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Transgene behavior in genetically modified teosinte hybrid plants: transcriptome expression, insecticidal protein production and bioactivity against a target insect pest

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Abstract

Background: In 2009, Spanish farmers reported a novel weed, now identified as a relative of maize's ancestor, teosinte, in their maize fields. Introgression of the insect resistance transgene *cry1Ab* from genetically modified (GM) maize into populations of this weedy Spanish teosinte could endow it with additional defense mechanisms. The aims of this study were: (1) to test if hybridization between GM maize and weedy plants from Spain is possible; (2) to understand the relationship between transgene transcription activity, concentrations of the expected transgene product (Cry1Ab protein) and the bioactivity of the latter on target insect pests following transgene flow from GM maize into Spanish teosinte plants.

Results: We demonstrated that hybridization between GM maize and the weedy Spanish teosinte is possible, with no observable barrier to the formation of crop/weed hybrids when teosinte served as pollen donor. When GM maize plants were used as pollen donors, significant crossing incompatibility was observed: hybrid plants produced only few "normal" seeds. Nevertheless, viable F1 seeds from GM pollen crossed onto teosinte were indeed obtained. The *cry1Ab* transgene was stably expressed as mRNA in all crossings and backgrounds. Similarly, toxicity on neonate *Ostrinia nubilalis*, presumably due to Cry1Ab protein, was consistently expressed in teosinte hybrids, with mortality rates 95% or higher after only 4 days exposure, similar to rates on parental GM maize plants. Nevertheless, no strong correlations were observed between transgene transcription levels and Cry1Ab concentrations, nor between Cry1Ab concentrations and insect mortality rates across all of the different genetic backgrounds.

Conclusions: Our results establish fundamental parameters for environmental risk assessments in the European context: first, we show that crop/weed hybridization in fields where maize and teosinte exist sympatrically can lead to potentially catastrophic transfer of resistance traits into an already noxious weed; second, our results question the viability of using gene dosage to model and predict ecological performance in either the intended crop plant or the undesired teosinte weed. Significant questions remain that should be addressed in order to provide a scientific, sound approach to the management of this novel weed.

Keywords: GM maize, Wild relatives, Emergent weed, Hybridization, Transgene flow, mRNA, *cry1Ab*, *Ostrinia nubilalis*, Spain

Background

Teosinte is the common name for a series of related species, including the wild ancestor of maize (*Zea mays* subsp. *mays*), native to Mexico and Central America [1].

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Teosintes are a group of perennial and annual species of the genus *Zea* and contain at least seven taxa divided into two sections and five species: *Zea diploperennis*, *Zea perennis*, *Zea luxurians*, *Zea nicaraguensis* and *Zea mays*. The last species, *Z. mays*, is divided into four subspecies: *Z. mays* subsp. *huehuetenanguensis*, *Z. mays* subsp. *mexicana*, *Z. mays* subsp. *parviglumis* and *Z. mays* subsp. *mays* [2]. The currently most accepted hypothesis is that maize (*Zea mays* subsp. *mays*) was domesticated roughly 9000 years ago from the annual teosinte *Z. mays* subsp. *parviglumis* in southern Mexico [3, 4].

In 2009, farmers in the Spanish Province of Aragón noticed a new, fast-spreading and highly destructive weed in their maize fields. By 2014, this novel noxious weed was also reported in maize fields in Cataluña [5]. Genomic analyses showed the weed to be of admixed origin, most likely involving *Zea mays* ssp. *mexicana* as one parental taxon, and an unidentified cultivated maize variety as the other [6]. We refer to this specific population (a putative taxon) as "Spanish teosinte". Spanish teosinte does not appear to group with any of the currently recognized teosinte taxa, including *Zea mays* ssp. *mexicana* or *Zea mays* ssp. *parviglumis*. Nevertheless, Le Corre et al. [7] added genomic information on teosinte plants that had been reported from French fields already decades ago suggesting that *Zea mays* ssp. *mexicana* is a likely ancestor, and also that the Spanish teosinte differs markedly from any putative Mexican ancestor [7]. Remarkably, teosinte in other locales, including Southern France, does not display the aggressive weedy behavior observed in Spanish teosinte.

Spain is the largest producer of genetically modified (GM) crops in Europe, growing over 90% of the only approved GM crop in the European Union, insect-resistant maize (event MON810) [8]. MON810 maize contains a transgene from the bacterium *Bacillus thuringiensis* (Bt) that enables GM maize plants to express the insecticidal Bt protein Cry1Ab, specifically targeting lepidopteran pest species. In 2017, around 37% of the total maize production area in Spain was GM maize, with most of it being cultivated in Aragón and Cataluña [9, 10], the primary areas impacted by the novel weedy teosinte species. Trtikova et al. [6] found that hybridization does occur between Spanish teosinte and cultivated maize in Spain, but is asymmetric, favoring the introgression of Spanish teosinte into cultivated maize, rather than vice versa. Similarly, Le Corre et al. [7] recently suggested that "elevated levels of genetic introgression" from European Dent maize has contributed to the adaptation of the teosintes in Europe, and presumably their eventual development as weeds there. For the French teosinte, the authors were able to prove gene flow from maize to French teosinte by using a conventionally bred herbicide resistance gene as a marker [7]. The possibility of introgression of the transgenic insecticidal Cry1Ab trait into Spanish teosinte can be expected to take place, which would increase the weediness of the Spanish teosinte population even more through acquisition of an additional defense mechanism against pest species.

Hybridization between maize and teosinte species can occur and has been reported in the past by others [11–14]. However, hybridization success is dependent on the taxa involved and on who serves as pollen donor for the crosses [11, 13]. For example, field hybrids with traits derived from landraces of maize transferred to *Zea luxurians* have been recently documented in southern Brazil [14].

Only a few studies have been carried out to date evaluating the fitness of transgenic vs. non-transgenic crop × wild hybrids in the crop maize system [12]. Studies focused on other crop species, such as sunflower [15], rice [16–18] and oilseed rape [19], have produced varied results, as in some cases the estimated hybrid fitness was decreased when compared with non-hybrid relatives, while it was higher in others. The Spanish teosinte case offers a unique opportunity to study fundamental questions with grave practical agroecological implications in a newly emergent weed/crop system.

The immediate goals of this study were to test if gene flow from GM maize to weedy Spanish teosinte is possible and, if so, to illuminate the relationship between transgene expression rates, concentrations of the transgene product (Bt protein) and the resulting bioactivity on target insect maize pests.

Material and methods

Plant material and crosses

Teosinte seeds were collected in the province of Zaragoza, Autonomous Region of Aragón, Spain, in summer 2015. Verification of the GM-free status of the teosinte seeds prior to production of the crosses was done by means of ImmunoStrip® enzyme-linked immunoassay tests (Agdia®, USA).

The GM variety used in this study (LG30490YG, event MON810) is a simple hybrid cultivated in Spanish fields for production of grain and silage. Genetic analysis shows a single functional copy of the *cry1Ab* transgene sequence in the genome of this variety, making it hemizygous for the *cry1Ab* transgene [20–23].

Crosses by controlled hand-pollination between GM maize and teosinte plants were produced in climate chambers (Kälte 3000 AG, Switzerland) at the ETH Zürich, Switzerland, as described in Fig. 1. The climate chamber conditions were: 12 h light, 25 °C, 50% rh, 12 h dark, 20 °C, 65% rh, with regular watering. Light conditions were: 100% ~ 28 klux ~ 356 PPDF

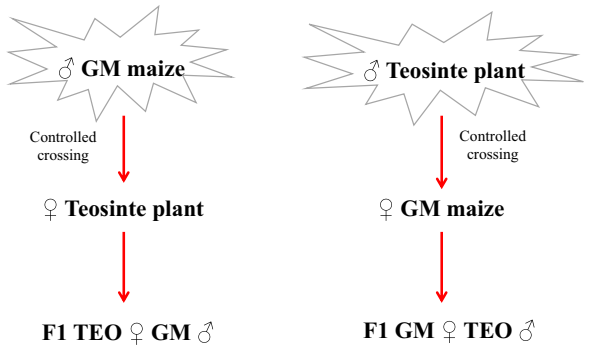

Fig. 1 Diagram indicating how the F1's crosses from Spain were obtained

[µmol m^{-2} s^{-1}] 1 m above plants. The F1 TEO ♀ GM ♂ was generated using GM maize as pollen donor and the teosinte as the female pollen recipient. Vice versa, when generating the F1 GM ♀ TEO ♂ crosses, teosinte was used as pollen donor and the GM maize as the female pollen recipient. The flowering period of the GM maize and teosinte plants, used to produce the respective teosinte hybrid plants, were similar with these climate chamber conditions above mentioned.

F1 populations were generated by growing maize and teosinte plants side by side in the climate chamber and removing immature tassels (detasseling) from female plants of interest to prevent natural fertilization with unwanted pollen. Plants were fertilized but no pesticides or herbicides were used.

Climate chamber conditions and ImmunoStrip® tests

During germination and growth of seedlings, the climate chamber conditions were the same as previously described. GM parent, teosinte and F1's seeds were planted in trays with 24 small pots filled with planting soil (Anzuchterde, Ökohum®, Germany). To prevent Sciaridae flies from laying eggs in the soil, pots were covered with a layer of quartz fine gravel (fire-dried; Carlo Bernasconi AG, Switzerland). Seedlings were qualitatively tested for the presence of Cry1Ab protein using enzyme-linked immunoassay ImmunoStrip® tests (Agdia®, USA) three weeks after planting. Only Bt-positive seedlings were transplanted into bigger pots (KREUWEL KC-Pflanzencontainer; V 4.4 L, Ø 21 cm) filled with potting soil (BioUniversalerde, Ökohum®, Germany). Results of Cry1Ab protein presence/absence tests in different genetic backgrounds are provided in Additional file 7: Table S1.

Experimental setup

All experiments were carried out over staggered time periods, from January to June 2018 in the same climate chamber conditions as mentioned above. The experimental design was a randomized complete block. The experiments were executed in three blocks and with different numbers of seedlings per different genetic background in each block, which ranged from one to six seedlings. In total, the GM parental maize and F1 GM ♀ TEO ♂ crosses had fifteen seedlings each, and the number of observed positive Bt seedlings in F1 TEO ♀ GM ♂ was only six seedlings (Additional file 7: Table S1). The seedlings were arranged randomly in the climate chamber.

Plant sampling

Two weeks after planting, all plants were sampled to determine transgene messenger ribonucleic acid (mRNA) expression, Cry1Ab concentration and insect toxicity via bioassays. At that time, the plants were between stages V5 and V7. The fifth fully developed leaf was sampled in each plant. Six circular leaf samples (Ø 1.5 cm) were cut out with a cork-borer, three on each side of the central part of the leaf but avoiding the main leaf vein (Additional file 1: Figure S1) [24]. Leaf disks assigned for transgene expression analysis were immediately flash-frozen in liquid nitrogen and subsequently stored at − 80 °C. Leaf disks designated for quantification of Cry1Ab concentration were kept on ice at − 20 °C during transport to storage on the same day. Leaf pieces designated for bioassay were used immediately, with feeding trials started on the same day that samples were taken.

Real-time polymerase chain reaction (qRT-PCR) and mRNA evaluation

Ribonucleic acid (RNA) was extracted from 36 leaf samples from the GM maize and teosinte plants (15 samples from GM maize; 15 samples from F1 GM ♀ TEO ♂ and 6 samples from F1 TEO ♀ GM ♂) following the protocol method of the RNA plant NucleoSpin® kit (Macherey-Nagel, Germany). RNA concentration was determined with a Qubit® fluorometer (Invitrogen™, USA) and the quality of extraction checked on an Agilent 2200 TapeStation System. Afterwards, the RNA was treated with deoxyribonucleic acid (DNA) digest buffer (RDD buffer) (Qiagen®, Germany) and deoxyribonuclease (DNAse) (Qiagen®, Germany) and inactivated with ribonuclease (RNase)-free H_2O. All assays were run with a standardized total RNA concentration of 5 µg/ml.

The cDNA synthesis was carried out using the QuantiTect® Reverse Transcription Kit (Qiagen®, Germany), including wipeout buffer and reverse transcriptase (RT) primer mix that contains a specially

optimized mix of oligo-deoxythymine (dT) and random primers that enable cDNA synthesis from all regions of RNA transcripts, even from 5′ regions.

Each plant sample was run in triplicate in a reaction volume of 10 µl using TaqMan® Gene Expression Master Mix (Applied Biosystems®, USA) and 1 µl of cDNA (Additional file 8: Table S2). The instrument used in the analysis was the ABI 7500 FastRT-PCR (7500 Software v2.0) from Applied Biosystems®, USA.

Primers and probe sequences for *cry1Ab* transgene were kindly provided by A. Coll (Institut de Tecnologia Agroalimentària, L'INTEA, Universitat de Girona). Additionally, three reference genes (*mep*, *ubcp*, *lug*), as recommended by Manoli et al. [25], were chosen to normalize the qRT-PCR data. TaqMan primers and probes for reference genes were designed based on the sequences obtained from the Maize Genetics and Genomics Database (http://www.maizegdb.org/) using Primer Express 3.0 software (Applied Biosystems®, USA).

The threshold cycles (Ct) for mRNA transcripts in the samples were calculated by means of Real-time PCR software, with data exported to Microsoft Excel. In cases where the Ct standard deviation for the triplicate group exceeded the default setting of the instrument (0.5), or in the presence of outliers, the samples were used in duplicate. Amplification efficiencies of the crosses were estimated using LinRegPCR software version 2012.3 [26] and the values are presented in Additional file 9: Table S3. The stability of the three reference genes was assessed using geNorm and for normalization ($M < 0.5$ and pairwise variance coefficient < 0.15) of the expression data, using the qbase + software Biogazelle®. The qbase + program facilitates the process of validating reference genes and performing state-of-the-art normalization using the geometric mean of multiple validated reference genes. Results were expressed as relative values, established by qbase + program, and using the comparative Ct method described in Bookout and Mangelsdorf [27].

A two-way ANOVA was performed to evaluate the significance of the effects of different genetic backgrounds and different blocks on transgene expression levels. However, outliers and the interaction between different genetic backgrounds and different blocks were excluded from the analyses.

Enzyme-linked immunosorbent assay (ELISA) and Cry1Ab concentration

Enzyme-linked immunosorbent assay (ELISA) was used to quantify the Cry1Ab concentration in the same leaf samples that were also used for the transgene expression analyses. This allowed simultaneous determination of Bt concentration and transgene activity. Between 5 and 10 mg of freeze-dried leaf material was ground using a FastPrep-24 Instrument (MP Biomedicals, USA) and homogenized in 1.5 ml of phosphate-buffered saline solution (PBST-buffer) (pH 7.4). After centrifugation supernatants were diluted 1:100 with PBST-buffer. Standards were prepared using freeze-dried Cry1Ab toxin (M. Pusztai-Carey, Case Western Reserve University) similar to the Cry1Ab protein expressed in the MON810 maize plants. Twelve Cry1Ab concentrations were used for the calibration curve ranging from 0 to 4.4 ng/ml dissolved in PBST-buffer. Cry1Ab concentrations in the different genetic backgrounds were determined using a commercial double antibody sandwich (DAS) ELISA kit (Agdia®, USA). Standards were added to a 96-well ELISA microplate in duplicates, and negative controls and samples were added in triplicates. The development of optical density at 650 nm was measured on a SPARK 10 M multimode microplate reader (TECAN®, USA).

Cry1Ab concentration was calculated using a linear regression equation for the standard curve including only triplicate samples with coefficient of variation less than 20%. Cry1Ab concentrations in the leaf plants from different genetic backgrounds were expressed in µg/g dwt (dry weight tissue).

Additionally, homogeneity of variances in Cry1Ab concentration between the different genetic backgrounds was calculated using the Fligner–Killeen test.

Bioassays

Insects

Ostrinia nubilalis (Hübner) is a target insect pest of GM maize plants that express the Cry1Ab protein [28]. *O. nubilalis* eggs were kindly provided by A. Herz (Institute for Biological Control, Julius Kuehn Institute, Germany) and kept in a growth chamber (Sanyo MLR 350) at 18 °C, at photoperiod of 16:8 h (light:dark) (L:D) and light intensity of 20% (fluorescent lamp FL40SS W/37). Larvae were reared in the laboratory until the start of the bioassays with second instar larvae. Larval diet contained deionized water, agar powder, organic corn semolina, wheat germ, yeast powder, benzoic acid, nipagin and ascorbic acid [29].

Mortality bioassays

Thirty-two-well trays were used to establish the bioactivity of Cry1Ab-containing leaf material on second-instar *O. nubilalis* larvae. In each well, moistened blotting paper (2 cm × 2 cm, distilled water) maintained sufficient humidity before fresh leaf test material was added together with one larva of *O. nubilalis*. Filled trays were closed with adhesive transparent lids including small perforations to allow for air circulation. Trays were placed in a climate chamber at 26 °C, a photoperiod of 16:8 h (L:D) and light intensity of 20%. Eight wells were designated for

each sampled leaf (Additional file 2: Figure S2). Different genetic backgrounds deriving from plant crosses were tested in three blocks, using tissue from between four and six experimental plant leaves from each of the GM parental maize and F1 GM ♀ TEO ♂, respectively, and between one and three experimental plant leaves from F1 TEO ♀ GM ♂. The number of live larvae was determined 4 days after the bioassays commenced. Trays with larvae were placed at − 20 °C for at least 48 h before disposal.

A general linear model (Binomial method) was used to analyze effects of the different genetic backgrounds on insect mortality rates.

Seed phenotypes from crosses

Cob phenotypes of the F1's crosses were visually compared to the cobs of their progenitors, i.e., teosinte and GM maize plants.

Seed phenotypes of F1 TEO ♀ GM ♂ and F1 GM ♀ TEO ♂ crosses were classified as "dry" or "normal" seeds. For the F1 TEO ♀ GM ♂ crosses, a seed was classified as "dry" when the color of its caryopsis was white, beige or even green, indicative of immature seeds, and the embryo was dry or empty. A seed was classified as "normal" when the color of its caryopsis was grey, black or brown, and the embryo was present and full. For the F1 GM ♀ TEO ♂ crosses, a seed was classified as "dry" when its caryopsis was dry and empty, and "normal" when the color of its caryopsis was yellow, typical of maize seeds.

Statistics

Due to non-normal distribution data, the correlation between transgene expression and Cry1Ab protein and the mortality rate and Cry1Ab protein were calculated using the Spearman rank correlation coefficient (*Rs*).

Analyses were conducted in R [30] and figures were produced using the package ggplot2 [31]. For multiple comparisons analyses the package multicomp [32] was used and for pairwise comparisons the package emmeans was used [33].

Results and discussion

Crosses and phenotypes

The first and most important finding of our crossing trials was that the GM Bt maize plants can indeed hybridize with Spanish teosinte plants. These Bt maize × teosinte hybrids can produce viable seeds which in turn can successfully germinate and grow into fertile Bt teosinte plants. In other words, we have shown a viable pathway for the acquisition of GM-derived traits in a newly emerged, noxious weed. Additionally, one positive F1 Bt teosinte hybrid plant (F1 TEO ♀ GM ♂) that was kept until maturity produced an F2 cob through self-pollination; this F2 cob exhibited characteristics of both parents, the teosinte and the GM maize plant (Additional file 3: Figure S3). Thus, we also demonstrated the possibility of viable generations beyond the F2 line. To our knowledge, this is the first confirmation of this route of unwanted spread of a transgene to a noxious, weedy relative of maize in Europe.

When teosinte plants were used as female pollen recipients and GM maize plants as male pollen donors, the cobs of F1 TEO ♀ GM ♂ crosses were similar to the cobs from parental teosinte plants (Additional file 4: Figure S4). In contrast, when GM maize plants were used as the female parent crossing with the teosinte as a male, the cobs from the F1 GM ♀ TEO ♂ crosses resembled those from parental GM maize plants (Additional file 5: Figure S5).

Crossings were not equivalent in either direction: in total, the mean percentage of "normal" (i.e., viable) seeds per plant were 92.8% in F1 GM ♀ TEO ♂ (i.e., teosinte as the pollen donor) and only 2.7% in F1 TEO ♀ GM ♂, (i.e., maize as the pollen donor, Table 1) (Additional file 6: Figure S6). Such a difference in maize × teosinte hybridization rates is consistent with prior studies, where this phenomenon has been attributed to the presence of the teosinte crossing barrier genes *Gametophyte factor1* (*Ga1*) and *Teosinte crossing barrier1* (*Tcb1*), preventing maize pollen from growing well in the female teosinte context [34]. The same genes do not prevent the teosinte pollen from successfully fertilizing maize plants [11].

Table 1 Mean results of the number (No.) of "dry" and "normal" seeds per plant in the F1's crosses

Genetic background	Plant	No. of "dry" seeds	No. of "normal" seeds	% "normal" seeds
F1 TEO ♀ GM ♂	1	258	32	11
	2	466	0	0
	3	143	7	4.7
	4	210	2	0.9
	5	251	8	3.1
	6	139	3	2.1
	7	974	0	0
	8	385	4	1
	9	583	11	1.9
Mean (/plant)		**378.8**	**7.4**	**2.7**
F1 GM ♀ TEO ♂	1	11	96	89.7
	2	11	105	90.5
	3	16	124	88.6
	4	10	126	92.6
	5	3	114	97.4
	6	3	148	98
Mean (/plant)		**9**	**118.8**	**92.8**

Across the range of teosinte species, reported hybridization rates with maize differ widely between teosinte taxa. In one study, researchers found that maize and the teosinte *Z. m.* spp. *mexicana* naturally hybridize at a low rate (< 1%), whereas maize and another teosinte, *Z. m.* ssp. *parviglumis,* can hybridize at a high rate (> 50%) [13]. Our results are compatible with that study, considering that the Spanish teosinte likely derives from *Z. m.* spp. *mexicana* [6].

Another study [11], also using *Z. m.* spp. *mexicana*, yielded similar results. There, teosinte ears produced fewer seeds per ear when pollinated with maize pollen than maize ears pollinated with teosinte pollen; between 90 and 95% of the fruit cases produced on teosinte fertilized by maize pollen were sterile, i.e., in the range of our own study [11].

Low rates of hybridization when Spanish teosinte is the maternal parent do not completely prevent gene flow. The easier production of seeds in the F1 hybrids when the teosinte plants are the pollen donors can serve as a "genetic bridge", as F1 hybrids can readily back-cross with teosinte plants, facilitating the transfer of maize genomic elements to the teosinte genome. We showed that even self-pollination of F1 Bt hybrid plants can produce viable F2 seeds.

While significantly reduced, a relatively low gene flow (2.7%) from (male) maize to (female) teosinte can still represent a significant ecological path for gene flow, especially in large populations as those found in the agro-ecosystems of Northern Spain. Given the large numbers of Spanish teosinte plants found in infested maize fields in Spain and the large numbers of seeds produced per plant, many F1 TEO ♀ GM ♂ plants can form in each field over the years. The ecological and agronomic risk produced by this situation is large, even when fitness and fertility of hybrids and their descendants remain to be firmly established in the field.

Transgene mRNA expression

The mean expression of transgene activity in leaves of different genetic backgrounds ranged in relative values compared to a standard between a relative mRNA level of 0.80 in F1 GM TEO and 1.12 in F1 TEO ♀ GM ♂ (Table 2).

There was no significant difference in relative transgene expression between different genetic backgrounds ($F = 2.628$; $df = 2$; $P = 0.09$). Thus, statistically, the *cry1Ab* transgene was expressed similarly in different genetic teosinte backgrounds compared to GM parental maize. In other words, the presence of the transgene was always reflected in production of the corresponding mRNA in the plants that carried it.

Table 2 Number of plants analyzed, mean and standard error (± SE) of relative transgene expression in different genetic backgrounds from Spain

Genetic background	No. of plants analyzed	Rel. transgene expression* ± SE
GM	15	1.00 ± 0.08
F1 TEO ♀ GM ♂	4	1.12 ± 0.16
F1 GM ♀ TEO ♂	15	0.80 ± 0.06

* ($F = 2.628$; $df = 2$; $P = 0.09$)

Table 3 Number of plants analyzed, mean and standard error (± SE) of Cry1Ab concentration in different genetic backgrounds from Spain

Genetic background	No. of plants analyzed	Cry1Ab concentration (µg/g dwt)* ± SE
GM	10	38.09 ± 3.82
F1 TEO ♀ GM ♂	3	32.40 ± 5.92
F1 GM ♀ TEO ♂	15	38.74 ± 3.02

*($F = 0.179$; $df = 2$; $P = 0.838$)

Cry1Ab concentration

The mean Cry1Ab concentration in leaves of different genetic backgrounds ranged between 32.40 µg/g dwt in F1 TEO ♀ GM ♂ and 38.74 µg/g dwt in F1 GM ♀ TEO ♂ (Table 3).

There was no significant difference in these values between any of the different genetic backgrounds studied ($F = 0.179$; $df = 2$; $P = 0.838$). The Cry1Ab concentration values in Spanish teosinte F1 crosses were similar to those in GM parental maize plants.

In addition, no significant difference in variances for Cry1Ab concentration was observed in the Fligner–Killeen test between different genetic backgrounds ($P = 0.753$). Thus, the variability of the measured Cry1Ab concentrations in the different genetic backgrounds was similar between them, even when compared to GM parental maize plants.

There are no prior studies relating transgene expression to the concentration of the corresponding transgenic protein in wild teosinte species after they become hybridized with GM maize, but a few studies have described effects of transgene outcrossing to wild relatives in other species, such as *Brassica* and rice. In *Brassica* species, the Bt protein was synthesized at similar levels in hybrid wild plants compared to their parental plants [35, 36], similar to what we describe here, although in wild *B. juncea*, significant differences in Cry1Ac protein concentration between F1 hybrid and backcrosses were found. In the 4–5-leaf stage of backcross 1 (BC1) *Brassica juncea*

hybrids, the Cry1Ac protein concentration was statistically lower than in the GM parental plant. In the other genetic backgrounds (F1 hybrids, and BC2 to BC5 crosses), Cry1Ac protein concentrations were similar compared to their GM parental plants. In those studies, the bolting and flowering stages, from F1 hybrid to BC5, showed statistically lower Cry1Ac protein concentrations compared to their GM parental plants. However, in the pod formation stage, from F1 hybrid to BC5, the plants showed statistically similar level of Cry1Ac concentration compared to GM parental plant [37]. In rice, other researchers also found that the concentration of Bt protein in F1 hybrid plants was expressed at similar concentrations as in their parental plants, with even higher concentrations of Bt toxin detected in some plants of the crop–wild hybrids and F2 progeny [38].

Correlation between transgene expression and Cry1Ab concentration

We did not observe a statistically significant correlation between the Cry1Ab concentrations and the transgene transcription level across different genetic backgrounds ($Rs = 0.20$, $P = 0.316$) (Fig. 2). The level of transgene transcription into mRNA, as determined here, had no apparent impact on the produced Cry1Ab toxin concentrations. Across the different genetic backgrounds, concentrations of Cry1Ab toxin differed between around 18 µg/g dwt and almost 62 µg/g dwt—more than threefold, while the transgene transcription level ranged between 0.3 and 1.4—a more than fourfold difference. The highest Cry1Ab concentrations occurred at transgene transcription level from around 0.8–1.2—a roughly 1.5-fold difference.

When separating the different genetic backgrounds, the data showed a correlation between Cry1Ab concentrations and mRNA level only in F1 GM ♀ TEO ♂ crosses ($Rs = 0.55$, $P = 0.04$). In other genetic backgrounds, the results did not reveal any correlation (Additional file 10: Table S4).

Overall, as previously observed in experiments with non-GM maize plants (i.e., hybrids and open-pollinated maize varieties, Lohn et al. [39]), the mRNA levels of the *cry1Ab* transgene in the crop–hybrid teosinte plants do not appear to determine directly, in any measurable way, the concentration of the produced Cry1Ab toxin. Based on other studies, the suggestion for such an apparently paradoxical behavior is that there are other plant regulatory processes influencing the final concentration levels of the Cry1Ab toxin, such as post-transcriptional, translational and protein degradation/synthesis regulation [40–43], or even promoter activity changes that could be influencing in the mRNA level changes [44].

Mortality rates of *O. nubilalis*

Mean mortality rates of *O. nubilalis* larvae fed on teosinte hybrid plants or GM maize were overall high with rates equal to or above 95% (Fig. 3). The mean mortality rates of *O. nubilalis* larvae fed with plant material from the different genetic backgrounds from Spain ranged between 26.67% on the teosinte parental plants and 100% on the F1 TEO ♀ GM ♂. Statistical analyses showed significant differences in mean mortality rates between the different genetic backgrounds ($F = 48.925$; $df = 3$; $P < 0.01$). As expected, there was a significant difference in the mean mortality rates between the teosinte parental plants and the GM parental maize plants ($Z = -6.351$; $P < 0.01$). However, mortality rates of *O. nubilalis* larvae did not differ between the F1 TEO ♀ GM ♂ ($Z = 0.010$; $P = 1.000$) and F1 GM ♀ TEO ♂ ($Z = -1.180$; $P = 0.501$) and the GM parental maize plants (Fig. 3).

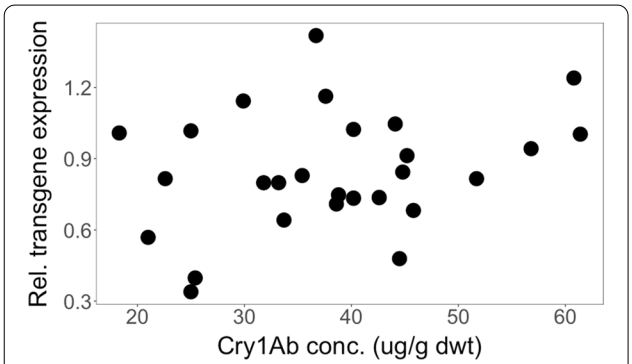

Fig. 2 Spearman rank correlation between relative transgene expression (transcription activity) and Cry1Ab concentration across different genetic backgrounds from Spain. ($Rs = 0.20$, $P = 0.316$)

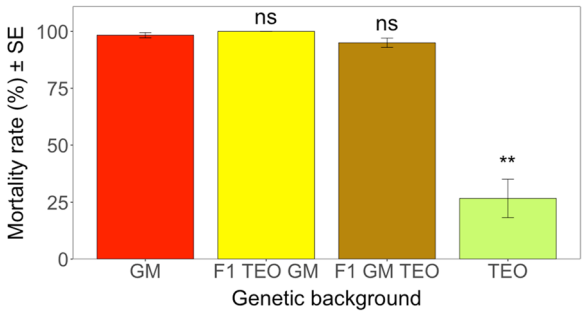

Fig. 3 Mean mortality rate (%) of *O. nubilalis* fed plant materials from different genetic backgrounds. Vertical bars show mean values, with the standard error (± SE) indicated as lines. Results of multiple comparisons of means with the group control GM parental maize (Dunnett's method). $^{**}P < 0.01$, ns = not significant. The *P*-values reported were adjusted by the single-step method

Snow et al. [15] reported that the *cry1Ac* transgene in back-crossed wild sunflower populations was active against its target pest insects and reduced herbivory damage significantly, leading to an increase in seed production and thus fitness. Halfhill et al. [35] revealed that the *cry1Ac* transgene reduced target pest herbivores when back-crossed onto wild oilseed rape populations. Another study involving transgenic *B. napus* also showed that all crosses, from F1 to BC5, had similar insecticidal efficacy as their parents against the insect pest species *Helicoverpa armigera* and *Plutella xylostella* [37]. These findings are critical for risk assessment as *O. nubilalis,* or other lepidopteran species considered "pests" on maize, would functionally serve as "biocontrol agents" on weedy teosinte. However, based on our bioassay data, it must be expected that Bt teosinte plants deriving from hybridization with Bt maize would be protected against feeding damage of *O. nubilalis* to the same degree as the parental Bt maize. Under these conditions, *O. nubilalis* herbivory would not serve as a biocontrol for the Bt teosinte weed; even more, herbivory would convey a selective advantage to Bt teosintes over non-Bt teosintes and foster the evolution of increasing numbers of Bt teosinte weeds, thus increasing its fitness and general noxious ecological behavior.

Correlation between mortality rate and Cry1Ab concentration

There was no statistically significant correlation between mortality rates and Cry1Ab toxin concentrations, where the toxin was found across different genetic backgrounds ($Rs = 0.11$, $P = 0.577$) (Fig. 4).

Nevertheless, presence of the *cry1Ab* transgene led to the production of bioactive Cry1Ab toxin and high mortality rates of *O. nubilalis* larvae, confirming a high susceptibility of these larvae to Cry1Ab in all measured concentrations.

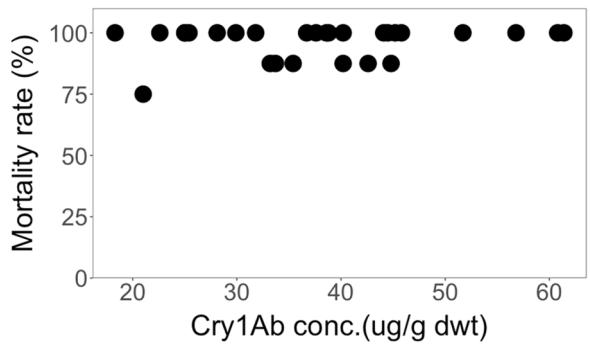

Fig. 4 Spearman rank correlation between mortality rate and Cry1Ab concentrations across different genetic backgrounds from Spain. ($Rs = 0.11$, $P = 0.577$)

However, we also observed that some young *O. nubilalis* larvae were able to survive on plants expressing the Cry1Ab toxin, raising some doubt that the expressed Cry1Ab concentration was not enough to kill 99% of the susceptible larvae, as required by insect resistance management (IRM) strategies [45].

Although there have been no reports of field resistance to Cry toxins in *O. nubilalis* from Europe or North America [46], some laboratory assays suggest that there are no intrinsic biological reasons why resistance may not eventually develop [47–49].

Conclusions

To our knowledge, this is the first report of successful outcrossing and expression of a transgene from maize into a teosinte species. Gene flow from the Spanish teosinte pollen to a maternal genome from cultivated GM maize seems all but unrestricted, while much lower rates of successful outcrossing were documented when GM maize was the pollen donor and Spanish teosinte the female recipient. Our results are in line with other reports documenting the existence of various degrees of crossing incompatibility when teosinte is the maternal parent [11, 34]. The *cry1Ab* transgene outcrossed into Spanish teosinte is expressed stably, producing the corresponding mRNA and yielding a fully bioactive Bt toxin. These results have important consequences in environmental risk-and ecological safety-assessments for places where GM maize plants and teosinte occur in sympatric populations.

Origin of a noxious weed with increased fitness

The Spanish teosinte situation poses two paradoxes: first, it is not self-evident that sympatric coexistence of domesticated maize and teosinte populations should drive the emergence of a noxious weed, as it in fact happened in Spain, since such coexistence has taken place for thousands of years in the native range of both, as well as in many other agro-ecosystems where they find themselves introduced. Much of Central America and North America has allowed for coexistence of teosinte with maize since the earliest domestication event ~ 9000 years ago, with teosinte populations thriving in those regions and forming hybrid swarms until today [13]. Even more, the regular hybridization of teosinte and maize in their native range has long been well documented [50]. In other areas, including vast expanses of maize cultivation in the American Corn Belt, India, Brazil and elsewhere, teosinte has been introduced intentionally or unintentionally [14, 51–56]. Indeed, situations where species of teosinte and maize coexist

are so numerous that the relevant question may be: why had a cross resulting in weedy behavior not been detected before? Indeed, it has been repeatedly shown [50] that while maize may be a beneficiary of hybridization events with teosinte, teosinte/maize hybridization had not been found to provide any fitness advantage to the wild relative, necessary for teosinte to become a weed [13].

The second, related paradox is that Northern Spain would be the specific place where the noxious hybrid of teosinte and maize should arise at all. Given that the genetic diversity basis of both maize and teosinte in Spain is much more reduced than in other regions where they meet, it may be considered paradoxical that a successful weed would not have emerged from the wider selection of genotypes available in the Americas than the much more limited diversity in Southern Europe.

The solution to this set of two paradoxes can only be speculative, despite its importance.

Two alternative hypotheses can be proposed: first, a purely **Heuristic Theory** can be posited, suggesting that Spain was simply the unfortunate, random location for an exceedingly unlikely—if also possible—scenario. This theory would be impossible to disprove as a null hypothesis, and it requires no further explanation beyond a blind "unfortunate draw of the luck" event.

A second hypothesis, which we call the **Supported Simplification Theory**, would have the two paradoxes of the Spanish teosinte situation, mentioned above, playing complementary roles in the generation of a novel, noxious weed, as follows.

We hypothesize that an alignment of conditions was made possible in the Northern regions of Spain in the late twentieth century, including (a) synchronized phenology between a homogenous population of maize and the teosinte population ancestral to the weedy Spanish teosinte; (b) conducive agronomic practices; (c) intense selective pressure favoring the emergence of teosinte with weedy behavior and (d) a highly reduced genetic base of populations in both maize and teosinte favoring the fixation of rare fitness traits in the potential weed.

The coincidence of these conditions in the Aragón and Cataluña regions is relatively recent, accompanying the emergence of industrial-scale maize production in Northern Spain in the second half of the twentieth century, driven by irrigation projects in arid lands using Pyrenean water and "elite hybrid" maize material from North America, which contrasts with the older and small-farm-scale production common in the Mediterranean basin going back to the fifteenth century. In the latter, a much wider genetic base still persists today, dominated by Caribbean stocks of maize admixed with Andean, North American and other materials [57].

How teosinte was introduced into Spanish or French fields remains unclear, although it is known that teosinte species have been promoted and introduced repeatedly as potentially promising forage species for cattle and even fish [14, 51]. A fecund grass that is easily adapted to dry and warm environments, teosinte species have seen intentional attempts at establishment since the early twentieth century in many areas of the Americas and the Caribbean, Southern Asia, the Middle East as well as Southern Europe, including Spain and France [14, 58]. Thus, the presence of teosinte in sympatry with maize in the region cannot be considered a limiting factor.

In Europe, synchronized phenology of teosinte and maize (condition (a) above, necessary for cross-pollination), seems to be coincidental only in the mid-latitude regions of Europe [59, 60]. We confirmed, using authentic materials from the region, that indeed both plants produced pollen and fertile tassels synchronously. Farmer reports also confirm this phenological coincidence in Northern Spain. This is not the case elsewhere in Europe, as Northern and North-Eastern maize production is accomplished with early-flowering varieties [59].

Given the industrialized, mechanized agronomic practices in the region (condition (b) above), early detection and negative selection of ancestral weedy teosinte individuals is not likely, since the weed has strong morphological resemblance to maize through the vegetative period of growth. Furthermore, mechanized harvesting in Northern Spain lends itself to positive selection of potential weed ancestors; in field conditions with both teosinte and maize, harvester machines carry a sieve that retains larger maize kernels, but shunts the smaller teosinte seed to a spreader that cast-seeds the teosinte in the field, effectively generating a seed-bank for the next years [5]. Teosinte seed can germinate immediately or remain dormant for years [61] exacerbating the problem of detection and early eradication of a potential weed ancestor.

It is important to note that most studies of hybrid fitness in the teosinte/maize system find little, if any, fitness increase in hybrids where teosinte is the rare recipient of maize genetics [13]. Under neutral, or even negative fitness change following hybridization, there is only a remote possibility of obtaining a potential new weed, as the early progeny from a putative hybridization event would be drowned-out by normal-fitness competitors. In other words, early hybrids would play an evolutionary bottleneck for the population to come out and spread as a higher-fitness weed. This is often presented as a reason to explain why a noxious weed may have not arisen from teosinte in the 9000 years since maize has been planted as a domesticated crop. Such explanations, however, do not include the effect of agronomic practices and human

selection pressure. In the Spanish teosinte situation of the late 20th Century, and under our Supported Simplification Theory, it can be said that farmers and farming practices might have inadvertently nourished the ancestral hybrid teosinte population(s) through an evolutionary bottleneck of neutral or depressed fitness, effectively subsidizing it with irrigation, fertilization and incremental re-planting (as described above) and selection for higher fitness traits (condition (c) above).

Such a human-supported "evolutionary bridge" through a depressed-fitness bottleneck would be greatly amplified and accelerated in a situation where populations of both teosinte and maize had highly homogenous genetics (condition (d) above) through the hybridization process. While a wide genetic basis still remains in most temperate and tropical environments where maize and teosinte may coexist, including elsewhere in Europe [59], recently introduced teosinte and maize populations in Northern Spain provide a relatively homogenous genetic background where rare traits might be fixed and amplified without the moderating effects of recombination due to crossing with a diversity of possible genetic backgrounds. The path through an evolutionary bottleneck towards fixation of the weedy traits in teosinte could have been greatly facilitated and accelerated by such a reduced-diversity condition in both maize and teosinte ancestral populations.

Policy and management observations

Our results and those recently published by Le Corre et al. [7] refute the conclusions by Devos et al. [62] and the European Food Safety Authority [63] that gene flow rates from maize to Spanish teosinte would be "too low" to allow significant gene flow to occur "even if teosinte is abundant". Gene flow has demonstrably occurred already and has led to increased weediness in Spanish teosinte by allowing the latter to adapt to local conditions in Spain [7]. While in a separate population of French teosinte, Le Corre et al. [7] documented the acquisition of an herbicide resistance gene from maize, with 43% of field collected teosinte plants rated as resistant against the herbicide cycloxydim. Considering the relatively recent introduction of teosinte species in Europe during the last century, and the even more recent introduction of herbicide resistant maize, these introgression processes have likely been happening repeatedly, and even frequently, sufficient to be well established and detectable in the field today.

Here, we provide further confirmation of the reality of GM maize-to-teosinte outcrossing in Spanish teosinte, even when pollination of teosinte by GM maize is demonstrably low, in the of 2% success rate. Such a limited rate cannot be considered a real limitation in the ecological reality of the field situation, where a large number of potential crossings takes place between intentionally planted monoculture maize and teosinte weeds undergoing explosive population growth. Indeed, if there is a surprising observation it is the possibility that the *cry1Ab* transgene has not yet been reported in the field. Such a paradoxical situation might simply be the result of extremely limited sampling (let alone genotyping-) efforts in the Spanish fields so far. There is no public record of any systematic monitoring for GM hybrid teosinte in the GM maize producing areas of Spain.

Regarding the functional susceptibility of plants and insects to the possible introgression of the *cry1Ab* transgene, our results cannot support the speculation by Devos et al. [62] and the European Food Safety Authority [63] that the Spanish teosinte would have already" high levels of pest resistance/tolerance" against European herbivores like *O. nubilalis*. Here, we show conclusively through feeding experiments that *O. nubilalis,* the main pest of maize in Spain, thrived equally well on leaf material from maize or teosinte. When those leaves contained the Bt toxin derived from activity of the *cry1Ab* transgene, insect larvae were also equally susceptible, regardless of whether they consumed maize or teosinte leaf material, as we also show here that both plant contexts lend themselves equally well to the expression of the toxin in their leaves. These results strongly suggest that Spanish teosinte, which is already an aggressive weed through its adaptation to the European agroecosystem, can become even more harmful through the acquisition of insect-resistant traits through crossing with local GM maize cultivated as a crop.

We must assume that the Bt toxin expressed in a teosinte background also works effectively against other herbivores, in particular the other target pest of Bt maize listed for Spain, *Sesamia nonagrioides* although increased resistance allele frequencies in Spanish Bt maize fields have already been reported for populations of this insect [64]. How the presence of Bt teosinte hybrids may accelerate the evolution of resistance in these pests will require extensive further research.

Future research will benefit from a note of caution stemming from results presented here: we show here that there is poor or no correlation between the active presence of the *cry1Ab* transgene, the corresponding levels of mRNA or Bt toxin in leaves, and the insecticidal bioactivity of the toxin. These results are similar to earlier findings in crossing experiments with open-pollinated varieties of maize, instead of teosinte plants [39]. As in those complementary studies, we confirm here again that *cry1Ab*-derived mRNA is not a good predictor of Cry1Ab concentration or insect mortality. Why such a lack of correlation might occur could be due to saturation of

the analytical methods (e.g., near-total insect mortality at all toxin concentrations) or to the intervention of cell regulatory processes, such as post-translation regulation. Whatever the cause, this lack of correlation should warn against assuming that a single measurement could be a faithful reflection of the chain of processes leading from transgene induction to insect death, and pest population control.

Our results open a range of important questions of evolutionary, ecological, agronomic, economic and social consequence. While the emergence of a new and noxious weed in Europe should already be of highest concern, we provide strong evidence for mechanisms that will likely lead to an even more aggressive weed behavior, in the European context at least, of this plant. More widely, our results should ring urgent caution in areas known to harbor native teosinte in Central America and Mexico, as well as the many areas around the planet where teosinte has already been intentionally introduced [14]. Particularly in cases of reduced genetic diversity of monoculture industrial production, bringing GM maize into sympatric continuity with teosinte populations can be reasonably suspected to result, sooner or later, in the acquisition of traits that can lead to a shift in ecological behavior of the teosinte towards weediness, as it has happened in Spain over a relatively short period of time. Furthermore, since teosinte can often back-cross into maize, teosinte populations could also become reservoirs of undesired GM traits even without GM maize in a locality. This last possibility is of great relevance in centers of origin and centers of diversity of maize (and teosinte) in Mexico, Central America and elsewhere [65, 66].

Abbreviations

ABI: Applied Biosystems®; Bt: *Bacillus thuringiensis*; cDNA: Complementary DNA; Ct: Threshold cycles; DNA: Deoxyribonucleic acid; DNAse: Deoxyribonuclease; dT: Deoxythymine; DWT: Dry weight tissue; *Ga1*: *Gametophyte factor1*; GM: Genetically modified; H_2O: Water; IRM: Insect resistance management; L:D: Light:dark; mRNA: Messenger RNA; No: Number; PBST-Buffer: Phosphate-buffered saline solution; qRT-PCR: Real-time polymerase chain reaction; RDD Buffer: DNA digest buffer; RNA: Ribonucleic acid; RNAse: Ribonuclease; *Rs*: Spearman rank correlation coefficient; RT: Reverse transcriptase; SE: Standard error; *Tcb1*: *Teosinte crossing barrier1*.

Supplementary Information

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Additional file 1: Figure S1. Sampling scheme.

Additional file 2: Figure S2. Bioassay tray (32 cells/tray).

Additional file 3: Figure S3. F2 infructescence derived from Bt positive teosinte hybrid (F1 TEO ♀ GM ♂).

Additional file 4: Figure S4. Infructescences of the Spanish teosinte, GM maize (*Z. mays* spp. *mays*) and their F1 hybrid. From left to right: GM maize infructescence, F1 TEO ♀ GM ♂ hybrid infructescence and Spanish teosinte infructescence.

Additional file 5: Figure S5. Infructescences of the Spanish teosinte, GM maize (*Z. mays* spp. *mays*) and their F1 hybrid. From left to right: Spanish teosinte infructescence, F1 GM ♀ TEO ♂ hybrid infructescence and GM maize infructescence.

Additional file 6: Figure S6. "Normal" and "dry" seeds from F1 TEO ♀ GM ♂ hybrid. From left to right: "Normal" seeds and "dry" seeds.

Additional file 7: Table S1. Number of positive and negative GM plants observed for different genetic backgrounds from Spain.

Additional file 8: Table S2. Components of the Master mix.

Additional file 9: Table S3. Sequences and amplification efficiencies of TaqMan primers and probes for the *cry1Ab* transgene and three reference genes in the Spain experiment.

Additional file 10: Table S4. Spearman's rank correlation and p-value between relative transgene expression and Cry1Ab concentration across different genetic backgrounds from Spain.

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Authors' contributions

All authors substantially contributed to the literature review, the drafting of the work, as well as the revision of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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