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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[1,2], and the possibility of unintentionally transferring traits of ecological relevance onto landraces and wild relatives have also been sources of concern[3,4]. The degree of genetic connectivity between industrial crops and their progenitors in landraces and wild relatives is a principal determinant of the evolutionary history of crops and agroecosystems throughout the world[5,6]. Recent introductions of transgenic DNA constructs into agricultural fields provide unique markers to measure such connectivity. For these reasons, the detection of transgenic DNA in crop landraces 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[7–9].

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 grain 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 grain samples of Yieldgard *Bacillus thuringiensis* (Bt)-maize (Bt1; Monsanto Corporation) and Roundup-Ready maize (RR1; Monsanto Corporation) obtained from leftover stock for the 2000 planting season in the United States. Using a polymerase chain reaction (PCR)-based approach, we first tested for the presence of a common element in transgenic constructs currently on the market—the 35S promoter (p-35S) from the cauliflower mosaic virus (CMV). The high copy number and widespread use of p-35S in synthetic vectors used to incorporate transgenic DNA during plant transformation make it an ideal marker to detect transgenic constructs[10–12].

We obtained positive PCR amplification using primers specific for p-35S in five of the seven Mexican maize samples tested (Fig. 1). Four criollo samples showed weak albeit clear PCR amplification, whereas the Diconsa sample yielded very strong amplification comparable in intensity to transgenic-positive Bt1 and RR1 controls. The historical negative control (data not shown) and the contemporary sample from Cuzco, Peru, were both invariably negative. Low PCR amplification from landraces was due to low transgenic abundance (that is, a low percentage of kernels in each cob), not to differential efficiency in the reaction, as demonstrated by internal control amplification of the maize-specific alpha zein protein 1 gene (Fig. 1, *zp1*). During the review period of this manuscript, the Mexican Government (National Institute of Ecology, INE, and National Commission of Biodiversity, Conabio) established an independent research effort. Their results, published through official government press releases, confirm the presence of transgenic DNA in landrace genomes in two Mexican states, including Oaxaca. Samples obtained by the Mexican research initiative from sites located near our collection areas in the Sierra Norte de Oaxaca also confirm the relatively low abundance of transgenic DNA in these remote areas. The governmental research effort analysed individual kernels, making it possible for them to quantify abundances in the range of 3–10%. Because we pooled all kernels in each cob, we cannot make such a quantitative statement, although low PCR amplification signal from criollo samples is compatible with abundances in this percentage range.

Using a nested primer system, we were able to amplify the weak bands from all CMV-positive criollo samples (Fig. 1) sufficiently for nucleotide sequencing (GenBank accession numbers AF434747–AF434750), which always showed at least 98% homology with CMV p-35S constructs in commercially used vectors such as pMON273 (GenBank accession number X04879.1) and the K1 sample (accession number AF434746).

Further PCR testing of the same samples showed the presence of the nopaline synthase terminator sequence from *Agrobacterium tumefasciens* (T-NOS) in two of the six criollo samples (A3 and B2; GenBank accession numbers AF434752 and AF434751, respec-

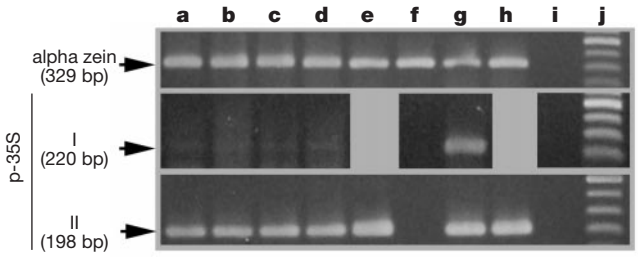

Figure 1 PCR amplification of DNA from the maize-specific alpha zein protein gene (top panel) and the CMV p-35S promoter (centre and bottom panels). The centre panel represents amplification protocol I (single amplification); the bottom panel indicates amplification protocol II (nested priming amplification). **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.

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tively) and the Diconsa sample (K1; accession number AF434753). We detected the *B. thuringiensis* toxin gene *cryIAb* 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[13]. 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. □

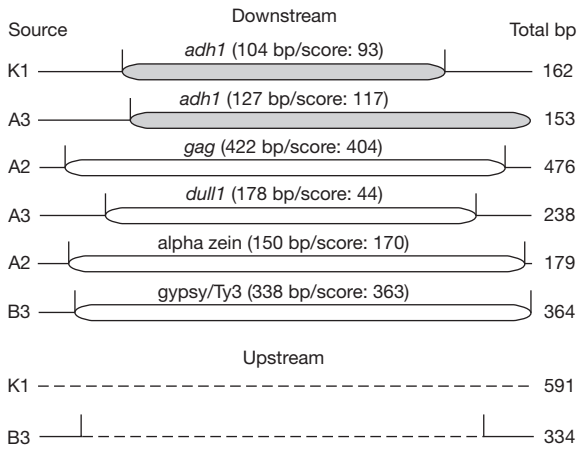

Figure 2 Inverse PCR sequences of flanking regions adjacent to the CMV p-35S in landraces (A2, A3 and B3) and in Diconsa seed (K1). The use of inverse primers results in the appearance of flanking regions as contained within CMV priming sites. For each sequence, best BLAST score is shown in parentheses after the number of nucleotides homologous to the assigned sequence. Vertical lines, end of known CMV sequence; shaded boxes, sequences believed to be derived from transgenic constructs; empty boxes, sequences believed to be native to the maize host; dashed lines, sequences with no homology in GenBank (see Supplementary Information for further details).

Methods

Extraction and purification of genomic DNA

For each cob (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. Before use with each sample, millers 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[10], 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 $MgCl_2$, 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/56 °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 1:250 dilution of the PCR products in a new reaction mix with partially or wholly nested primers for 10–15 cycles. Primers cm01 (5′-CACTACAAATGCCATCAT TGCGATA-3′) and cm02 (5′-CTTATATAGAGGAAGGGTCTTGCGA-3′)[10] were used to detect CMV 35S promoters with protocol I. With protocol II we used nested primer pairs mp3 (5′-TCATCCCTTAC GTCAGTGGAGATAT-3′) and mp4 (5′-GATAAAGGAAAGGCCATCGTTGAAG-3′)[12], and cm02 and mp4. Primers cm01 and cr02 (5′-CTCTCGGCGTAGATTTGGTACA-3′)[10] were used to detect the *cryIAb* synthetic gene. Primers zp01 (5′-TGCTTGCATTGTTCGC TCTCCTAG-3′) and zp02 (5′-GTCGCAGT GACATTGTGGCAT-3′)[10] 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[14,15]. Genomic DNA for iPCR was digested with *Eco*RV (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 iCMV1 (5′-ACGTCTTCAAA GCAAGTGGA-3′), iCMV2 (5′-AGTGACAGATAGGATCGGGAAT-3′) or iCMV3 (5′-GGAGAGGACACGCTGAAATC-3′), iCMV4 (5′-TAGTGGGATTGTGCGTCATC-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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Competing interests statement

The authors declare that they have no competing financial interests.

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Kranz anatomy is not essential for terrestrial C_4 plant photosynthesis

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An important adaptation to CO_2-limited photosynthesis in cyanobacteria, algae and some plants was development of CO_2-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 CO_2 reduction, climate change, geological trends, and evolutionary diversification of species[2]. In plants, this is achieved through a biochemical inorganic carbon pump called C_4 photosynthesis, discovered 35 years ago[3]. C_4 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 C_4 photosynthesis[4]. We provide evidence that C_4 photosynthesis can function within a single photosynthetic cell in terrestrial plants. *Borszczowia aralocaspica* (Chenopodiaceae) has the photosynthetic features of C_4 plants, yet lacks Kranz anatomy. This species accomplishes C_4 photosynthesis through spatial compartmentation of photosynthetic enzymes, and by separation of two types of chloroplasts and other organelles in distinct positions within the chlorenchyma cell cytoplasm.

CO_2-concentrating mechanisms (CCM) have evolved that increase the level of CO_2 at the site of fixation by the C_3 photosynthetic pathway via the enzyme ribulose 1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco); these CCMs also negate the counterproductive oxygenase activity of Rubisco. Plants that lack a CCM directly fix atmospheric CO_2 in their photosynthetic cells via Rubisco. They are called C_3 plants[5] 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 C_4 dicarboxylic acid pathway; thus they are called C_4 plants. C_4 plants actively take up CO_2 from the atmosphere and concentrate it around Rubisco for assimilation into organic matter. This requires spatial separation of fixation of atmospheric CO_2 (via phosphoenolpyruvate carboxylase) into C_4 acids, and donation of CO_2 from C_4 acids (via C_4 acid decarboxylases) to RuBP carboxylase of the C_3 pathway.

Photosynthesis has been thought to occur in all terrestrial C_4 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 cells[4,6]. Whereas the CCM in terrestrial plants occur via a C_4 dicarboxylic acid pathway, cyanobacteria and algae employ different mechanisms[1]. Besides C_3 and C_4 plants, some vascular plants fix atmospheric CO_2 at night through a C_4 pathway and further process the carbon via the C_3 pathway during the day (called crassulacean acid metabolism or CAM)[7]. This results in a temporal separation of the process rather than a spatial separation, such as in Kranz anatomy, as occurs in C_4 plants. The process of photosynthesis in C_3 and CAM plants is achieved within a single photosynthetic cell, without Kranz anatomy.

Our evidence that Kranz anatomy is not essential for C_4 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 C_4 or obligate CAM plants[8]. The Chenopodiaceae family has the largest number of C_4 species among dicotyledonous plants[4]; it has high diversity in evolution of C_4 photosynthesis, including five variants of Kranz anatomy[8,9] and two variants of C_4 biochemistry[10–12].

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 C_4 salsoloid type as demonstrated by the C_4 plant *S. laricina* Pall (Fig. 1b)[10]. *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 starch[10].

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

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