

Photo-oxidative stress

The inhibition of photosynthesis is also an early event during chilling stress. Under optimal growth conditions, light energy absorbed by leaves is used primarily for the assimilation of carbon in the process of photosynthesis. When plants experience suboptimal growth temperatures in the field, as is almost always the case during the early growing season in northern Europe, light absorbed by the leaves cannot be used efficiently for photosynthesis, and becomes potentially damaging as the excessive electrons react with the abundantly present oxygen. Together with the electron transport chain of mitochondria, chloroplasts are the main site of AOS production (Figure 3). Electrons passing through the transport chain in the photosystems and mitochondria can react with oxygen to form superoxide radicals ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). In the glyoxisomes, H_2O_2 is produced during fatty acid degradation in the glyoxylate cycle. In the peroxisomes, photorespiration is responsible for H_2O_2 production. Although neither $O_2^{\cdot-}$ nor H_2O_2 seem particularly harmful at physiological concentrations, their toxicity *in vivo* is intensified by a metal ion-dependent conversion into hydroxyl radicals (OH·), one of the most reactive species known in chemistry.

$$O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + 2\ OH\cdot$$

(Haber-Weiss reaction)

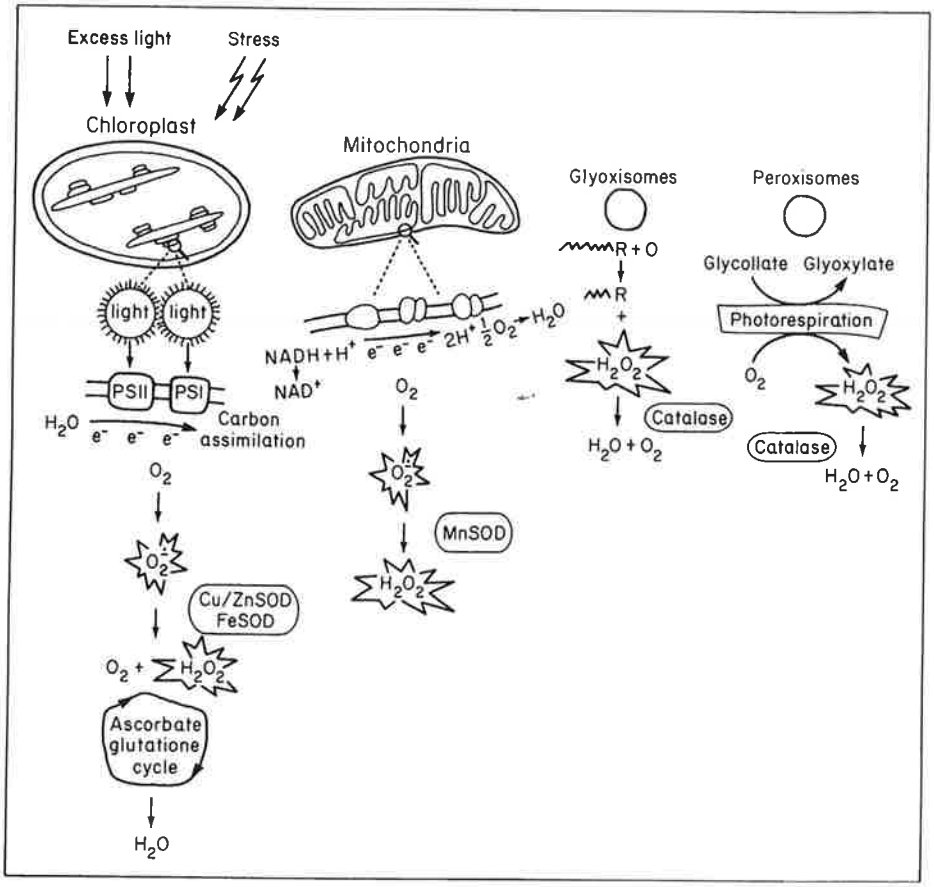

Figure 3. Subcellular production and scavenging of AOS in plants. The electron transport chains of mitochondria and chloroplasts are the main sites of AOS production. In the peroxisomes, H_2O_2 is mainly produced during photorespiration. Fatty acid degradation in the glyoxylate cycle in the glyoxisomes also generates H_2O_2. Abbreviations: PS, photosystem; SOD, superoxide dismutase; ∧∧∧R, β-fatty acids.

Hydroxyl radicals are capable of reacting indiscriminately to cause lipid peroxidation, protein denaturation and DNA mutation.

Fortunately, plants have the capacity to cope with these aggressive agents by eliminating them with an AOS-scavenging system. Under moderate stress conditions, the produced radicals can be efficiently scavenged. During periods of more severe stress, however, the scavenging systems become saturated with the increased rate of radical production. The presence of excessive AOS results in damage to the photosynthetic apparatus (photoinhibition), bleaching of the leaves (by oxidation of the pigments), and hence, severe yield losses (for a complete overview, see Foyer and Mullineaux[8]). AOS are produced in a wide variety of stresses (drought, heat, salt, pollution, chilling, ozone). These, at first sight unrelated, stresses all have in common the electron leakage from electron transport chains in chloroplasts and mitochondria.

AOS are also used in a beneficial way. For example, oxygen radical species are involved in the oxidative burst during the hypersensitive response upon pathogen infection. Furthermore, AOS might also serve as secondary messengers responsible for the induction of pathogen defence-related genes.

In maize, H_2O_2 can act as an inducer of protection against chilling-induced oxidative stress in dark-grown seedlings. H_2O_2 levels rose dramatically in maize seedlings after two to four hours of chilling stress (14°C) and induced several metabolic events (enhanced enzyme activities of a catalase and peroxidase) that led to a subsequent chilling tolerance at 4°C of the (acclimated) seedlings.[9]

AOS scavengers

Plants have evolved non-enzymatic and enzymatic protection mechanisms

Table 1. Non-enzymatic antioxidants in plants.

Antioxidants	Subcellular localization	Main function
Ascorbate (Vitamin C)	Highest concentration in chloroplasts	H_2O_2 removal (via ascorbate peroxidase) Direct destruction of AOS ($O_2^{\cdot-}$, H_2O_2, $OH^{\cdot}$, singlet oxygen) Protection of thiol-modulated enzymes Recycling α-tocopherol Formation of zeaxanthin
Glutathione	Highest concentration in chloroplasts	Recycling ascorbate (via glutathione-ascorbate pathway) Redox balance Protein disulfide reductant Heavy metal detoxification
α-tocopherol (Vitamin E)	Photosynthetic membranes	Direct antioxidant effect Oxidative stress protection via VitE/VitC complex Chain reaction terminator during lipid photo-oxidation Membrane permeability and fluidity
Carotenoids	Chromoplast-chloroplast membranes	Quenching of chlorophyll triplets Accessory pigments for photosynthesis Colouring ABA precursor Energy dissipation (zeaxanthin)

which efficiently scavenge AOS.[10] The most important non-enzymatic antioxidants are ascorbic acid, glutathione, α-tocopherols, and carotenoids. These antioxidants are present in high concentrations within the plant cell. Table 1 summarizes the role of various non-enzymatic antioxidant defence mechanisms.[11]

Hydroxyl radicals are far too reactive to be controlled directly; aerobic organisms prefer to eliminate the less reactive precursor forms (superoxide radical and H_2O_2) as efficiently as possible and prevent their coming into contact with each other by the enzymatic system. Superoxide radicals are scavenged by superoxide dismutase. H_2O_2 is eliminated by catalases and peroxidases.[12] Ascorbate peroxidases are thought to be the most important H_2O_2 scavengers operating both in cytosol and chloroplast (via the ascorbate-glutathione pathway).[13] Other enzymes of the AOS system are monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase

Table 2. Genes or cDNAs, coding for maize AOS defence enzymes.

Enzyme	Gene	Reference
MnSOD	*sod3.1*	Zhu and Scandalios (1994)[a]
	sod3.2	
	sod3.3	
	sod3.4	
FeSOD		F. Van Breusegem (unpublished results)
Cu/ZnSOD (cytosolic)	*sod2*	Cannon *et al* (1987)[b]
	sod4	Kernodle and Scandalios (1996)[c]
	sod4A	
Catalase	*cat1*	Guan and Scandalios (1996), and references therein[d]
	cat2	
	cat3	
Ascorbate peroxidase (cytosolic)		Van Breusegem *et al* (1995)[e]
Glutathione reductase		F. Van Breusegem (unpublished results)

[a] D. Zhu and J.G. Scandalios, 'Differential accumulation of manganese-superoxide dismutase transcripts in maize in response to abscisic acid and high osmoticum', *Plant Physiology*, Vol 106, 1994, pp 173–178.
[b] R.E. Cannon, J.A. White and J.G. Scandalios, 'Cloning of cDNA for maize superoxide dismutase 2 (SOD2)', *Proceedings of the National Academy of Sciences of the USA*, Vol 84, 1987, pp 179–183.
[c] S.P. Kernodle and J.G. Scandalios, 'A comparison of the structure and function of the highly homologous maize antioxidant Cu/Zn superoxide dismutase genes, *Sod4* and *Sod4A*', *Genetics*, Vol 144, 1996, pp 317–328.
[d] L.Q. Guan and J.G. Scandalios, 'Molecular evolution of maise catalases and their relationship to other eukaryotic and prokaryotic catalases', *Journal of Molecular Evolution*, Vol 42, 1996, pp 570–579.
[e] F. Van Breusegem, R. Villarroel, M. Van Montagu and D. Inzé, 'Ascorbate peroxidase cDNA from maize', *Plant Physiology*, Vol 107, 1995, pp 649–650.

Superoxide dismutases

Superoxide dismutases (SOD; superoxide: superoxide oxidoreductase; EC 1.15.1.1: SOD) can be considered as the key enzymes within the antioxidative stress defence mechanism.[14] These enzymes directly determine the cellular concentration of $O_2^{\cdot-}$ and H_2O_2, because they dismutate superoxide into O_2 and H_2O_2.

$$2H^+ + 2O_2^{\cdot-} \xrightarrow{SOD} H_2O_2 + O_2$$

Besides a few exceptions, SODs are present in all aerobic organisms and in all subcellular compartments that have to deal with AOS. They are classified according to their metal co-factor as copper-zinc (Cu/Zn)-, iron (Fe)-, and manganese (Mn)-containing isozymes. Experimentally, these three types can be distinguished by differential sensitivity towards cyanide and H_2O_2.

The subcellular distribution of these isozymes is also distinctive. MnSOD is found in the mitochondria of all eukaryotic cells, including plants, whereas Cu/ZnSODs are found in both the cytosol and chloroplasts of higher plants. FeSODs are present in prokaryotes and in chloroplasts of plants. The amount and relative abundance of the SOD isozymes varies within each organism. Developmental control and environmental stresses which generate the production of AOS (UV, ozone, air pollutants, low temperatures, salt stress, drought, heat shock) are shown to induce plant *SOD* genes.[15] Also maize *SOD* genes are differentially regulated and react specifically to diverse stress factors. Indigenous enhanced SOD levels in maize correlate with more resistant phenotypes. In chilling-tolerant maize lines, higher MnSOD and Cu/ZnSOD activities are present during chilling and post-chilling periods.[16] Total SOD levels are constitutively increased in chilling- and drought-tolerant maize varieties when compared with sensitive ones.[17] These data clearly indicate that by upregulating AOS-scavenging capacities (eg by genetic engineering), higher tolerance can be conferred on crop plants.

In maize, ten different SOD isozymes are known: four cytosolic Cu/ZnSODs, a chloroplast-associated Cu/ZnSOD, four mitochondria-associated MnSODs, and a novel type of chloroplastic FeSOD (see also Table 2). Cu/Zn SODs are dimeric proteins and are located in the chloroplast and cytosol. *Sod2* was the first maize Cu/ZnSOD cDNA to be isolated. Besides the fact that *Sod2* mRNA is present in higher levels in scutella than in leaves of endosperm tissue, little is known on the regulation of this isoform.

Sod4 and *Sod4A* code for two very similar cytosolic Cu/ZnSODs. Only the 3' untranslated part of the cDNAs is completely different. The coding regions share a great degree of homology with *Sod2*. The cytosolic *Sod4* and *Sod4A* transcripts are found in most tissues of the maize plant. Both genes are induced, albeit in different ways, by several stress conditions. The promoters of both genes contain several known regulatory elements (ABA-responsive element, heat shock element, cold stress-responsive element). The corresponding cDNA or gene for another cytosolic Cu/ZnSOD SOD-5 is not yet cloned.

In addition to cytosolic Cu/ZnSOD, many plants have been found to contain an isoform located in the chloroplasts. These enzymes

are nuclear encoded and subsequently targeted to the organelle by an amino-terminal chloroplast transit peptide. In tomato, tobacco, and pea chloroplastic Cu/ZnSOD is developmentally and stress regulated. In maize, the chloroplastic *Cu/Znsod1* cDNA is not yet cloned.

As for the other SODs, several oxidative stress-generating conditions induce MnSOD. MnSODs are nuclear encoded and imported in the mitochondria via a transit peptide. Plant MnSOD cDNAs and/or genes have been isolated from different species. In maize, MnSOD proteins are approximately 90 kDa and are encoded by a multigene family: *Sod3.1*, *Sod3.2*, *Sod3.3*, and *Sod3.4*. All four deduced amino acid sequences have a mitochondrial transit peptide (31 residues) and the first nine amino acids (matrix-targeting sequence) are conserved. The different genes are spatially and developmentally regulated at the transcriptional level. Interestingly, three of the four maize *MnSOD* genes are induced by ABA. The specific mechanism whereby ABA affects expression of the *MnSOD* genes is not known. Probably, ABA induces major metabolic changes, which, in turn, regulate expression of MnSOD.[18]

FeSOD and MnSOD probably have a common ancestor and can be considered as structural homologues. The metal cofactor of MnSODs and FeSODs is generally specific, but is interchangeable within a few prokaryotic organisms. FeSOD is accepted as the oldest SOD because anaerobic prokaryotes have an FeSOD, but no MnSOD. In prokaryotes, eukaryotic algae, protozoa, mosses, and a few dicotyledonous seed plants, FeSOD could be identified. Because of the absence of FeSODs in fungi and animals, and because of the specific sequence similarities between plant and cyanobacterial FeSODs, it is thought that the *Fesod* gene was acquired by plants through endosymbiotic uptake of the *Fesod* gene from the chloroplast ancestor.

In maize leaves, *Fesod* transcripts are induced upon growth at low temperatures (after one day). The induction is most pronounced in younger, still healthy leaves. This observation is in contrast with expression levels of a cytosolic Cu/ZnSOD (*Sod4*) during the same stress regimes. *Sod4* was only induced in older, bleached leaves after several days at chilling temperatures. In these leaves, FeSOD mRNA levels dropped back to undetectable levels.[19] A similar observation is reported by Kernodle and Scandalios.[20] *Sod4* transcript levels increased dramatically in seedlings that were treated with high concentrations of ethephon, which promotes early senescence and wilting of the leaves, or with paraquat. This suggests that *Fesod* is responding early during chilling stress and *Cu/Znsod4* transcripts only accumulate in irreversibly damaged maize leaves.

Catalases

A second class of important AOS scavengers in plants are catalases (CAT; H_2O_2:H_2O_2 oxidoreductase; EC 1.11.1.6). They are found in the peroxisomes, glyoxisomes, and possibly mitochondria of plants. Catalases are able to directly dismutate H_2O_2, or they can oxidize substrates (R), such as methanol, ethanol, formaldehyde, and formic acid.

$$2\,H_2O_2 \rightarrow 2\,H_2O + O_2$$
$$H_2O_2 + RH_2 \rightarrow 2H_2O + R$$

Our laboratory showed that catalases can be divided into three classes. Class 1 is most prominent in photosynthetic cells, where they are involved in the removal of H_2O_2 produced during photorespiration. In maize, relatively low amounts of photorespiration-associated catalase are present. This can be explained by the reduced photorespiratory activity in C_4 plants. Class 2 catalases are highly expressed in vascular tissues. The maize isoform is biochemically different from other plant catalases: it has an enhanced peroxidatic activity and it is possibly localized within the mitochondria. CAT2 might play a role in lignification, but its exact biological role remains unknown. The third class is formed by catalases that are highly abundant in seeds and young plants. Their activity is linked with the removal of excessive H_2O_2, produced during fatty acid degradation in the glyoxylate cycle in glyoxisomes. Because catalase isozymes are rapidly induced by UV-B and ozone, they may also play a direct role in stress protection.[21]

Ascorbate peroxidase

Peroxidases are ubiquitous enzymes found in plants. Besides the peroxidases, whose oxidation products

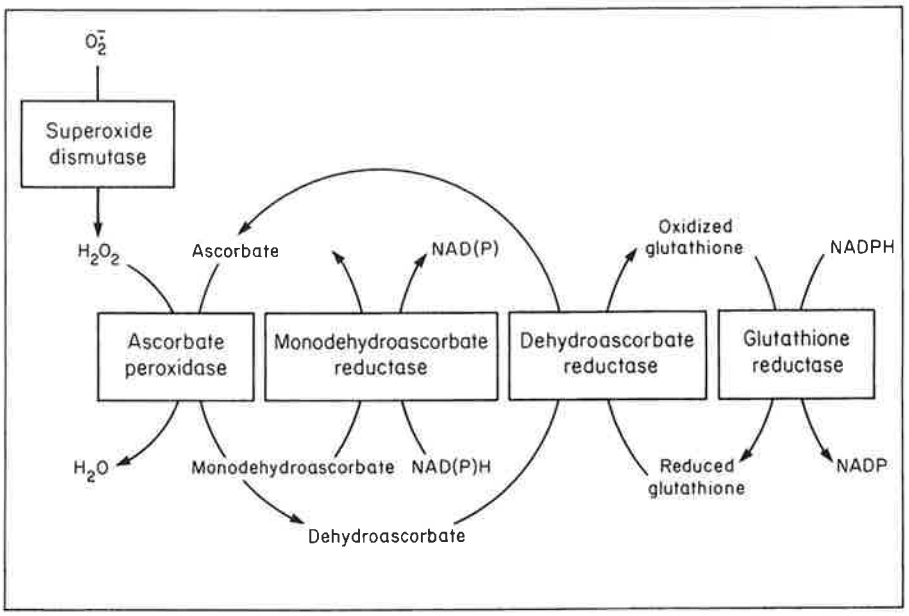

Figure 4. The ascorbate–glutathione cycle. Superoxide radicals are dismutated by superoxide dismutases. H_2O_2 is removed by APx and ascorbate is regenerated by the ascorbate–glutathione cycle. Ascorbate is oxidized to monodehydroascorbate. Monodehydroascorbate can spontaneously disproportionate into ascorbate and dehydroascorbate. Dehydroascorbate reductase recycles ascorbate using reduced glutathione. Oxidized glutathione is regenerated by glutathione reductase in a NADPH-dependent reaction.

Table 3. Transgenic plants with higher and lower AOS-scavenging enzyme levels.

A. Plants with enhanced AOS-scavenging capacities

Transgenic plant	Target enzyme	Ectopic location	Stress assessment	Tolerance	Reference
Tobacco	Petunia chloroplastic Cu/ZnSOD	chloroplast	ozone	no	1
			paraquat	no	2
	N. plumbaginifolia MnSOD	chloroplast	methyl viologen	less sensitive (light and dark)	3
		mitochondria	ozone	reduction visible injury	4
	A. thaliana FeSOD	chloroplast	methyl viologen	increased tolerance	5
	Pea glutathione reductase	cytosol	methyl viologen	increased tolerance	6
		chloroplast	methyl viologen	increased tolerance	
		cytosol and chloroplasts	ozone	less sensitive	
	Spinach chloroplastic Cu/ZnSOD	chloroplast	methyl viologen	increased tolerance	7
	Pea chloroplastic Cu/ZnSOD	chloroplast	chilling	higher photosynthetic rates	8
			methyl viologen	increased tolerance	
	Pea cytosolic Cu,ZnSOD	cytosol	ozone	partial resistance to foliar necrosis	9
	A. thaliana APx	chloroplast	aminotriazole	complete protection	10
			ozone	no protection	11
	Pea APx	chloroplast	ozone	no protection	12
	E. coli GR × rice Cu/ZnSOD	cytosol	methyl viologen	increased tolerance	13
Tomato	Petunia Cu/ZnSOD	chloroplast	chilling	no	2
			low CO_2	no	
Potato	Tomato cytosolic and chloroplastic Cu/ZnSOD	cytosol and chloroplasts	methyl viologen	less chlorosis and wilting	14
				enhanced root growth	
Alfalfa	*N. plumbaginifolia* MnSOD	chloroplast and mitochondria	acifluorfen	increased tolerance	15, 16
			freezing	faster regrowth	
Poplar	*E. coli* GR	chloroplast	methyl viologen	increased antioxidant capacity	17
Maize	*N. plumbaginifolia* MnSOD	chloroplast	paraquat	increased tolerance	18
			chilling	tendencies for better growth	
	A. thaliana FeSOD	chloroplast	paraquat	increased tolerance	18
			chilling	better growth	

B. Plants with lower scavenging capacities

Transgenic plant	Target enzyme	Effect	Reference
Tobacco	APx	higher susceptibility to ozone injury	19
Tobacco	GR	increased paraquat sensitivity	20
Tobacco	CAT	development of necrosis	21, 22, 23

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have physiological roles (lignification, cross-linking of cell wall matrices, etc), a second class forms part of the AOS defence system. Ascorbate peroxidases (APx; EC 1.11.1.11) destroy harmful H_2O_2 (mostly produced by the dismutation of superoxide) via the ascorbate–glutathione pathway in chloroplasts of plants (Figure 4).

The ascorbate–glutathione pathway provides protection against oxidative stress by a series of coupled redox reactions. APx removes deleterious H_2O_2 via the following reaction:

$$2 \text{ ascorbate} + H_2O_2 \xrightarrow{APx} 2 \text{ monodehydroascorbate} + 2\ H_2O$$

There are at least four types of APx in plants: a cytosolic, a chloroplastic stromal, a thylakoid membrane-bound, and a glyoxysomal APx. The different isoforms share the same molecular and enzymatic properties, but they differ in molecular weight, pH optimum and stability.[22] APx proteins and corresponding cDNAs have been characterized from *A. thaliana*, pea, spinach, tea and maize. In pea and radish, APx activities are induced by salt and drought stress. Winter acclimated pine needles contain up to 65-fold more APx than summer needles. Transgenic tobacco plants overproducing cytosolic APx in the chloroplasts show enhanced resistance towards oxidative stress.[23] Transgenic plants, expressing antisense RNA with reduced APx

activity, display increased ozone injury following even low levels of ozone exposure (Table 3). Until now, only two different cytosolic APxs have been characterized in maize. No detailed expression data are yet available.[24]

Glutathione reductase

Glutathione reductase (GR; EC 1.6.4.2) has an essential role in the ascorbate-glutathione pathway. GR recycles oxidized glutathione (γ-glutamyl-cysteinyl-glycine; GSSG) to the reduced (GSH) form, in a NADPH-dependent reaction (Figure 4). Besides the major role of GSH in sulphur transport and detoxifying xenobiotics, the ratio of GSH/GSSG acts as a redox balance within the cell. It is thought that this redox balance serves as a sensing element for oxidative stress.

GR has been characterized in a wide variety of organisms. In plants, GR is found in three subcellular compartments: the cytosol, mitochondria, and chloroplasts. Within the chloroplasts, GR is mainly located in the stroma. GR isoforms are induced by several oxidative stress-associated conditions (chilling, salt stress, heavy metals, paraquat, flooding).[25] Transgenic tobacco with decreased activity of GR exhibited enhanced sensitivity to the AOS-producing herbicide, paraquat (Table 3), indicating an important role for GR within the AOS-scavenging system. In maize, GR has mainly been studied in correlation with drought and chilling stress. Chilling-tolerant maize lines possess higher levels of GR.[26]

Transgenic plants overproducing AOS scavengers

Because of the involvement of AOS in a wide variety of environmental stresses, the defence enzymes are interesting molecular targets for the production of new plant varieties that can cope with these stresses. Several antioxidative stress enzymes have been genetically engineered into plants to assess their potential capacities for enhancing oxidative stress tolerance (Table 3). The beneficial effects observed in some of these transgenic plants could lead to interesting agronomic applications. The tolerance towards oxidative stress is often *in vitro* tested with methyl viologen (paraquat). This herbicide strongly enhances the formation of superoxide radicals and is a fairly good mimic of the process of superoxide formation as it occurs *in vivo* in illuminated chloroplasts.

The observed tolerance in laboratory conditions sometimes correlates with enhanced stress tolerance in field conditions. Overexpression of *Nicotiana plumbaginifolia MnSOD* in alfalfa confers resistance to freezing stress, whereas tobacco plants overexpressing the same construct, confer visible ozone tolerance. Underproduction of antioxidative stress enzymes leads to increased sensitivity towards the experienced stress (Table 3). Tobacco plants with decreased APx or GR activity, obtained via antisense technology have enhanced susceptibility to ozone stress and paraquat, respectively (Table 3). These experiments provide further evidence that overproduction of AOS scavengers by genetic engineering is a valuable strategy to make plants less sensitive to several environmental stress situations.

Stable transformation of maize

Most dicotyledonous species are susceptible to *Agrobacterium tumefaciens* infection and have been routinely transformed for several years. Monocotyledonous species have a strong recalcitrant behaviour towards *Agrobacterium tumefaciens* infection. Only recently, research groups reported on successful transformation of monocotyledonous plants (maize and rice). Until now, direct gene transfer has been the most frequently used method for the transformation of maize. Particle gun bombardment and electroporation are the most popular methods.[27]

Direct gene transfer

Monocotyledonous plants are genetically transformed by direct uptake of DNA into regenerable protoplasts or into intact tissue, which is cultured *in vitro*. In the past, the most used gene transfer methods were particle bombardment, electroporation, and polyethylene glycol-mediated uptake. With a particle gun, microparticles (gold or tungsten) coated with DNA are bombarded into the plant cell. In electroporation experiments, a high voltage is briefly applied to the plant tissue. The high current pulse allows DNA to pass through the cell walls before integrating into the genome.

With these methods it is possible to transform various cell types, becoming independent from protoplast or cell culture systems. With both transformation systems, DNA can also be delivered into intact plant cells. Now, all major cereals can be genetically transformed using particle bombardment. A disadvantage of the direct transfer methods is that the introduced DNA often undergoes rearrangements and that multiple copies are integrated into the genome. The presence of multiple transgene copies often negatively correlates with the expression levels of the transgene. This disadvantage can make the screening for transgenic individuals with high expression levels of the transgene, a laborious job.

Agrobacterium tumefaciens-mediated transformation

The advantages of *A. tumefaciens*-mediated transformation are that segments of DNA can be transferred with little or no rearrangements and that low numbers of transgene copies integrate into the plant chromosome. Despite several reports claiming successful routine transformation with *A. tumefaciens*, it is still not a commonly used transformation method for maize.[28] Several independent factors seem to influence the efficiency of transformation: the type of vector, the developmental stage of the tissue, the *A. tumefaciens* strain, the maize genotype, etc. Better knowledge of the molecular base of plant regeneration might bring solutions for this problem and for the burden of empirical approaches.

Engineering stress tolerance in maize

One of the research projects within our laboratory focuses on the role of SODs and APx in maize during chilling stress. The working hypothesis is that maize lines with an improved scavenging system for oxygen radicals will be better protected against chilling stress. To this end, the fundamental insights into the molecular biology of the oxidative

stress defence systems explored in model plants, such as tobacco and *Arabidopsis* (see also Table 3) are applied to a more economically important crop, maize. Target enzymes for transformation were: MnSOD (*N. plumbaginifolia*), FeSOD (*A. thaliana* and Z. *mays*), and APx (*Z. mays*).

By particle bombardment, transgenic maize has been generated that overproduces a *N. plumbaginifolia* MnSOD cDNA fused to a chloroplast transit peptide from pea under control of the cauliflower mosaic virus 35S promoter. The recombinant MnSOD was correctly targeted to chloroplasts and its enzymatic activity could be distinguished on SOD activity gels. One transgenic line showed enhanced tolerance to the herbicide paraquat. The growth characteristics of transgenic maize lines were followed during growth at ambient and chilling temperatures (25/22°C and 17/15°C). Although the transgenic lines in all experiments had a growth advantage compared to the wild-type lines, no statistically significant increase in growth could be observed.

To extrapolate the *in vitro* oxidative stress tolerance to improved growth effects during environmental stress conditions, such as chilling, we initiated field trials at different locations in Europe in order to scale up the experiment and to check the behaviour of the transgenic plants under natural environmental stress conditions. Immunolocalization experiments revealed that in transgenic maize, the recombinant MnSOD is mainly (although not exclusively) located in the chloroplasts of the bundle sheath cells. The low levels of MnSOD in the mesophyll cells could be attributed to a different expression capacity of the CaMV 35S promoter, but post-transcriptional or post-translational regulation cannot be excluded. Run-off experiments as well as *in situ* hybridizations could answer this question.

Wilson *et al*[29] have also shown the vascular-specific expression of β-glucuronidase activity in leaves of maize plants transformed with a CaMV 35S-β-glucuronidase fusion. In maize, a clear partitioning of the AOS defence system between the mesophyll and bundle sheath cells is present.[30] SOD and APx activities are restricted to the bundle sheath cells, whereas GR and dehydroascorbate reductase activities could only be detected in the mesophyll tissue. This differential localization correlates with the need for NADPH of the respective enzymes. Because NADPH is limited in the bundle sheath cells, GR and dehydroascorbate reductase activities are rate limited in this compartment (Figure 4). This differential distribution of antioxidants determines the capacity of maize plants to deal with oxidative stress. By overproducing AOS scavengers specifically within each cell type it could be possible to restore this natural imbalance and, hence, confer a higher tolerance against chilling-associated oxidative stress on maize plants. Exclusive presence of the transgenic MnSOD in the bundle sheath cells will probably have influenced the expected protective effect of MnSOD in the transgenic maize plants during chilling stress.

Preliminary experiments with transgenic maize lines overproducing an *Arabidopsis thaliana* FeSOD in the chloroplast are more encouraging. Transgenic plants suffered less paraquat damage than controls, as indicated by decreased membrane leakiness and by higher photosynthetic activity. Also the transgenic maize plants exhibited a significantly increased growth rate at low temperatures (as estimated from fresh weight and 'summed leaf length' determinations). These and previous results in tobacco suggest that FeSOD is a better candidate enzyme for protection of plants against oxidative stress.

Perspectives

The production of crop plants that can cope with adverse environmental conditions is a very important research objective within the agro-industry. Improved production rates of crops during stress situations, such as drought and chilling, or resistance against pathogen attacks will certainly improve the life quality of the ever-growing world population in the next century. The rationale for the production of chilling-tolerant maize lines is to reduce the yearly losses during the early growing season and to expand the growing areas within northern Europe.

In the production process of chilling-tolerant maize, two main routes can be followed. The enhancement of lipid desaturation leads to a higher fluidity of the plant cell membranes during chilling stress. Ishizaki-Nishizawi *et al*[31] have shown that by overproducing an acyl lipid desaturase from *Anacystis nidulans*, transgenic tobacco plants were better protected against chilling stress. The second route is the study (and eventually modification) of the antioxidants in maize. Besides the production and evaluation of transgenic lines, we are now focusing on a detailed characterization of the AOS-scavenging machinery in maize. The apparent discrepancy in SOD activity between bundle sheath and mesophyll cells could be a major cause for the chilling susceptibility of maize. Enhancing the levels of SOD in the mesophyll cells could lead to chilling-tolerant maize lines.

Recent evidence indicates a dual role for H_2O_2. In maize, it can either serve as a signal molecule to induce AOS-scavenging enzymes during chilling acclimation or, when present at higher levels, to start acting as a destructive molecule again. As in animal systems, H_2O_2 seems to play an important role in the signal transduction cascade of stress responses. Modulation of H_2O_2 levels is, hence, an interesting route to unravel and to improve the oxidative stress signal transduction pathway(s).

The isolation of novel isoforms of AOS enzymes in maize will also lead to better insights into the defence mechanism of maize. The molecular characterization of several chloroplastic APxs in *Arabidopsis thaliana* clearly shows that the AOS-scavenging enzyme families in plants are larger than previously thought. We also characterized a novel chloroplastic FeSOD in maize. This isozyme is located in the chloroplast and is transcriptionally induced during growth in chilling conditions. Until now, it was thought that FeSODs were limited to dicotyledonous plants. With the help of rapid screening methods, such as the different display technique, or by the outcome of several genome and cDNA sequence initiatives, new isoforms of the different AOS-scavenging enzyme families will certainly be discovered. Together with the characterization of the signal transduction pathways involved in oxidative stress, these novel isozymes

will open new opportunities for the engineering of stress tolerance in plants.

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