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Expression of an evolved engineered variant of a bacterial glycine oxidase leads to glyphosate resistance in alfalfa

A. Nicolia[a,*], N. Ferradini[a], G. Molla[b,c], E. Biagetti[a], L. Pollegioni[b,c], F. Veronesi[a], D. Rosellini[a]

[a] Dipartimento di Scienze Agrarie, Alimentari e Ambientali, University of Perugia, Borgo XX Giugno, 74, 06121 Perugia, Italy
[b] Dipartimento di Biotecnologie e Scienze della Vita, Università degli studi dell'Insubria, via J.H. Dunant 3, 21100 Varese, Italy
[c] Centro Interuniversitario di Ricerca in Biotecnologie Proteiche The Protein Factory, Politecnico di Milano, ICRM CNR Milano and Università degli studi dell'Insubria, via Mancinelli 7, 20131 Milano, Italy

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ABSTRACT

The main strategy for resistance to the herbicide glyphosate in plants is the overexpression of an herbicide insensitive, bacterial 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). A glyphosate resistance strategy based on the ability to degrade the herbicide can be useful to reduce glyphosate phytotoxicity to the crops. Here we present the characterization of glyphosate resistance in transgenic alfalfa (*Medicago sativa* L.) expressing a plant-optimized variant of glycine oxidase (GO) from *Bacillus subtilis*, evolved *in vitro* by a protein engineering approach to efficiently degrade glyphosate. Two constructs were used, one with (GO^{TP+}) and one without (GO^{TP-}) the pea *rbcS* plastid transit peptide. Molecular and biochemical analyses confirmed the stable integration of the transgene and the correct localization of the plastid-imported GO protein. Transgenic alfalfa plants were tested for glyphosate resistance both *in vitro* and *in vivo*. Two GO^{TP+} lines showed moderate resistance to the herbicide in both conditions. Optimization of expression of this GO variant may allow to attain sufficient field resistance to glyphosate herbicides, thus providing a resistance strategy based on herbicide degradation

1. Introduction

Glyphosate (*N*-phosphonomethyl-glycine) is the most used herbicide worldwide. Glyphosate formulations have a broad-spectrum herbicidal activity with minimal human and environmental toxicity. Glyphosate-based herbicides have been used since the 1970s for weed control (in the absence of vegetating crop plants, or *via* directed application avoiding contact with crop plants) or crop desiccation, and for non-agricultural uses. Starting in 1996, transgenic glyphosate resistant crop plants were introduced, allowing over-the-top application of the herbicide to remove emerged weeds without crop damage.

Glyphosate tolerance in crops is presently obtained by overexpression of the bacterial 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS, EC 2.5.1.19) isolated from *Agrobacterium* sp. CP4, or a variant of EPSPS (T102I/P106S double mutant called TIPS) that is not inhibited by glyphosate, as well as a mutated maize EPSPS (Pollegioni et al., 2011). A different mechanism is represented by conversion of glyphosate into *N*-acetylglyphosate (which is not herbicidal and does not inhibit EPSPS) by a glyphosate acetyltransferase from *Bacillus licheniformis* modified by DNA shuffling; it was used to produce canola and soybean plants tolerant to glyphosate (Castle et al., 2004; Siehl et al., 2007). Notably in canola a mechanism based on the co-expression of the *gox* (glyphosate oxidoreductase–GOX) gene from *Ochrobactrum anthropi*, and the CP4 EPSPS, was employed with the benefits of having reduced phytotoxicity of glyphosate thanks to the action of the metabolizing enzyme.

Soil microorganisms can metabolize glyphosate by the cleavage of the carbon—phosphorus bond (yielding phosphate and sarcosine: C–P lyase pathway) or by the oxidative cleavage of the carbon—nitrogen bond on the carboxyl side (yielding aminomethylphosphonic acid – AMPA – and glyoxylate: the AMPA pathway) (Pollegioni et al., 2011). Notably, although no glyphosate degrading enzyme has been identified in plants, high level of AMPA was found in seeds of a glyphosate resistant soybean (by CP4 EPSPS) (Duke et al., 2003; Duke, 2011).

Abbreviations: EPSPS, enolpyruvylshikimate-3-phosphate synthase; GO, glycine oxidase; GOX, glyphosate oxidoreductase; AMPA, aminomethylphosphonic acid.

* Corresponding author at: Department of Plant Breeding, Swedish University of Agricultural Sciences, Box 101, 230 53 Alnarp, Sweden. Tel.: +39 3394610994.
E-mail address: alessandro.nicolia@gmail.com (A. Nicolia).

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Glycine oxidase (GO, EC 1.4.3.19) from *Bacillus subtilis* was able to convert glyphosate into AMPA and glyoxylate, with low efficiency and a reaction mechanism different from that of GOX (Pedotti et al., 2009; Pollegioni et al., 2011). GO is a flavoprotein oxidase consisting of four identical subunits of 42 kDa, each containing one molecule of noncovalently bound FAD (Job et al., 2002a, 2002b). The physiological role of this flavoenzyme in bacteria is the oxidation of glycine in the biosynthesis of the thiazole ring of thiamine. Its structure was solved as free enzyme and in complex with the inhibitor glycolate to 1.8 Å resolution (Settembre et al., 2003; Mörtl et al., 2004): from the structural viewpoint, GO is a member of the glutathione reductase family (subfamily GR_2) of flavoproteins. GO catalyzes the oxidation of short chain D-amino acids and primary or secondary amines (*e.g.* glycine, sarcosine) to yield the corresponding α-keto acids and hydrogen peroxide (Job et al., 2002a, 2002b; Molla et al., 2003).

We used a rational design approach, based on both site-saturation and site-directed mutagenesis, to generate a GO variant more active on glyphosate than on the physiological substrate glycine. The G51S/A54R/H244A GO variant (triple variant) possesses a specific activity of 1.5 U mg^{-1} protein on glyphosate and reaches an up to 210-fold increase in catalytic efficiency on glyphosate and a 15,000-fold increase in the specificity constant (*i.e.* the k_{cat}/K_m ratio between glyphosate and glycine) as compared to the wild-type counterpart (Pedotti et al., 2009). The inspection of the 3D-structure of the GO triple variant showed that the α2–α3 loop (containing two of the mutated residues) assumes a novel conformation and that the newly introduced residue Arg54 stabilizes glyphosate binding while destabilizing glycine positioning in the binding site, thus selectively increasing the catalytic efficiency on the herbicide (Pedotti et al., 2009).

Here we report the production and evaluation of glyphosate resistant plants expressing the evolved GO triple variant. Alfalfa, an important leguminous forage crop studied in the Authors' lab, was used in this proof-of-principle type of work.

2. Materials and methods

2.1. Vector construction

The mutant *go* gene coding for the GO triple variant (G51S/A54R/H244A) described in Pedotti et al. (2009) was codon-optimized for *Medicago truncatula* (L.), a species closely related to alfalfa, and synthesized by GeneArt® GeneArt, Suppl. Seq.). In the expression cassette, provided in the vector pMA-GO^{TP+}, the gene was fused with the pea *rbcS* chloroplast transit peptide (rubisco TP), and placed under the control of the dual-enhancer CaMV35S (d35S) promoter and the nopaline synthase terminator (*nos*T) (GeneBank accession no. KF874823).

The expression cassette (2342 bp) was excised from the vector pMA-GO^{TP+} with *Xma*I (New England Biolabs) and cloned in the binary vector pCAMBIA2300 (CAMBIA, Australia) previously digested with the same enzyme, thus obtaining pCAMBIA2300-GO^{TP+} (including the rubisco TP, 11078 bp) (Suppl. Fig. 1). Clones containing the expression cassette in the desired head-to-head orientation with the selectable marker gene *npt*II, were identified by colony PCR using the primers NosRTFor (5′-AATCCTGTTGCCGGTCTTGC-3′) and VbRBRev (5′-GAAGACGGCTGCACTGAACG-3′); thermal cycling was: 94 °C for 5 min, 30 cycles at 94 °C for 20 s, 65 °C for 20 s and 72 °C for 30 s.

The rubisco TP was removed from the *go* coding sequence by amplifying the gene using the primers 5′-AAAA**CCTCAGC**ATGAAGAGGCATTATG-3′ (introducing a *Bbv*cI site, in bold) and 5′-AAAA**ACTAGT**TCAAATCTGAACAGCC-3′ (introducing a *Spe*I site, in bold), with HF buffer, 0.2 mM dNTP, 0.5 mM primers and 1 U of Phusion HF polymerase (Finnzymes); thermal cycling was: 98 °C for 30 s, 30 cycles at 98 °C for 10 s and 72 °C for 1 min. The PCR product was cloned into the vector pCR2.1 (TOPO TA cloning kit—Life Technologies) and verified by sequencing. After digestion with *Bbv*cI (New England Biolabs) and *Spe*I (New England Biolabs), the expected fragment (1123 bp) was ligated into the pCAMBIA2300-GO^{TP+} digested with the same enzymes, thus obtaining pCAMBIA2300-GO^{TP-} (10907 bp, not shown) (GeneBank accession ID no. KF874824).

2.2. In vitro test for glyphosate sensitivity

To determine glyphosate optimal concentration for *in vitro* selection, leaf explants of the alfalfa genotype RSY1 (Bingham, 1991) were used for somatic embryo regeneration, as described previously (Ferradini et al., 2011), in the presence of 0.01, 0.025, 0.05, 0.1, 0.2, 0.4 or 0.8 mM glyphosate (Micropoli, Italy).

To assess whether GO expression conferred *in vitro* glyphosate resistance, leaf explants from GO^{TP+} and GO^{TP-} PCR-positive plants were subjected to the same regeneration protocol on media containing 0.2 mM glyphosate; controls without glyphosate were performed. In both trials, somatic embryo production was estimated visually after 4 weeks.

2.3. Plant transformation

The binary vectors pCAMBIA2300-GO^{TP+} and pCAMBIA2300-GO^{TP-} were separately electroporated into the *Agrobacterium tumefaciens* strain AGL1. *Agrobacterium* liquid cultures and alfalfa transformations were carried out as described previously (Samac and Temple, 2004; Ferradini et al., 2011), using 30 mg l^{-1} kanamycin (Kan, Sigma) or 0.2 mM glyphosate as selective agents. After co-culture, 400 mg l^{-1} cefotaxime (Micropoli, Italy) was used to control *Agrobacterium* growth. In the transformations with pCAMBIA2300-GO^{TP+}, 370 leaf explants of the genotype RSY1 were used: 310 for transformation (150 and 160 explants under Kan and glyphosate selection, respectively); 20 for positive control (no selection and no transformation); 40 for negative control (no transformation), equally distributed on Kan and glyphosate selection.

In the transformations with pCAMBIA2300-GO^{TP-}, 310 leaf explants were used: 250 for transformation (200 and 50 explants under Kan and glyphosate selection, respectively); 20 for positive control without transformation or selection; 40 for negative control without transformation (20 on Kan and 20 on glyphosate selection).

Mature, green somatic embryos obtained from Kan-selected transformed tissues were picked, trying to avoid duplicated events (*i.e.* one embryo for each leaf explant), and converted into plants, as well as non transgenic embryos from the control plates. Total genomic DNA (gDNA) was extracted from young leaves of non transgenic and putative transgenic plants using the GeneElute Plants Genomic DNA Miniprep Kit (Sigma).

PCR was performed using 30 ng of gDNA with the primer pairs TPFor (5′-GCGGCCTCAAATCCATGACT-3′) + GOscRev (5′-CCAGCCCAAAATCTGTCCAC-3′) and with GOscFor (5′-CCCCATCTGGTGATGTTTGG-3′) + GOscRev, for GO^{TP+} and GO^{TP-} putative transgenic plants, respectively. For the former, thermal cycling was: 94 °C for 5 min and 35 cycles at 94 °C for 20 s, 63 °C for 20 s and 72 °C for 30 s; for the latter: 94 °C for 5 min and 30 cycles at 94 °C for 30 s, 64 °C for 30 s and 72 °C for 1 min. All the PCR reactions were performed using 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM primers, 1 U Taq (Sigma). PCR reactions were analyzed by electrophoresis in 1.2% agarose gel and digital pictures of PCR products were acquired.