Transgenic DNA introgressed into traditional maize landraces in Oaxaca, Mexico

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Concerns have been raised about the potential effects of transgenic introductions on the genetic diversity of crop landraces and wild relatives in areas of crop origin and diversification, as this diversity is considered essential for global food security. Direct effects on non-target species, and the possibility of unintentionally transferring traits of ecological relevance onto landraces and wild relatives have also been sources of concern. The degree of genetic connectivity between industrial crops and their progenitors in landraces and wild relatives is a principal determinant of genetic connectivity. For these reasons, the detection of transgenic DNA in landrace genomes is of critical importance. Here we report the presence of introgressed transgenic DNA constructs in native maize landraces grown in remote mountains in Oaxaca, Mexico, part of the Mesoamerican centre of origin and diversification of this crop.

In October and November 2000 we sampled whole cobs of native, or ‘criollo’, landraces of maize from four standing fields in two locations of the Sierra Norte de Oaxaca in Southern Mexico (samples A1–A3 and B1–B3), more than 20 km from the main mountain-crossing road that connects the cities of Oaxaca and Tuxtepec in the Municipality of Ixtlán. As each kernel results from ovule fertilization by individual pollen grains, each pooled criollo sample represents a composite of ~150–400 pollination events. One additional bulk sample (K1) was obtained from the local stores of the Mexican governmental agency Diconsa (formerly the National Commission for Popular Subsistence), which distributes subsidized food throughout the country. Negative controls were cob samples of blue maize from the Cuzco Valley in Peru (P1) and a 20-seed sample from an historical collection obtained in the Sierra Norte de Oaxaca in 1971 (H1). Positive controls were bulk

![Image](https://example.com/image.png)

**Figure 1** PCR amplification of DNA from the maize-specific alpha zein protein gene (top panel) and the CMV p-35S promoter (centre and bottom panels). The central panel shows amplification protocol I (single amplification); the bottom panel indicates amplification protocol II (nested priming amplification). a–d, Criollo maize samples. Samples A2 (a), A3 (b), B2 (c) and B3 (d) are shown. e, Sample K1 from Diconsa store. f, Negative control P1, from Peru. g, Roundup-Ready maize RR1. h, Bt-maize Bt1. i, Internal negative control for PCR reaction. j, DNA ladder (100 base pairs (bp), 500 bp marker at the top in each panel. Expected size for each fragment is marked on the left.)
tively) and the Diconosa sample (K1; accession number AF434753). We detected the B. thuringiensis toxin gene cry1Ab in one criollo sample (B3) (data not shown). We confirmed all of the PCR results through repeated testing.

We performed inverse PCR (iPCR) to reveal the various genomic contexts in which the CMV construct was embedded in the Oaxacan criollo maize. This method enabled us to sequence unknown DNA regions flanking the known p-35S sequence in each of the samples. For each sample, iPCR yielded 1–4 DNA fragments differing in size. We isolated these fragments from electrophoresis gels and attempted to sequence them individually, yielding sequences in eight cases (GenBank accession numbers AF434754–AF434761; Fig. 2). Sequences adjacent to the CMV p-35S DNA were diverse, suggesting that the promoter was inserted into the criollo genome at multiple loci. When compared with GenBank (BLAST, February 2001), two sequences were similar to synthetic constructs containing regions of the adh1 gene found in transgenic maize currently on the market, such as Novartis Bt11 (Fig. 2, samples A3 and K1). Notably, these two sequences had high homology with each other. Other sequences represented maize-native genomic DNA, including retrotransposon regions, whereas others showed no significant homology with any GenBank sequence (Fig. 2). The diversity of transgenic DNA constructs present in criollo samples suggests the occurrence of multiple introgression events, probably mediated by pollination. In some of these events, the introgressed DNA appeared to have retained its integrity as an unaltered construct (as with adh1 (ref. 10), whereas in others the transgenic DNA construct seemed to have become re-assorted and introduced into different genomic backgrounds, possibly during transformation or recombination.

The apparent predominance of re-assorted sequences obtained in our study might be due to PCR bias for amplification of short fragments, as intact functional constructs are expected to be much longer.

Our results demonstrate that there is a high level of gene flow from industrially produced maize towards populations of progenitor landraces. As our samples originated from remote areas, it is to be expected that more accessible regions will be exposed to higher rates of introgression. Our discovery of a high frequency of transgene insertion into a diversity of genomic contexts indicates that introgression events are relatively common, and that the transgenic DNA constructs are probably maintained in the population from one generation to the next. The diversity of introgressed DNA in landraces is particularly striking given the existence in Mexico of a moratorium on the planting of transgenic maize since 1998. Whether the presence of these transgenes in 2000 is due to loose implementation of this moratorium, or to introgression before 1998 followed by the survival of transgenes in the population, remains to be established. The intentional release of large amounts of commercial transgenic seed into the environment since the mid-1990s represents a unique opportunity to trace the flow of genetic material over biogeographical regions, as well as a major influence on the future genetics of the global food system.

Further study of the impact of the gene flow from commercial hybrids to traditional landraces in the centres of origin and diversity of crop plants needs to be carefully considered with respect to the future of sustainable food production. Long-term studies should establish whether, or for how long, the integrity of the transgenic construct is retained, and whether the relatively low abundance of transgene introgression detected in the 2000 harvest cycle in Oaxaca will increase, decrease, or remain stable over time.

Methods

Extraction and purification of genomic DNA

For each ear (sample), all kernels (152–384 kernels per cob) were ground to a fine powder using a steel miller to obtain a pooled sample. Three hundred seeds were also ground from each of the bulk samples, except for the historical negative control, which consisted of 20 seeds. DNA was extracted with use each sample, microbes were thoroughly washed, soaked in 10% sodium perchlorate for 30 min, rinsed and then autoclaved. Genomic DNA was extracted from 100 mg of the powder as described elsewhere, with an added purification step using a Geneclean I Kit (Bio 101).

Polymerase chain reaction

For protocol I, amplification reactions using 50–100 ng of extracted genomic DNA were carried out in 25 μl containing 1× PCR buffer (Promega), 2.5 mM MgCl2, 0.2 mM of each dNTP, 0.5 μm of each primer and 0.625 U of Platinum Taq Polymerase (GibcoBRL). We used a water negative control to verify that reactions were free of contamination. Amplifications were performed on a PTC-100 thermal cycler (MJ Research) with the following parameters: initial denaturation at 95 °C for 2 min; 40 cycles each with denaturing at 95 °C for 45 s, annealing for 1 min at 60°C for CMV/NOS, respectively, extension at 72°C for 1 min; and a final extension for 5 min at 72°C. For protocol II, where low amplicon yields were obtained, amplification was repeated as in protocol I but with only 20–25 cycles, followed by a re-amplification or nested amplification of a 1:25 dilution of the PCR products in a new reaction mix with partially or wholly nested primers for 10–15 cycles. Primers cm01 (5′-GCCAGGTGCATTTGGAATAAGC-3′) and cm02 (5′-TCTATGATAGAAGGGGTCTGG-3′) were used to detect CMV 35S promoters with protocol I. With protocol II we used nested primer pairs mp3 (5′-TGATCTACCTGCTGATAGAT-3′) and mp4 (5′-GCCAGGTGCATTTGGAATAAGC-3′), followed by a second round of nested PCR with primers cm01 and cm02 (5′-CTCTCGCCGGTAGTTGACAG-3′) to detect the cry1Ab synthetic gene. Primers zp01 (5′-TTCTCCTGACATTGGTTCCT-3′) and zp02 (5′-GATCGATTGGATATGACATG-3′) were used to amplify the maize-specific alpha zein protein 1 gene (zp1) as an external control for the presence of maize DNA and the efficiency of the reaction.

Inverse PCR

Inverse PCR reactions were modified from previously described protocols. Genomic DNA for PCR digestion with EcoRV (Promega), which targets a single digestion site internal to the p-35S. Restriction fragments were self-ligated with T4 DNA Ligase (Promega) (14°C, 18 h), followed by heat inactivation (75°C, 15 min) and phenol extraction. The purified, circularized products were resuspended in 10 μl 1:10 TE (10 mmol l−1 Tris, pH 8.5; 1 mmol l−1 EDTA). PCR amplification was performed using primers designed specifically for the CMV 35S promoter iCMV (5′-AGCTTCTCCTAAA GCAAGTGG-3′), iCMV2 (5′-ATGACACTAGATGGCTGCGATAT-3′) or iCMV3 (5′-GAA GGCTGCGATATGGCTGCGATAT-3′). These primer pairs were designed to amplify outwards of the 35S promoter, downstream and upstream, respectively.

Nucleotide sequencing

All nucleotide sequencing was carried out at the University of California at San Francisco Comprehensive Cancer Center, Genome Analysis Core Facility. All sequences mentioned above are available on the NCBI GenBank server (http://www.ncbi.nlm.nih.gov).

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Kranz anatomy is not essential for terrestrial C₄ plant photosynthesis

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An important adaptation to CO₂-limited photosynthesis in cyanobacteria, algae and plants was development of CO₂-concentrating mechanisms (CCM)¹. Evolution of a CCM occurred many times in flowering plants, beginning at least 15–20 million years ago, in response to atmospheric CO₂ reduction, climate change, geological trends, and evolutionary diversification of species². In plants, this is achieved through a biochemical inorganic carbon pump called C₄ photosynthesis, discovered 35 years ago³. C₄ photosynthesis is advantageous when limitations on carbon acquisition are imposed by high temperature, drought and saline conditions. It has been thought that a specialized leaf anatomy, composed of two distinctive photosynthetic cell types (Kranz anatomy), is required for C₄ photosynthesis⁴. We provide evidence that C₄ photosynthesis can function within a single photosynthetic cell in terrestrial plants. Borszczowia aralocaspica (Chenopodiaceae) has the photosynthetic features of C₄ plants, yet lacks Kranz anatomy. This species accomplishes C₄ photosynthesis through spatial compartmentation of photosynthetic enzymes, and by separation of two types of chloroplasts and other organelles in distinct positions within the chloroplast membrane cytoplasm.

CO₂-concentrating mechanisms (CCM) have evolved to increase the level of CO₂ at the site of fixation by the C₄ photosynthetic pathway via the enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RUBP) carboxylase/oxygenase (Rubisco); these CCMs also negate the counterproductive oxygenase activity of Rubisco. Plants that lack a CCM directly fix atmospheric CO₂ in their photosynthetic cells via Rubisco. They are called C₃ plants⁵ because the initial product of fixation is a three-carbon compound, and they show high rates of photorespiration owing to the oxygenase activity of Rubisco. CCM in terrestrial plants occur via a C₄ dicarboxylic acid pathway; thus they are called C₄ plants. C₄ plants actively take up CO₂ from the atmosphere and concentrate it around Rubisco for assimilation into organic matter. This requires spatial separation of fixation of atmospheric CO₂ (via phosphoenolpyruvate carboxylase) into C₄ acids, and donation of CO₂ from C₃ acids (via C₄ acid decarboxylases) to RUBP carboxylase of the C₃ pathway.

Photosynthesis has been thought to occur in all terrestrial C₄ plants by the cooperative function of two types of photosynthetic tissue: an inner layer called Kranz or bundle sheath cells, and an outer layer of palisade cells⁶,⁷. Whereas the CCM in terrestrial plants occur via a C₄ dicarboxylic acid pathway, cyanobacteria and algae employ different mechanisms⁸. Besides C₂ and C₄ plants, some vascular plants fix atmospheric CO₂ at night through a C₄ pathway and further process the carbon via the C₃ pathway during the day (called crassulacean acid metabolism or CAM)⁹. This results in a temporal separation of the process rather than a spatial separation, such as in Kranz anatomy, as occurs in C₄ plants. The process of photosynthesis in C₂ and CAM plants is achieved within a single photosynthetic cell, without Kranz anatomy.

Our evidence that Kranz anatomy is not essential for C₄ plant photosynthesis in terrestrial species is based on studies with the monotypic genus Borszczowia. Borszczowia aralocaspica Bunge (subfamily Salsoloideae, family Chenopodiaceae) grows in salty depressions of Central Asian semi-deserts. It is a succulent species with unusual chlorenchyma, and its carbon isotope composition is like that of C₄ or obligate CAM plants⁹. The Chenopodiaceae family has the largest number of C₄ species among dicotyledonous plants⁹; it has high diversity in evolution of C₄ photosynthesis, including five variants of Kranz anatomy⁹,¹⁰ and two variants of C₄ biochemistry¹⁰–¹².

Figure 1 shows the leaf anatomy of B. aralocaspica, Salsola laricina and Suaeda heterophylla, all in subfamily Salsoloideae, family Chenopodiaceae. The general leaf anatomy of B. aralocaspica (Fig. 1a), looks similar to the C₄ salisloid type as demonstrated by the C₄ plant S. laricina Pall (Fig. 1b)¹³. S. laricina has a central, main vein surrounded by water storage parenchyma, and Kranz anatomy with distinctive peripheral layers of palisade and Kranz cells. The Kranz cell chloroplasts have grana and accumulate starch, whereas the palisade chloroplasts have reduced grana and lack starch¹⁰.

However, in contrast, B. aralocaspica has only a single layer of unusual palisade-shaped chlorenchyma cells, which are located between the central water storage tissue and the hypodermal cells (Fig. 1a, also see ref. 8). These radially elongated chlorenchyma cells have a large, central vacuole and a layer of peripheral cytoplasm with few chloroplasts in the distal (from the vascular bundle) part of the cell and a high density of cytoplasm with numerous chloroplasts and large mitochondria (the latter observed by electron microscopy) in the proximal position (see arrows in Fig. 1a). Chloroplasts


Supplementary Information accompanies the paper on Nature’s website (http://www.nature.com).