Quantitative genetic analysis of Cry1Ab tolerance in *Ostrinia nubilalis* Spanish populations

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Abstract

Tolerance to *Bacillus thuringiensis* Cry1Ab toxin in Spanish *Ostrinia nubilalis* populations was analyzed by quantitative genetic techniques, using isolines established from field-derived insects. F_1 offspring was tested for susceptibility to trypsin activated Cry1Ab using a concentration that caused a mean larval mortality of 87% (± 17% SD). The progeny of the most tolerant isolines (that had shown mortalities lower than 60%) was crossed to obtain the F_2 generation that was exposed to the same Cry1Ab concentration. A clear reduction in mortality (62% ± 17% SD) was observed. The upper limit for heritability was estimated to range between 0.82 and 0.90, suggesting that a high part of phenotypic variation in tolerance to Cry1Ab was attributable to genetic differences. An estimate of the minimum number of segregating factors indicated that the *loci* involved in tolerance to Cry1Ab were at least two. The role of the *cadherin* gene, which is a *B. thuringiensis* resistance gene in Lepidoptera, was assessed in the most tolerant isolines by using an EPIC-PCR marker specifically developed for this study. Association between *cadherin* and tolerance was obtained in one tolerant isoline; however it could be not confirmed by segregation analysis in the F_2 progeny because F_2 offspring was not viable. Our results indicate that the tolerance trait is common in Spanish field populations. Quantitative genetic techniques may be helpful for estimating the influence of genetic factors to Cry1Ab tolerance in *O. nubilalis*.

1 INTRODUCTION

There is a wide consensus that the European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae), is one of the most devastating pests of maize in temperate climate regions. Larvae of this species tunnel through the stem of the plant and feed within the stalk until pupation, causing yield losses. Since 1996, *O. nubilalis* has been efficiently controlled by transgenic maize expressing the insecticidal Cry1Ab protein from *Bacillus thuringiensis* (Berliner) (Bt). The worldwide adoption of Bt maize is continually increasing, leaded by the US where plantings of Bt maize reached the 67% of the total maize acreage in 2012 (NASS, 2012). In the European Union (EU), the cultivation of Bt maize expressing Cry1Ab started in 1998 and to date it is the only Bt crop allowed for cultivation. A few EU countries have occasionally grown Bt maize for commercial purposes, but only in Spain the Bt cultivated surface has been steadily rising since its introduction. In 2012, Spanish Bt maize area covered around 116,000 hectares (Ministerio de Agricultura, Alimentación y Medio Ambiente, 2012) and represented the 30% of the total maize-cultivated surface. This percentage substantially increases when some Spanish regions with higher adoption rates (such as the northeast corner) are considered (Farinós et al., 2011).

The continuous growing of Bt maize exercises a high selection pressure on the target pests. It might drive to the arising of the resistance trait in *O. nubilalis* field populations, particularly in those areas where the cultivation of Bt maize has been effectively implemented. In fact, field-evolved resistance of certain lepidopteran pests to Cry toxins have been reported after massive cultivation of Bt crops (Dhurua and Gujar, 2011; Kruger et al., 2011; Storer et al., 2010). Laboratory selection experiments have shown that increased levels of resistance to Cry1Ab could be achieved in *O. nubilalis* colonies after few generations (Crespo et al., 2009; Coates et al., 2008; Alves et al.,

2006; Farinós et al., 2004). Furthermore, differences in susceptibility to Cry1Ab protein, attributable to natural variation in sensitivity to the toxin, have been reported for *O. nubilalis* field populations (Saeglitz et al., 2006; Farinós et al., 2004; Siegfried et al., 2001; Marçon et al., 1999). However, in spite of the intense selection pressure exerted by the Bt maize and the potential to evolve resistance displayed by laboratory experiments, resistance monitoring plans carried out in North America and Spain did not detect a decrease in susceptibility of *O. nubilalis* larvae to Cry1Ab after the introduction of Bt maize (Siegfried and Hellmich, 2012; Farinós et al., 2004).

In most cases, resistance to Cry toxins in Lepidoptera fit with a single *locus* model (Ferré et al., 2008). However, the genetic characterization of three *O. nubilalis* resistant strains revealed that laboratory-selected resistance to Cry1Ab had a polygenic inheritance in this species (Crespo et al., 2009; Alves et al., 2006). Quantitative genetic techniques have been applied to study insecticide resistance in Lepidoptera (Alinia et al., 2000; Firko and Hayes, 1991; Tabashnik and Cushing, 1989) and they may be used to study the genetic basis of resistance in *O. nubilalis* field populations. These techniques provide estimates of genetic parameters whatever the number of genes involved since they only require an observed or assumed continuous distribution of phenotypes (Firko and Hayes, 1990; Tabashnik and Cushing, 1989). Natural variation in resistance phenotype can be parted in environmental and genetic components, and the overall heritable genetic variation can be quantified.

The mode of action of the Cry1A proteins is very complex but the binding to a receptor located in the larval midgut (as cadherin, cdh) is a key step (Vachon et al., 2012). High levels of resistance can be achieved by the alteration of the receptor binding (Ferré et al., 2008) and in three lepidopteran species mutations in the *cdh* gene have been genetically linked with the resistance trait (Gahan et al., 2001; Morin et al.,

2003; Xu et al., 2005). Contribution of *cdh* gene to the Cry1Ab resistance in *O. nubilalis* may be assessed by using molecular markers, such as PCR-RFLP (Coates et al., 2005) or exon-primed intron crossing (EPIC) PCR. This last technique detects intron polymorphisms by using primers designed on the flanking exons (Lessa, 1992). The EPIC-PCR markers are co-dominant, segregate according the Mendelian inheritance, and have been developed and used for genetic surveys in organisms that lack sufficient DNA sequence data (Arias et al., 2009; Bierne et al., 2000) or for gene mapping (Wydner et al., 1994).

The objective of this study was to examine the variation in tolerance to Cry1Ab in *O. nubilalis* through a quantitative genetic approach, by using a selection strategy based on single-pair mating isolines derived from larvae collected in Spanish commercial maize fields. Moreover, the association between *cdh* gene and Cry1Ab tolerance was assessed using an EPIC-PCR marker specifically developed for this study.

2 MATERIALS AND METHODS

2.1 Insect collection, rearing and bioassay

The *O. nubilalis* populations used in the present study were obtained from diapausing last instar larvae collected in 2004 from two important Spanish maize growing areas, one (E) located in the Northeast (Ebro Valley) and another (B) in the Southwest (Badajoz), by dissecting damaged stalks from commercial maize fields. The larvae were moved to the laboratory to establish laboratory field-derived populations. The process used for the rearing is described in detail by Farinós *et al*. (2004).

For single-pair mating, each adult pair was confined in an oviposition cage consisting of a plastic cup (15.5 cm high x 6 cm diameter) with a fine mesh at its end,

placed over and opposed to another plastic cup (7 cm high x 7 cm diameter). Cotton soaked with a 10% honey solution was placed in the oviposition cages for feeding. The top of each cage was covered with a black waxed paper sheet for the oviposition. After 3 and 7 days, egg masses were collected and transferred to plastic boxes containing moistened filter paper until hatching. Cages for mating, oviposition and eggs incubation were placed in a growth chamber (Sanyo MLR-350H, Sanyo, Japan) maintained at 25 ± 0.5 °C, 70 ± 5% RH, and with photoperiod of 16:8 h (L:D).

Bioassays were performed with trypsin activated Cry1Ab toxin solubilized in CAPS buffer (pH 10.5), kindly provided by the laboratory DLR Rheinpfalz (Neustadt/Weinstrasse, Germany). For testing larval tolerance, we decided to use a threshold Cry1Ab concentration (40 ng/cm^2) which was expected to cause about 90% of mortality according to our previous data (Farinós *et al*., 2004), to ensure the survival of only the more tolerant individuals. We used the term tolerance in the sense defined by Finney (1971), to refer to a quantitative measure of resistance that is normally distributed among individuals within a population. The toxin dilution was applied on the surface of artificial diet solidified in the wells of a 128-well plastic tray (Bio-Ba-128, Color-Dec Italy, Capezzano Pianore, Italy). Trays were let dry in a laminar flow hood and immediately five to seven neonate larvae were placed in each well and confined with a cover (Bio-Cv-16, Color-Dec Italy, Capezzano Pianore, Italy). Trays were incubated in the growth chamber at 25 ± 0.3 °C, 70 ± 5% RH, and constant dark. Larval mortality was recorded after 7 days. The larvae were considered dead if they did not show any reaction when prodded. In this study, we refer to the larvae that survived to the Cry1Ab screening as tolerant. Control mortality never exceeded 5%.

2.2 Isoline establishment and screening for the Cry1Ab tolerance genetic basis

A full siblings experimental design was used to investigate the genetic basis of tolerance to Cry1Ab (Figure 1). We started with 110 crosses between two adults, each one from one of the two different geographical locations (B x E and reciprocal cross E x B). The offspring (F_1) of the pairs that oviposited more than 80 eggs was divided into two parts within the isolines: the bioassayed and the control groups. The bioassayed group consisted in about 125 neonates per isoline that were exposed to the Cry1Ab protein. After seven days, mortality was recorded and tolerant larvae were moved to non-supplemented artificial diet, where they were reared until the third instar. At this point, tolerant larvae from isolines which showed a percentage of mortality higher than 60% were frozen while tolerant larvae from isolines with mortality less than 60% (called I_T isolines) were reared until the adult stage. Neonate larvae of the control group were reared on non-supplemented artificial diet until the third instar, and then frozen.

Tolerant adults from I_T isolines were single-pair mated within each isoline (forming the sib-I_T subfamilies) to obtain the next generation (F_2). Again, the F_2 progeny of each sib-I_T subfamily was divided in two groups: the bioassayed and the control groups. The bioassayed larvae were challenged with the same concentration of Cry1Ab toxin used in the bioassays with the previous generation, following the procedure described before. Larvae from control group were reared until the third instar without Cry1Ab exposure and then frozen.

2.3 Data analysis

The estimate of the heritability of tolerance to Cry1Ab was determined considering the mortality recorded after Cry1Ab exposure as a threshold character with tolerance as underlying continuous variable (Falconer and Mackay, 1996) since our methodology could not directly measure the tolerance level of each individual insect. The heritability of tolerance to Cry1Ab was estimated by using two different methods:

the first one based on variances among families in the unselected F_1 generation (Tabashnik and Cushing, 1989; Bull *et al*., 1982) and the other one based on the actual response to selection from one generation to the next (Firko and Hayes, 1990).

The first method, called the contingency method, was initially described by Bull and coworkers (1982) and later adapted by Tabashnik and Cushing (1989) to estimate heritability of insecticide resistance. As the proportion (*p*) of the population expressing the tolerance trait (i.e., proportion of survivors) in the unselected generation F_1 was different from 0.5, the ρ_x value was determined from tables for computing bivariate normal probabilities (Owen, 1962), using the formula described by Bull *et al.* (1982). The heritability of tolerance was calculated as $2\rho_x$ for full siblings. In this full siblings analysis, variation between families is often increased by the common environment and the dominance variance; thus our estimate of heritability only sets an upper limit (Falconer and Mackay, 1996).

In the second method, we used the proportion of tolerant individuals (in this case the survivors to the Cry1Ab treatment belonging to families with mortality lower than 60%) in F_1 and F_2 generations, to calculate the regression coefficient of offspring on one parent (*b*). In this model, the estimate of heritability of tolerance is obtained from the formula:

$$h^2 = 2b = 2\mu^{'}/\mu_s$$

where μ_s represents the mean of the underlying trait (tolerance) of the larvae that survived the treatment with the insecticidal toxin Cry1Ab and had offspring, and μ' represents the mean of the tolerance in the F_2 generation. Both values were obtained applying the method described by Hartl and Clark (1989) that uses the proportion of tolerant individuals scored in each generation. The interpretation of the correlation of tolerance between relatives in terms of heritability is subject to uncertainties about

resemblances due to the common environment (Falconer and Mackay, 1996), thus also in this case the estimate of heritability represents the upper limit.

The minimum number of independently segregating genes with equal and additive effects on the tolerance to Cry1Ab was calculated using the method proposed by Lande (1981) to estimate the effective number of genetic factors (n_E) influencing a particular trait within a population. The estimate was obtained with the formula:

$$n_E = \frac{\left[\sum_{i=1}^{n} \sigma_i^2\right]^2}{\sum_{i=1}^{n} \left(\sigma_i^2\right)^2}$$

where σ^2 is the variance recorded in each generation and *n* is the number of generations. To find the sampling variance of the underlying trait (tolerance) which mean is the normal deviate *x* for the corresponding *p* (proportion of the population expressing the tolerance trait) we used the formula:

$$\sigma_x^2 = (1-p)/i^2 A$$

where *i* is the mean deviation of tolerant individuals from the population mean (tabulated by Falconer and Mackay, 1996) and A is the number of tolerant individuals.

To compare the mortality values among groups of isolines (total isolines and I_T isolines) and generations we used the non-parametric Mann-Whitney test implemented in the GraphPad Prism 5 software (GraphPad software, La Jolla, CA, USA). The same test was used to detect differences in mortality in F_1 generation among isolines depending from the origin of the founders. We used ANOVA to determine if the arcsine transformed mortality frequencies were significantly different among sib-I_T subfamilies depending from the I_T isoline of origin.

In the association studies between *cdh* alleles and tolerance we compared the allelic frequencies between tolerant and control groups within each I_T isoline by the χ^2 test.

2.4 DNA extraction, PCR reactions and cloning

Total genomic DNA was extracted from the thorax of frozen parental adults or from frozen whole larvae, using the PrepMan^{TM} Ultra reagent (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. DNA quality was checked by agarose gel 1% staining with ethidium bromide.

The EPIC marker used to study *cdh* allele segregation was selected for the maximum size variability, after comparing all the *cdh* regions (data not shown). We previously demonstrated that Bt related *cdh* from *O. nubilalis* is a single copy nuclear gene (Bel et al., 2011), suitable to be studied in inheritance analysis. Primers 3.14 L (5'-CCTAGGCGAGGAGTCTAT - 3') and 3.14 R (5' - CGAGCCGTACTTCGTCATGGAGAG - 3') anneal on the exons 31 and 32 of the *cdh* gene and the amplification results in a fragment containing only one intron. Both primers were designed with Primer3 software (Whitehead Institute for Biomedical Research, MA, USA) and purchased from GenScript Corporation (Piscataway, NJ, USA). Amplification reactions were carried out by using ≤ 1 µg of total DNA as template, in a final volume of 25 µl containing 0.4 U of Expand High Fidelity Taq polymerase (Roche Diagnostics, Mannheim, Germany), 0.2 mM of each dNTP, 0.3 µM of each primer and the buffer provided with the Taq enzyme, using an Eppendorf Thermal Cycler (Hamburg, Germany). The reaction conditions included an initial step of denaturation at 94°C for 5 min, 35 cycles composed by denaturation at 94°C for 30 sec, primers annealing at 55°C for 30 sec and elongation at 72°C for 2 min, and a final elongation step at 72°C for 5 min. Amplification products were separated in 1% agarose gel and stained with ethidium bromide to detect size variations. Each amplified marker was sequenced to confirm the identity. For this purpose, PCR amplicons were gel purified using the GFX^{TM} PCR DNA and Gel Band Purification Kit (GE Healthcare,

Buckinghamshire, UK) following the manufacturer's instructions, and cloned in the pGEM-t Easy Vector (Promega, Madison, WI, USA) with ligation O/N at 4°C. The resulting constructs were cloned in competent *Escherichia coli* cells DB3.1 and the positive colonies were screened by colony PCR. The plasmids were purified with the High Pure Plasmid Purification Kit (Roche Diagnostics) and sequenced at the IBMCP sequencing facilities (Valencia, Spain) using M13 primers. Sequences were aligned and analyzed with the SeqMan software (DNAStar, Madison, WI, USA). In the EPIC-PCR markers variability analysis, only indels (insertion/deletion) were scored, while SNPs identified were not used for allele characterization.

3 RESULTS

3.1 Genetic basis of Cry1Ab tolerance

One hundred and ten single-pair matings were established from field-derived *O. nubilalis* populations from two representative Spanish Bt maize areas. Fifty-three pairs laid eggs enough to establish an isoline and to test the offspring, and the mean of the bioassayed larvae was 123 (± 58 SD) per isoline (Table 1). The mortality caused by Cry1Ab toxin in the F_1 generation varied extensively among families, ranging from 24 to 100% (Figure 2), with a mean mortality per isoline of 87% (± 17% SD) (Table 1); nine isolines (17% of the total) had no survivors after the treatment. The overall mortality observed was similar to the expected, based on our previous analyses of field Spanish populations (Farinós *et al.*, 2004). As we used a high toxin dose causing about 90% of mortality, the distribution of the mortality frequencies was significantly different from a normal distribution (Shapiro-Wilk test, $W = 0.7301$, $P < 0.0001$). Sex bias in the mortality values due to the origin of the founders of the isolines was not evidenced, because the mean mortalities of the progenies derived from the reciprocal

parental crosses did not significantly differ (B x E = 88 ± 16% SD; E x B = 86 ± 19% SD; Mann-Whitney test, $U = 263$; P = 0.1426).

Mortality values recorded in F_1 generation varied continuously from 70 to 100%, with the exception of five isolines (#1, #7, #76, #81 and #109), the 9% of the total (Figure 2). These isolines, called I_T isolines, exhibited a larval mortality rate after the treatment with Cry1Ab below 60%. The mean mortality of the I_T isolines was 44% (± 14% SD) significantly lower than the one of the whole F_1 generation (87 ± 17%) (Mann-Whitney test, $U = 14.5$, $P<0.01$) (Table 1). To test if tolerance exhibited by the I_T isolines was a genetically determined trait, tolerant insects from I_T isolines were single-pair mated within each isoline and the offspring (corresponding to F_2 generation) was challenged with the same Cry1Ab doses used in the previous assays. From I_T isolines #1 and #7 was possible to make only three single-pair matings for each one and no viable offspring was obtained. The other three I_T isolines (#76, #81 and #109) allowed the establishment of 31 single-pair matings which laid enough eggs (314 ± 81) for the bioassays (Table 1). Mortality per subfamily in F_2 generation ranged from 28 to 89% with a mean of 62% (± 17% SD) (Figure 2) that means a significant reduction with respect to the overall mortality showed by the previous generation (87 ± 17%) (Mann-Whitney test, $U = 203$, $P<0.01$) (Table 1). In addition, 100% of mortality in F_2 subfamilies was never obtained, whereas in the F_1 generation the 17% of the isolines had no survivors. The distribution of mortality frequencies in F_2 generation was essentially continuous and, differently from the F_1 generation, did not deviate from a normal distribution (Shapiro-Wilk test, $W = 0.9712$, P = 0.5526). The overall results evidenced a shift of the tolerance level after only one generation that indicates a fast response to the selection pressure (Figure 2). Within the F_2 generation, ANOVA did not detect differences among mortality means depending on the isoline of origin of the sib-

I_T subfamilies (Table 1) (F = 0.1901, P = 0.347), indicating a similar response of all isolines to the Cry1Ab selection.

The upper limit for heritability of tolerance to Cry1Ab in our *O. nubilalis* sample was estimated to be 0.90, using the method described by Bull *et al.* (1982) based on variance among families. This value indicates that up the 90% of the phenotypic variation in the population may be due to additive genetic variation. When the heritability of tolerance was estimated using the method based in response to the selection, the result was 0.82. Also in this second case, the common environment where insects were kept could inflate the estimate and 0.82 represents the upper limit for heritability.

Analysis with the Lande's method indicated that tolerance was not controlled by a single major gene. Lande's formula based on population variances recorded along the selection yielded an estimate of 2 for the minimum number of effective segregating factors with equal effect on Cry1Ab tolerance.

3.2 Segregation of the cadherin alleles in the I_T isolines

The *cdh* EPIC-PCR amplifications performed on the DNA from ten parental adults (I_T isolines founders) resulted in the identification of five alleles (called allele A to E) that showed length polymorphism plus a non-amplified fragment (null allele, allele N) (Table 2). The amplicon sizes of the alleles A to E ranged from 758 to 1444 bp, and sequenciation evidenced that size differences were based on indels in the intronic region. Six out of ten individuals analyzed were heterozygous for the *cdh* gene. The level of heterozygosity obtained in the amplifications with our marker (0.6) did not differ from the expected heterozygosity (0.8) based on the Hardy-Weinberg equilibrium, as indicated by the P-value of the Fisher's exact test (0.626). Mendelian inheritance of *cdh* alleles in the control progenies was observed in all the I_T isolines analyzed (Table

3) since the observed genotypes did not significantly deviated from expected ratios, although we detected a non-amplifying EPIC-PCR marker. This fact suggested that its sequence was unambiguous and associated to only one *cdh* allele. This data set pointed out that the EPIC-PCR marker selected in this work was appropriate for the inheritance analysis of the *cdh* gene.

The I_T isoline #7 was discarded from the analysis of *cdh* segregation because resulted not informative since all the individuals of the F_1 generation were heterozygous for both the N and C alleles (Table 3). In three out of the other four I_T isolines, the genotype ratios observed in control group compared with tolerant individuals were not statistically different, neither in F_1 nor in the F_2 generations, indicating no association between their *cdh* alleles and the tolerance trait (Table 3). However, the isoline #1 showed statistically different genotype ratios between control and tolerant insects, suggesting association between tolerance and *cdh* alleles present in this isoline (Table 3). This observation could not be confirmed by the analysis of *cdh* segregation in F_2 generation, because single-pair matings of F_1 tolerant adults from isoline #1 did not produce any viable offspring. It should be noted that the predominant genotype in the group of tolerant larvae from the isoline #1 was CD. However, the alleles C and D were present in the genotypes of tolerant individuals from other I_T isolines, for which no differences were observed in genotype ratios between control and tolerant larvae.

4 DISCUSSION AND CONCLUSIONS

Previous genetic analysis of three different laboratory-selected *O. nubilalis* resistant strains described the resistance to Cry1Ab as incompletely recessive and controlled by more than one *locus* (Crespo et al., 2009; Alves et al., 2006), in contrast to what happens in the majority of the resistant strains of other lepidopteran species selected

with Cry toxins (Ferré *et al*., 2008). In addition, variations in susceptibility to Cry1Ab have been reported in field populations of *O. nubilalis* (Saeglitz et al., 2006; Farinós et al., 2004; Siegfried et al., 2001; Marçon et al., 1999). These two evidences suggested that tolerance to Cry1Ab, present in varying degrees in *O. nubilalis* field populations, may be studied from the genetic point of view of a quantitative trait. Our results are consistent with the hypothesis that some of the variation in tolerance within *O. nubilalis* populations has a polygenic inheritance, since we found significant heterogeneity in mortality among isolines that could be reduced in the F_2 generation after selection. In fact, the variation in tolerance determined by polygenic inheritance is normally distributed within a population and essentially continuous (Tabashnik and Cushing, 1989), resulting in a wide range of responses as we have observed in the present study.

The estimates of the upper limit of heritability for tolerance to Cry1Ab made by using two different methods were 0.90 and 0.82. Both values indicate that high levels of additive genetic variation in larval tolerance to Cry1Ab toxin were present in the *O. nubilalis* Spanish field-derived populations under study. The full siblings approach used in this study only provides an upper limit for heritability because the common environment and the dominance variance could inflate the estimates (Falconer and Mackay, 1996). However, Tabashnik and Cushing (1989) found that the overestimation bias introduced by experiments with full siblings may be expected as < 10%. Therefore, the heritability of tolerance to Cry1Ab estimated in this study is can be considered high, but comparable with the highest heritability estimates of insecticide resistance obtained in other insect pests reviewed by Omer and coworkers (1993). In the case of the *B. thuringiensis* toxins, a heritability of tolerance to Cry1Ab of 0.52 was found for a small sample of *Chilo suppressalis* from Philippines (Alinia et al., 2000), and Tabashnik (1994) reported that realized heritability of resistance to Bt products in seven different

lepidopteran species ranged from 0.04 to 0.61.. However, it should be noted that realized heritability after the first generation of selection often underestimates the heritability in the base population (Falconer and Mackay, 1996).. In *O. nubilalis,* the estimates of the realized heritability for Dipel resistance in five different field-collected colonies ranged from 0.16 to 0.46 in the first six generations but decreased to 0 – 0.08 in the last part of the selection process, when authors supposed to have obtained a homogeneous resistance (Huang et al., 1999).

Estimates of genetic parameters like heritability are specific to the conditions under which they were made, which means that heritability is not only a property of a genetic trait but also of the population, the environment and the experimental measurements (Falconer and Mackay, 1996; Firko and Hayes, 1990). Therefore, predictions based on heritability estimates should be made with caution (Alinia et al., 2000). If the heritability of tolerance in *O. nubilalis* populations under field conditions is as high as the one estimated in our study, then the long term use of Bt products for controlling this pest (like Bt maize expressing Cry1Ab toxin) would be seriously threatened by the resistance apparition. So far, the strategy widely used for delaying evolution of insect resistance to Bt crops is based on the creation of refuges for the random mating between susceptible and resistant insects and on the expression of high concentration of Cry toxins that reduce the dominance of the resistance and result into a decrease of the heritability sufficient to decrease the response to selection (Tabashnik et al., 2004). Up to now, this strategy has been highly effective in delaying apparition of *O. nubilalis* resistant individuals under field conditions as no resistance incidences have been reported (Farinós et al., 2004; Siegfried et al., 2001) or no major resistance alleles have been detected (Hellmich and Siegrfried, 2012; Engels et al., 2011).

In the present study, the 9% of the isolines, challenged with a Cry1Ab dose that caused an overall mean mortality of 87%, showed a significant higher survival rate. This means that the occurrence of the Cry1Ab tolerance trait is frequent in our Spanish field-derived populations. Previously, some authors reported that partial resistance alleles were common in field populations from France and USA (Bourguet et al., 2003; Andow et al., 2000). Larvae carrying partial resistance alleles could not complete the development on Bt maize expressing Cry1Ab, but they caused more injury to Bt plants than totally susceptible larvae (Andow et al., 2000). The additive effect of these alleles (together with the environment) could result into a continuous variation of the tolerance phenotype similar to the one detected in our study. Under this point of view, the common presence of partial resistance alleles in *O. nubilalis* populations might favor the evolution of a polygenic resistance under laboratory selection conditions (as the one emerged in this study or the ones described by Crespo et al., 2009 and Alves et al., 2006). However, Bt maize expressing a high dose of Cry1Ab toxin may only lead to a monogenic resistance caused by rare alleles, which has never been detected in *O. nubilalis* species (Siegfried and Hellmich, 2012). In accordance with this, up to now no resistant larvae of *O. nubilalis* strains selected with Cry1Ab were able to develop on commercial Bt maize plants (Crespo et al., 2009; Siqueira et al., 2004). The common occurrence of partial resistance alleles in natural populations should be considered, to avoid false early resistance detection in case of small changes in susceptibility to Cry1Ab toxin. Besides, it would be interesting to investigate if these partial tolerance alleles could act as modifier loci in the action of a major resistance allele, influencing the emergence of resistance (Andow et al., 2000).

Monogenic inheritance of tolerance is associated with a decrease of the standard deviation of the mortality values along the selection process, because a single genotype

would be quickly selected. In the case of several genes involved in the tolerance, the standard deviation would remain constant, and the selection response would be weak. Our results showed similar standard deviation values among isolines and generations, suggesting a polygenic inheritance of the tolerance. However, the fast response obtained to the selection (shown by a clear reduction in the mean mortality in just one generation) pointed out to few genes involved. Analysis of the minimum number of freely segregating factors by using the Lande's method estimated that tolerance to Cry1Ab was influenced by at least two *loci*. Previous studies with laboratory Cry1Ab resistant colonies of *O. nubilalis* estimated (with Lande's method and direct and indirect tests based on mortality recorded in backcross progeny) that the number of genes involved in resistance of the SKY colony was from 2.6 to 5 (Crespo et al., 2009), while in the resistant colonies Europe-R and RSST-R was higher than 10 (Alves et al., 2006). These results only provide indirect evidences for the additive contribution of a multiple number of *loci* to the tolerance or resistance phenotype in the *O. nubilalis* laboratory selected strains studied. Only linkage mapping could provide the direct evidence of a polygenic inheritance of the Cry1Ab tolerance. Nevertheless, to the date no genes have been genetically associated with resistance in *O. nubilalis*.

One of the candidate Cry resistance genes is *cdh*, which was genetically linked to resistance in three lepidopteran species: *Heliotis virescens*, *Pectinophora gossypiella* and *Helicoverpa armigera* (Gahan et al., 2001; Morin et al., 2003; Xu et al., 2005). Its role in the toxicity process caused by Cry1Ab in *O. nubilalis* was demonstrated by an *ex vivo* study which correlated the expression of the *O. nubilalis cdh* in sf9 cells with the increased susceptibility to Cry1Ab (Flannagan et al., 2005). However, several studies with *O. nubilalis* resistant strains did not clarify the involvement of the *cdh* in resistance to Cry1Ab toxin (Bel et al., 2009; Khajuria et al., 2009; Coates et al., 2008). We

investigated *cdh* allele segregation in the isolines selected for the tolerance trait using an EPIC-PCR marker specifically developed for this study. This marker showed a random pattern of indels and followed the Hardy-Weinberg equilibrium. Only the presence of a null allele could affect its usefulness; however we observed that it did not interfere with the Mendelian segregation. We detected six size alleles whose differences were due to intron indels, confirming the genetic variation for the *cdh locus* that was described in previous reports (Bel et al., 2009; Coates et al., 2005). One out of the five tolerant I_T isolines (isoline #1) showed significant differences in the genotype ratios of tolerant insects compared with controls, being CD the predominant genotype in tolerant individuals. However, the alleles C and D were also present in other I_T isolines that did not exhibit such association, even though never combined together as CD genotype. It is worth to note that the association between *cdh* and tolerance could not be confirmed by segregation analysis in the F_2 generation, because isoline #1 did not produce viable F_2 progeny. Although these findings appear to reject the implication of the C and D *cdh* alleles in the tolerance, it cannot be completely discarded since their whole sequences have not been determined.

In summary, the analysis of field-derived *O. nubilalis* populations collected in Spanish maize fields has revealed the common occurrence of Cry1Ab tolerance trait, which can be studied with quantitative genetic techniques. Analysis of the effective number of genetic factors indicated that few *loci* (at least two) contribute to the tolerance phenotype and that *cdh* could be one of them; further studies should be performed to confirm this implication and to identify other *loci* involved in tolerance. The results obtained in the present work have a special relevance from the resistance management point of view. The monitoring of field evolved resistance should be aware about the relevance to track and deal with the *O. nubilalis* tolerance trait since it may

confer to the insects the ability to survive on Bt plants during short periods of time, which would enhance the conditions for a potential development of resistance.

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FIGURE CAPTIONS

Figure 1. Diagram of the selection process.

Figure 2. Distribution of mortality per isoline, obtained in F_1 and F_2 generations. Mortality was recorded after 7 days of Cry1Ab exposure. White bars indicate F_1 generation whereas grey bars indicate F_2 generation. Arrow heads indicate I_T isolines (selected to found the F_2 generation because they had shown less than 60% of mortality in F_1 generation).

TABLES

Table 1. Larval survival of field-derived *O. nubilalis* isolines exposed to 40 ng of Cry1Ab per cm^2 of artificial diet

	F_1 generation		**F_2 generation**			
	Overall Isolines	**I_T isolines[a]**	**Overall Sib-I_T subfamilies**	**Sib-I_T subfamilies #76**	**Sib-I_T subfamilies #81**	**Sib-I_T subfamilies #109**
No. of single-pair matings	110	-	52	15	20	11
No. of isolines screened	53	5	31	11	15	5
No. of eggs laid	15242	1478	9743	4173	4390	1180
(mean ± SD)[b]	(288 ± 78)	(246 ± 83)	(314 ± 81)	(379 ± 66)	(293 ± 65)	(236 ± 41)
No. of Cry1Ab treated larvae	6506	760	3410	1443	1675	292
(mean ± SD)[b]	(123 ± 58)	(152 ± 42)	(110 ± 50)	(131 ± 51)	(112 ± 45)	(58 ± 25)
Mortality (%)[c]	87 ± 17	44 ± 14	62 ± 17	60 ± 16	66 ± 17	55 ± 16
(min-max)	(24-100)	(24-56)	(28-89)	(29-87)	(28-89)	(35-73)

[a] Isolines that showed mortality lower than 60% in the F_1 progeny, used to found the Sib-I_T subfamilies;

[b] mean ± SD per isoline;

[c] Data (mean ± SD per isoline) recorded after 7 days of Cry1Ab exposure.

Table 2. EPIC-PCR *cdh* alleles detected in the founders of the I_T isolines

Allele name	**GenBank acc. number**	**PCR product length**	**Intron length**
A	HQ185401	1444 bp	1287 bp
B	HQ185402	1219 bp	1062 bp
C	HQ185403	1024 bp	867 bp
D	HQ185404	827 bp	670 bp
E	HQ185405	758 bp	601 bp
N	-	No amplifying	-

Table 3: *cdh* genotypic ratios observed in the F_1 and F_2 generations of *O. nubilalis* I_T isolines

Isoline	**Generation (Mean mortality)**	**n[a]**		**Parental genotypes**	**Offspring Genotype**	**Frequency**		**P-value (df)[b]**
		Control larvae	**Tolerant Larvae**	♀ x ♂		**Control larvae**	**Tolerant Larvae**	
#1	F_1 (58.8%)	20	20	AD x CC	AC	0.65	0.3	0.027 (1)
					CD	0.35	0.7	
#7	F_1 (37.1%)	10	10	NN x CC	CN	1	1	1 (1)
#76	F_1 (56.3%)	10	22	DN x BN	BD	0.5	0.3	0.943 (3)
					BN	0.1	0.1	
					DN	0.2	0.3	
					NN	0.2	0.3	
#81	F_1 (23.8%)	10	30	CE x AB	AC	0.2	0.2	0.661 (3)
					AE	0.2	0.1	
					BC	0.3	0.3	
					BE	0.3	0.4	

#109	F_1 (44.3%)	10	10	AA x CN	AC	0.5	0.4	0.653 (1)
					AN	0.5	0.6	
***#76*-P8**[c]	F_2 (28.8%)	10	10	NN x DN	DN	0.3	0.4	0.639 (1)
					NN	0.7	0.6	
***#81*-P11**[c]	F_2 (27.6%)	10	10	AC x AC	AC	0.5	0.5	1 (2)
					AA	0.2	0.2	
					CC	0.3	0.3	
***#109*-P10**[c]	F_2 (39%)	10	10	AN x AC	AA/AN	0.6	0.6	0.565 (2)
					AC	0.1	0	
					CN	0.3	0.4	

[a] Number of individuals analyzed for *cdh* segregation;

[b] P-values obtained with χ^2 test, comparing the genotype ratios recorded in the control and in the larvae tolerant to Cry1Ab;

[c] SibI_T-subfamilies showing the lowest mortality value within each isoline

P

1 ♀ X **1**♂

> 80 eggs

F_1

Untreated control
(reared to 3rd instar and then frozen)

Cry1Ab screening

Mortality > than 60%

Larvae reared to 3rd instar and then frozen

$\underline{I_T}$ isolines

Mortality < than 60%

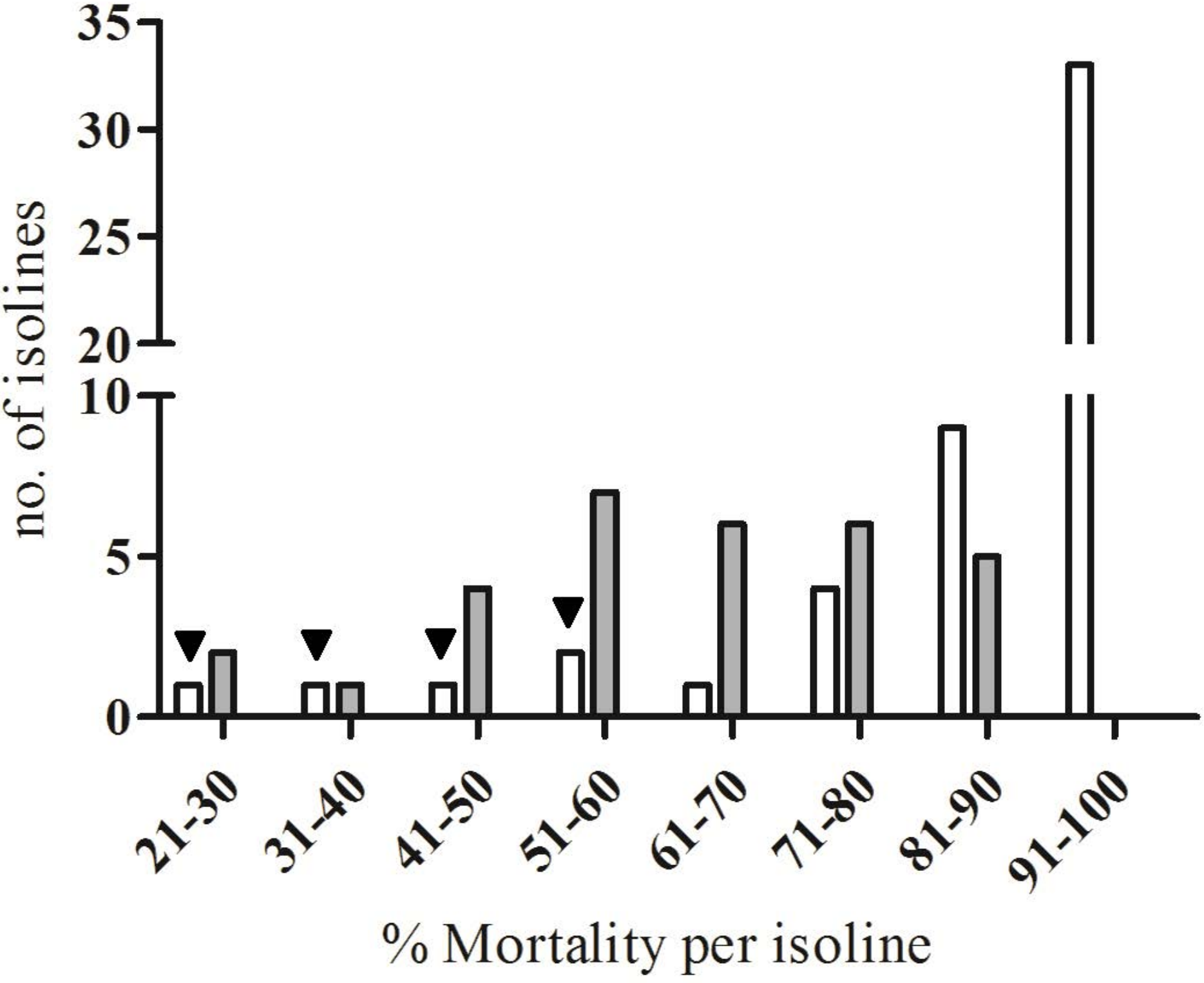
1 ♀ X **1**♂
(Cry1Ab tolerant larvae)

$\underline{Sib\text{-}P_T}$ sufamilies

F_2

Untreated control
(reared to 3rd instar and then frozen)

Cry1Ab screening
(reared to 3rd instar and then frozen)


35
30
25
20
10
5
0
no. of isolines
21-30
31-40
41-50
51-60
61-70
71-80
81-90
91-100
% Mortality per isoline