Protection from oxidative stress in transgenic plants

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Introduction

Reactive oxygen species such as singlet oxygen (\(\cdot{O}_2\)), superoxide radicals (\(O_2^-\)), hydrogen peroxide (\(H_2O_2\)) and hydroxy radicals (\(OH^+\)) are inevitably formed as by-products of aerobic metabolism. The accumulation of these oxides is toxic, because they cause damage to DNA, lipids and proteins [1]. In order to remove reactive oxygen species, plant cells are equipped with protective systems composed of low-molecular-mass antioxidants such as ascorbate, GSH and \(\alpha\)-tocopherol and enzymes such as superoxide dismutases (SOD), catalases and peroxidases.

SODs efficiently dismutate \(O_2^-\) to \(H_2O_2\) and molecular oxygen [2]. Different isoforms of SOD, distinguished by different prosthetic metals in the reaction centre, are localized in mitochondria (Mn-SOD), cytosol (CuZn-SOD), peroxisomes (Mn-SOD or Fe-SOD) and chloroplasts (CuZn-SOD or Fe-SOD) [3]. \(H_2O_2\) produced in peroxisomes and mitochondria is scavenged by catalases [4]. In the cytosol and in chloroplasts, \(H_2O_2\) detoxification is mediated by peroxidases, using specifically ascorbate as reductant [5].

Ascorbate serves also as an antioxidant in many other detoxification reactions [6]. Therefore, it is vitally important to maintain ascorbate in its reduced state. This is achieved by enzymes reducing the products of ascorbate oxidation, namely by monodehydroascorbate radical reductase and dehydroascorbate reductase. Dehydroascorbate reductase restores ascorbate at the expense of oxidizing two molecules of GSH, thereby generating GSSG. The regeneration of GSH is achieved by glutathione reductase (GR) using NADPH as reductant. Thus, GR activity completes the sequence of reactions involved in the recycling of reduced ascorbate (ascorbate-GSH pathway) [7].

It is well documented that unfavourable environmental conditions such as drought stress, high light intensities in combination with low temperatures, air pollutants, etc., can result in an increase of oxidative stress to plants [8]. Strong oxidative threats may overwhelm the protection capacity of antioxidative compounds and enzymes present in plant tissues and, thus, may finally reduce yield in agriculture and decrease vitality of forest trees. Plant cultivars have been identified that confer enhanced tolerance to oxidative stress [9]. The stress-resistant biotypes contained higher activities of protective enzymes than less-resistant cultivars [9]. Consequently, when techniques of molecular biology became available, plants were transformed with native plant or bacterial genes encoding antioxidative enzymes with the aim to improve stress tolerance and to understand the factors regulating the production of antioxidants and activities of antioxidative enzymes.

Abbreviations used: GR, glutathione reductase; SOD, superoxide dismutase.

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Stress compensation in transgenic plants overexpressing protective enzymes

In recent years, transgenic plants overexpressing protective enzymes such as peroxidases [10,11], SOD and GRs have become available from several species (Table 1). To improve the stress resistance of plants, SOD or GR was targeted to different subcellular compartments (Table 1) and the resulting plants were tested by applying various forms of oxidative stress. However, increased stress tolerance was not observed consistently. Therefore, it is important to look more closely at the experimental conditions which were chosen to test stress resistance. In the present survey, we will discuss only those stress factors that were employed by several research groups, i.e. paraquat, ozone, and high light intensities in combination with low temperature.

In transgenic tobacco plants overexpressing GR, Aono et al. [13,14] found an increased resistance towards paraquat. Paraquat is known to divert electrons from photosystem I to molecular oxygen, yielding \( O_2^- \). The protective effect of elevated GR activity disappeared when leaf disks were exposed to paraquat concentrations higher than 0.8 \( \mu \text{M} \) [4].

Foyer et al. [12] reported that GR-overexpressing tobacco plants did not show improved tolerance to paraquat treatment. However, they exposed leaf disks to a concentration of 5 \( \mu \text{M} \) paraquat, which abolished oxygen evolution completely. Still, the redox status of glutathione remained largely reduced in controls as well as in transgenic plants [12]. Therefore, Foyer et al. [12] concluded that the level of GR activity occurring naturally is greater than that required for maximum operation of the ascorbate-GSH pathway.

Aono et al. [13,14] observed that tobacco plants overexpressing GR in the cytosol or the chloroplasts were not more tolerant to ozone (500 p.p.b. for 4 h) than wild-type plants. Also tobacco plants overexpressing SOD did not show increased resistance to ozone than wild-type plants (300 p.p.b. for 6 h) [16]. In contrast, Van Camp et al. [20] reported an increased protection from ozone injury in tobacco plants overexpressing SOD in the chloroplasts or the mitochondria when they were exposed to near-ambient ozone fluctuations of 40–120 p.p.b. with an average dose of 59 p.p.b. for 7 days. The degree of damage, i.e. 25% necrosis, observed on older leaves was reduced 2–4-fold. The reason why SOD activity in chloroplasts or mitochondria mediated ozone tolerance in this study remains obscure. As the initial reactions of ozone take place in the aqueous phase of the cell walls [8], primary protection measures from ozone injury would be expected in this compartment rather than the chloroplasts or mitochondria.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Enzyme overexpressed</th>
<th>Localization</th>
<th>Increase in activity (-fold)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotiana tabacum</td>
<td>GR</td>
<td>Cytosol</td>
<td>2–10</td>
<td>[12]</td>
</tr>
<tr>
<td>N. tabacum</td>
<td>GR</td>
<td>Cytosol</td>
<td>2–4</td>
<td>[13]</td>
</tr>
<tr>
<td>N. tabacum</td>
<td>GR</td>
<td>Chloroplast</td>
<td>3</td>
<td>[14]</td>
</tr>
<tr>
<td>Poplar hybrid</td>
<td>GR</td>
<td>Cytosol</td>
<td>2–10</td>
<td>[15]</td>
</tr>
<tr>
<td>Poplar hybrid</td>
<td>GR</td>
<td>Chloroplast</td>
<td>Up to 100</td>
<td>[15]</td>
</tr>
<tr>
<td>N. tabacum</td>
<td>CuZn-SOD</td>
<td>Chloroplast</td>
<td>Up to 15</td>
<td>[16]</td>
</tr>
<tr>
<td>N. tabacum</td>
<td>CuZn-SOD</td>
<td>Chloroplast</td>
<td>3</td>
<td>[17, 18]</td>
</tr>
<tr>
<td>Nicotiana plumbaginifolia</td>
<td>Mn-SOD</td>
<td>Chloroplast</td>
<td>ND</td>
<td>[19]</td>
</tr>
<tr>
<td>N. plumbaginifolia</td>
<td>Mn-SOD</td>
<td>Mitochondria</td>
<td>ND</td>
<td>[19]</td>
</tr>
<tr>
<td>N. plumbaginifolia</td>
<td>Mn-SOD</td>
<td>Chloroplasts</td>
<td>2–4</td>
<td>[20]</td>
</tr>
<tr>
<td>N. plumbaginifolia</td>
<td>Mn-SOD</td>
<td>Mitochondria</td>
<td>8</td>
<td>[20]</td>
</tr>
<tr>
<td>Lycopersicum esculentum</td>
<td>CuZn-SOD</td>
<td>Chloroplasts</td>
<td>5–20</td>
<td>[21]</td>
</tr>
<tr>
<td>Medicago sativa</td>
<td>Mn-SOD</td>
<td>Chloroplast</td>
<td>~2</td>
<td>[22]</td>
</tr>
<tr>
<td>M. sativa</td>
<td>Mn-SOD</td>
<td>Mitochondria</td>
<td>~2</td>
<td>[22]</td>
</tr>
<tr>
<td>Solanum tuberosum</td>
<td>CuZn-SOD</td>
<td>Cytosol/chloroplast</td>
<td>ND</td>
<td>[23]</td>
</tr>
</tbody>
</table>
When testing plants for stress tolerance, it is important to consider the mode of action of the respective stress factor. Employing paraquat in the light, Bowler et al. [19] observed a higher degree of protection in tobacco overexpressing SOD in chloroplasts than in mitochondria. When leaf disks were incubated with paraquat in darkness, a similar degree of protection was observed in both types of transgenic plants. However, the protective effect was only observed in old, bolting plants [19]. In younger stages of plant development, the increase in SOD activity was much less pronounced in SOD-overexpressing tobacco plants, and their sensitivity to paraquat treatment was even higher than observed with wild-type controls [19].

Chilling temperatures combined with high light intensities have also been used to test stress resistance in transgenic plants overexpressing SOD. Tepperman and Duinsmuir [21] found no protective effect of elevated SOD in transgenic tomato plants. In contrast, Sen Gupta et al. [18] observed a significant protection of photosynthetic rates in tobacco overexpressing SOD, especially in older leaves. An important observation was that ascorbate peroxidase activity increased together with SOD activity [18]. Therefore, Sen Gupta et al. [18] assumed that protection was achieved, because increased \( \text{H}_2\text{O}_2 \) production by SOD under stress conditions was compensated by an adequate increase in ascorbate peroxidase activity. Other enzymes such as dehydroascorbate reductase and GR activities were not affected. In transgenic tobacco overexpressing GR activity, modulations in ascorbate peroxidase activity were not observed [12].

Although the picture as to whether elevated activities of antioxidant enzymes can improve protection is still confused, some common patterns emerge. First of all, the expression of the newly introduced genes is dependent on leaf age and the developmental stage of the plant. Therefore, protection may be confined to specific developmental stages. The available data show improved protection predominately in older leaves in which the antioxidant capacity is normally much lower than in young foliage. The amplitude of the stress factor is also important, as exposure to strong stress pulses caused similar damage in transgenic and wild-type plants, whereas some protection was observed under moderate stress conditions. A balanced increase in other components of the antioxidative system may also be required.

**Stress compensation in transgenic plants overexpressing enzymes of antioxidant synthesis**

Transfer of *Escherichia coli* from anoxic to oxic conditions results in an increase of glutathione contents by one order of magnitude [24]. Exposure of plants to various forms of oxidative stress is often connected with increased cellular levels of low-molecular-mass antioxidants such as ascorbate, glutathione and/or \( \alpha \)-tocopherol [25,26]. It can therefore be assumed that elevated contents of low-molecular-mass antioxidants can increase the capability of plants to withstand oxidative stress.

High cellular levels of antioxidants in plants may be achieved with molecular biology techniques by increasing the activity of key enzymes of antioxidant synthesis. As a first attempt into this direction, glutathione metabolism and stress sensitivity of poplar plants overexpressing bacterial glutathione synthetase (GSH-S) were studied. Although transgenic lines exhibited 15–60-fold higher activities than wild-type controls, total glutathione levels were similar (Table 2). Apparently, glutathione synthesis *in vivo* was limited by the availability of cysteine (M. Strohm, L. Jouanin, K. J. Kunert, C. Pruvost, A.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-type</th>
<th>Transformants</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH-S activity (nkat GSH·mg of protein(^{-1}))</td>
<td>0.023–0.04</td>
<td>0.39–2.14</td>
</tr>
<tr>
<td>Total glutathione (nmol·g of fresh weight(^{-1}))</td>
<td>141–563</td>
<td>146–385</td>
</tr>
<tr>
<td>GSSG (nmol·g of fresh weight(^{-1}))</td>
<td>15.4–44.9</td>
<td>4.7–36.8</td>
</tr>
<tr>
<td>GR activity (nkat·mg of protein(^{-1}))</td>
<td>1.5–3.5</td>
<td>0.2–1.</td>
</tr>
</tbody>
</table>
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Polle, C. H., Foyer and H. Rennenberg, unpublished work). Even when cysteine was supplied in excess amounts, glutathione levels in leaves of the transformants did not exceed wild-type controls by more than a factor of 3–6, because the limiting formation of γ-glutamylcysteine provided an upper limit for the cellular glutathione content (M. Strohm, L. Jouanin, K. J. Kunert, C. Pruvost, A. Polle, C. H. Foyer and H. Rennenberg, unpublished work). Surprisingly, GR activity was reduced in the transformants without affecting the GSH/GSSG ratio of the cells (Table 2). Transgenic poplar lines overexpressing bacterial GR, however, showed increased cellular glutathione contents in the leaves [15]. First fumigations with ozone (200–300 p.p.b for 3 days) of transgenic poplar plants overexpressing GSH synthetase or GR did not provide evidence for an improved protection from oxidative stress, compared with wild-type controls (data not shown).

Conclusions

From the data currently available, we conclude that the physiological window in which improved stress resistance is manifested is rather narrow; as a consequence, enhancement of just one component of the complex antioxidant system is usually insufficient. As a first step, it may be worthwhile to introduce both SOD and GR genes together. This idea is supported by the finding that maize cultivars with elevated SOD and GR activities, obtained by conventional cross-breeding, were more resistant towards oxidative stress than their respective parental lines [27]. Whether it will also be useful to elevate steady-state concentrations of low-molecular-mass antioxidants such as ascorbate, glutathione, or α-tocopherol can presently not be evaluated and requires further research. Given that antioxidative systems are important for plant adaptation to changing and stressful environmental conditions, plants with elevated levels of enzymes or antioxidants may have important implications for future plant breeding, especially in tree species which have a long lifetime in which they must cope with a wide range of adverse conditions.

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Antibody production in transgenic plants
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Introduction
In recent years, methods have been developed for the expression of immunoglobulin-encoding genes in a wide range of heterologous organisms, including non-lymphoid mammalian cells, insect cells, yeast, bacteria and plants. The isolation of antibody-encoding genes from hybridoma cells and their expression in these heterologous hosts has led to a revolution in monoclonal antibody technology, in which recombinant DNA techniques have been used to alter the structure, properties, specificity, function and immunogenicity of antibodies. Developments in recombinant antibody (recAb) engineering and recAb gene expression in heterologous systems have extended so far that it is now possible to express entire immune repertoires on bacteriophage surfaces [1]. Once displayed on phage surfaces, specific antibodies can be isolated and improved by mutation in processes that mimic immune selection. These methodologies by-pass hybridoma technology and even immunization [1].

The relative ease with which specific antibody-encoding genes can be isolated and expressed in heterologous systems has opened up a vast array of applications of recAb technology. As far as plant expression of antibodies (plantibodies) is concerned, these applications can be broadly divided into two categories. Firstly, crop plants may be used for the large-scale production of commercially valuable antibodies, a process known as antibody farming. Secondly, antibodies may be produced in plant cells such that the activity, function or mobility of the cognate antigen may be modified in the expressing cells, a phenomenon called immunomodulation. Both types of applications require that functional, antigen-binding antibodies be able to accumulate in appropriate cellular compartments.

Abbreviations used: MAb, monoclonal antibody; recAb, recombinant antibody; \(V_{H}\), variable heavy chain; \(V_{L}\), variable light chain.
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The production of a recombinant antibody in plants was first described by Hiatt et al. in 1989 [2]. A two-step strategy was adopted in order to produce transgenic tobacco plants synthesizing an intact immunoglobulin derived from the catalytic IgG1 antibody, 6D4. The first step involved production of separate transgenic lines expressing either the \(V_{H}\) heavy chain or the \(V_{L}\) light chain of 6D4. The second step of the strategy involved the sexual crossing of individual plants expressing either heavy- or light-chain cDNA, in order to produce progeny that expressed cDNAs encoding both chains. Upon isolation, the plant-produced 6D4 antibody was found to display antigen-binding activity comparable with that of the parental monoclonal antibody. Tobacco plants expressing both chains accumulated assembled antibody at levels of up to 1% of soluble leaf protein [2,3].

Intact immunoglobulin proteins are formed by the association of at least four polypeptide chains, some of which are post-translationally modified by glycosylation. Accumulation of functional 6D4 antibody in transgenic tobacco plants was found to be dependent upon signal-sequence-mediated translocation of nascent immunoglobulin chains to the endoplasmic reticulum [2,3]. Consequently, the assembled, intact antibody proteins were (by default) secreted into the cell-wall space, the apoplast [3]. Although this subcellular location may be suitable for antibody-farming applications, it will clearly limit the scope of immunomodulation strategies.

A number of small and structurally simple recombinant antibody fragments have been shown to be synthesized in heterologous hosts more readily than intact immunoglobulins [4]. The Fv, formed by the association of two separate polypeptides, the variable heavy chain (\(V_{H}\)) and the variable light chain (\(V_{L}\)) retains the complete antigen-binding site of an intact immunoglobulin. The single-chain Fv (scFv), in which the \(V_{H}\) and \(V_{L}\) domains of an immunoglobulin are linked together by a pep-