Large genetic variability of maize leaf palatability to european corn borer : metabolic insights

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Maize is the most-produced cereal in the world, but its production faces constraintssuch as parasitic attacks from stem-borers . We evaluated the resistance of a core-collection of 18 maize lines by measuring their palatability to European Corn Borer (ECB) larvae fed on maize leaf discs. Usingan original consumption test device that takes into account the variability of larvae behavior, we were able to phenotype the resistance of the 18 maize lines. We matched consumption data to existing enzymatic and metabolomic data that characterized the maize core-collection and identified some metabolites such as caffeoyl-lquinate, trocopherol, digalactosylglycerol and tyrosine that are positively or negatively correlated with the palatability to ECB larvae. Altogether, our results confirm the metabolic complexity involved in the establishment of plant defenses. Metabolic changes mainly related to membrane and cell wall composition, particularly those related to the phenylpropanoids pathway, were observed independently of plant developmental pace and earliness.[condensée:]Metabolic changes associated to leaf palatability mostly concern membrane and cell wall composition. Some of them, pointing-out to the phenylpropanoids pathway, were observed independently of plant developmental pace and plant earliness.

maize| European Corn Borer| feeding bioassays| plant genetic variability|plant metabolism

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Introduction

Maize is the leading cereal in terms of production volume, before wheat and rice (25) and plays diverse roles in global agrifood systems, including human alimentation. Worldwide, 6% to 19% of global maize production is lost each year due to insects and other herbivores preying (50). More than 90 insect species are known to feed on cultivated maize (64). Among them European Corn Borer (ECB) *Ostrinia nubilalis* (Hübner), and Mediterranean corn borer (MCB) *Sesamia nonagrioides*, damage maize by boring tunnels within the stems of the plant. Fodder maize plots infested by the European Corn Borer can show up to 80% of plants and 40% of cobs damaged (7), while a single larvae per plant (?)may cause 6% loss in an average grain yield on maize hybrids (9). In the course of the evolutionary arms race between plants and pests, plants developed many different defense strategies, including physical defenses to minimize the entry of pathogens like cell wall or spines, and biochemical defenses that can be repulsive or toxic (67). They can be constitutive or induced with different resource allocation costs (53). The setting-up of chemical plant defenses is a paradigm of biological complexity. It begins with the exogenous signal perceived from the pathogen and continues with signal perception and signal transduction that may result in the reprogramming of cellular metabolism towards the biosynthesis of secondary metabolites (59). Signal transduction is regulated by hormones (38) and results in a coordinated response mediated by a crosstalk between phytohormones and transcription factors (26, 47).

Indeed, the setting-up of plant chemical defenses has a metabolic cost and mobilizes resources that could have been allocated to other functions like growth or reproduction (32, 33). It may result in trade-offs (29) between different life-history traits. The cost of defenses can affect the carbon-nutrient balance (54), the growth rate (68), or the growth-differentiation balance (62). Those hypotheses are difficult to test (63). Indeed, manifestation of detectable trade-offs may depend on the strength of resources limitation or other factors (73). For example, in brown algae, phlorotannins play a role in both primary and secondary metabolism and cannot serve as a reliable indicator (4). However, a recent meta-analysis over a wide range of plants species showed that herbivory reduced growth, photosynthesis and reproduction, but not carbohydrate contents (28).

In maize, resistance to European Corn Borer encompasses both the synthesis of specific antibiosis molecules like DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) (14, 60) and changes in the molecular composition of cell wall. In particular, phenolic acids like ferulic or p-coumaric acids may increase leaf toughness (8). More generally, variations in cell-wall phenylpropanoids are associated with resistance to corn borers (30).

One way to measure plant resistance to phytophageous insects is to measure leaf disks' palatability to insect larvae. Such method can also be used to evaluate the antifeedent properties of specific chemicals (1, 41, 48, 49, 57). Feeding preference tests are efficient screen-tests to select resistant plant varieties (19, 65). For example, those methods have been used to measure the palatability of Brassicae plants

for *Microtheca punctigera* larvae (45). They allowed for the identification of rice varieties resistant to the lepidoptera *Cnaphalocrocis medinaiset* (6).

Prime to the identification of specific traits associated to resistance or tolerance, it is necessary to evaluate the extent of genetic variability for this trait within the plant species/genetic group colorpink using a small number of varieties that represent the genetic diversity within a species or a collection (12, 23). In maize, the evaluation of a panel of 85 inbred lines representing the diversity of the varieties cultivated in Europe allowed for the identification of specific inbreds resistant to *Sesamia nonagrioides* and *Ostrinia nubilalis* after artificial infestation (43), but also for the identification of inbreds able to maintain the plant yield despite pest pressure (44).

In the present study, we used a core-panel of 18 maize inbred lines chosen to represent the genetic diversity of maize varieties cultivated in Europe and North America (11), but also a range of variation for the resistance to European Corn Borer (3, 70) and for cell-wall digestibility (24, 72). Most of the inbred lines from the panel were already shown to present a wide genetic diversity for a large set have already been shown to exhibit a wide rangeof physiological, enzymatic and metabolic data (18). We used an original consumption test (57) to measure the genetic variability of leaf-disks palatability to ECB larvae. Making use of the availability of this large dataset, the objectives of the present paper were (i) to assess the amount of genetic variability of maize leaf palatability to European Corn-Borer, and (ii) to seek for correlations between leaf palatability and metabolic or physiological traits that characterized the inbred lines.

Methods

Insects rearing. *Ostrinia nubilalis* Hbn.eggs were obtained from Bioline AgroSciences (France). Hatched larvae were maintained in Petri dishes on an artificial diet (1.32l water, 27g agar powder, 224g corn flour, 60g dried yeast, 56g wheat germ, 12g L-ascorbic acid, 4g vitamin mixture and minerals (Réf.0155200), 0.8g chlortetracycline, 2g hydroxybenzoic acid methyl, 1.6g sorbic acid and 4g benzoic acid), under 16 :8 (light: dark) photoperiod at 70% humidity and at 26°C. Second instar larvae (10 days old) were used for the feeding bioassays.

Plant material: core collection. The plant material comprised 18 maize inbred lines (Table 1). 13 of them belong to a core-panel of 19 lines representative of the genetic diversity of modern varieties cultivated in North-America and Europe (11, 15). Those 13 lines were previously characterized at two developmental stages for their variability for central carbon metabolism enzymes activities, metabolites concentrations and a set of physiological traits (18). Among them, two inbred lines (B73, Mo17) are already known for their sensitivity to pyralids attacks (42, 70). The panel was completed with three inbred lines (F66, F271, CM494) exhibiting differences for cell wall digestibility (24, 72), and two inbred lines (F618, F918) known for their tolerance to pyralids attacks (3). All the lines are maintained in the *Centre de Ressources Biologiques INRAEdes lignées de maïs* at Saint Martin de Hinx, France. Female flowering time (FFT, TO:0000359 from the Plant Ontology (69)) was predicted by combining data from (11) and yearly measurements at the INRAE field station from Saint Martin de Hinx, France (see Supplementary Methods S1). It was measured in degree-days, by transforming the time-scale into Accumulated Thermal Time (51). As shown in Table 1, the 18 inbred lines belong to four different maize genetic groups and show a wide range of flowering time variation.

Plant material: growing conditions. : To compare the inbred lines at the same developmental stage, flowering time was used to constitute four different sowing groups (Table 1) and to plan three to ten different sowing dates by group. Altogether, sowing were realized between october 1st 2019 and november 1st 2019 (Supplementary Methods S1). For each sowing date, six seeds per line were pre-germinated on sowing plates until 3-4 visible leaves. Then each plant was repoted in 4L pots containing Jiffy® premium substrate and deposited on a shelf that contained plants from the 18 inbred lines at the same developmental stage and constitute a replicate for the feeding bioassays (Fig 1a). Plants were cultivated in a greenhouse under 16:8 (light: dark) photoperiod with 70% humidity and a temperature comprised between 21 and 24°C. Shelves were watered two times a week. The position of the pots in the shelf was randomized.

Feeding Bioassays. Delayed sowing according to known flowering time allowed to grow four replicates with three plants per inbred lines at approximately the same developmental stage. Only three replicates were used for the feeding bioassays. Because the data were to be compared with metabolic and physiological data collected by (18), we chose the vegetative developmental stage between GRO:0007011 (tassel initiation) and GRO:007013 (ear initiation) from the Cereal Plant Development Ontology (69). This corresponds to plants having between 5 (V5) and 7 (V7) visible leaf collars.

For each inbred line, one replicate consisted of a 50-wells (5x10) plate with 50 leaf discs punched from the sixth leaf of the three plants. Each plate was first filled with agarose 1% to avoid the drying out of the leaf discs. Then, a single L2 instar larva was placed in each well on each leaf disc (10) following (57) (Fig. 1b). The feeding bioassays experimental set-up has a limited capacity of only six plates at a time. Also, the disposal of the leaf discs and the larvae in the plates is time-consuming. Therefore, the 18 lines from the same replicate have been distributed into three batches (see Supplementary Methods S1) that were run one at a time during three consecutive days.

Leaf disc consumption by L2 larvae was monitored for each individual cell for 48h and generated image stacks. Image stacks were analyzed using the plugins *RoitoRoiArray* and *Areatrack* from the image software Icy (20). Plugins serve at delimiting wells and leaf discs in a semi-automated approach, then follow the changes through time in the number of pix-

Line	Pedigree	Group	FFT
F64	Argentina PI-186.223	EF	79^A
SA24U	Pop-corn	CBD	77^A
HP301	Pop-corn	EF	76^A
F918	**F618** x **F630**	SS	75^A
B73	Iowa Stiff Stalk synthetic BSSSC5	SS	74^A
Lo32	Isola Basso	EF	74^A
Mo17	CI187-2 x C103	CBD	73^B
MBS847	Pioneer 3901	CBD	71^B
Lo3	Nostrano dell'Isola	EF	71^B
F618	(**A166** x B37) x B37	SS	70^B
NYS302	Black Mexican	NF	66^C
C105	Purple Flour #626 x Ohio Early Yellow Dent inbred #25	NF	66^C
F252	F186 x Co125 -8.2.3.3.4	CBD	63^D
F271	Co125 x W103 (19.3x8.5) -4.4.1.1.1.1	CBD	62^D
Cm484	(Canada-Morden-1989)200-2-1		62^D
F2	Lacaune -2.9.1.1.3	EF	61^D
F66	Sost -15.8	EF	61^D
ND36	Manitoba Yellow Flint	NF	59^D

Table 1. Description of the maize panel Pedigree and genetic group of each 18 inbred lines from the panel. Genetic groups are Corn Belt Dent (CBD), European Flint (EF), Northern Flint (NF) or Stiff-Stalk (SS). Lines in **bold** were selected for their tolerance to pyralids attacks. The average female flowering time FFT is expressed in [PAS des degrés-jour, plutôt des jours après semis à Saint Martin de Hinx]degree- days after sowing. The letter above indicates the sowing group for feeding bioassays.

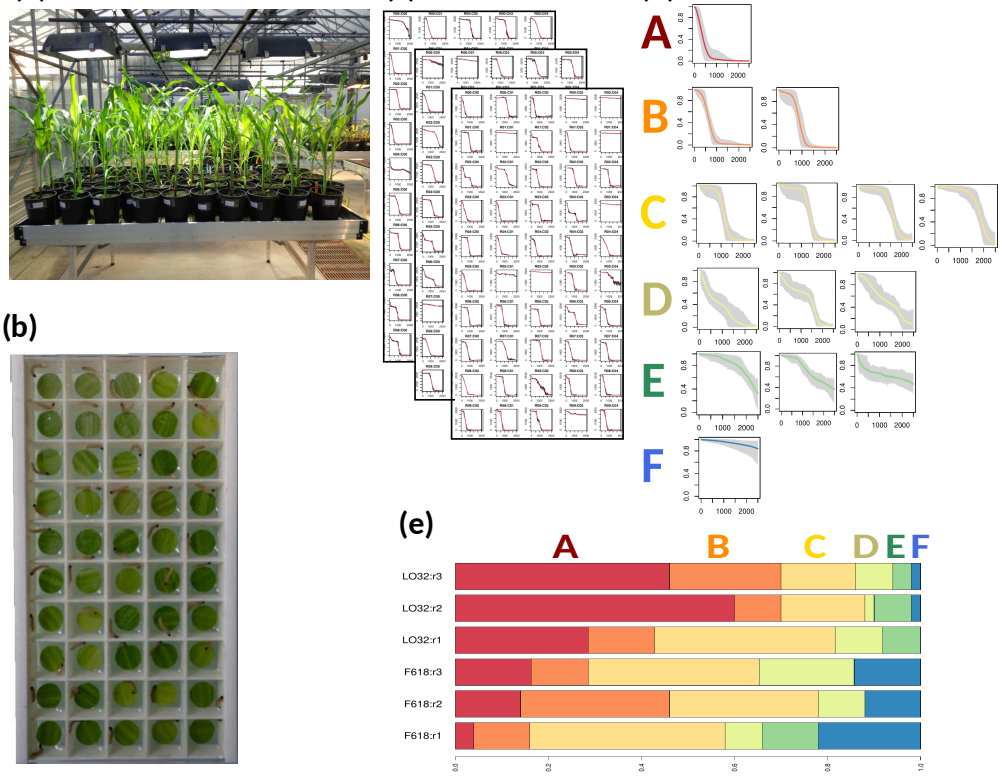

Fig. 1. Overview of the larvae feeding bioassays. (a) Plants from the 18 inbred lines of the panel were grown in a greenhouse. Delayed sewing allowed to sample plants at the same developmental stage for each replicate. **(b)** For each inbred line/replicate, 50 leaf discs were arrangedinto a 50-cells plate. A single L2 larva was deposited into each cell at the beginning of the monitoring. **(c)** The consumption of 50 leaf discs by L2 larvae was monitored during approximately 24h. Image analysis allowed to measure the proportion of leaf discs consumed at each time step. **(d)** Clustering methods allowed to classify the consumption curves into six insect larvae behavioral groups, from consumers (group A) to haters (group F). **(e)** The output was the proportion of each behavioral type in each replicate. As an example, the three replicates from the two most extreme lines $F2$ and $Lo32$ are shown

centring

els from each leaf disc. Image analysis generates measures (pixel per minute) for each leaf disc in each well that is exported as an Excell spreadsheet. Data are converted into CSV flat files and further analyzed with the R software (52).

Statistical analyses of feeding bioassays: behavioral types. As in (57), data analyses were conceived as a two-stage procedure. The first stage consisted in describing the variability of individual larvae feeding behaviors and classi-

fying them into six behavioral types. At the end of this stage, each inbred line/replicate is characterized by the distribution of behavioral types. The full procedure and corresponding R scripts are available online (55).

Briefly, the R scripts generates pdf files representing consumption curves for each larva in each well (Fig. 1c). Different larvae are not expected to have exactly the same behavior even when submitted to the same conditions. A non-supervised hierarchical classification of the 2700 individual consumption curves corresponding to the 3 replicates of the 18 inbred lines was realized using the SOTA algorithm (34). It ended-up with 14 clusters identified by a letter from a to n. To reduce the number of groups, each cluster was characterized by summary statistics like the average times T20, T50 and T80 to consume 20, 50 or 80% respectively of the leaf disc area, or the total consumption. Based and those summary statistics, the Kmeans algorithm (31) along with some manual grouping were used to group the 14 clusters into six ordered feeding behavioral types, named from A to F. Average feeding behavioral profiles and their range of variation are presented on Fig. 1d.

- A, B, C behavioral types mainly differ in the existence or not of a lag-time before consuming, and in the length of the lag-time. Generally, the leaf is fully consumed at the end of the experiment. A types correspond to fast consumers.
- D, E, F behavioral types are reluctant to consume. Consumption rate is low. Generally, the leaf is not fully consumed. Type F larvae are non-consumers.

At the end of the process, each consumption curve, corresponding to a single well in a single plate is attributed a behavioral type, from A to F. For each inbred line i and each replicate j, the distribution of behavioral types can be counted. We called Z_{ij}^k be the number of observations of behavioral type $k \in \{A,B,C,D,E,F\}$ from inbred line i in replicate j. Fig. 1e shows three examples of the Z_{ij} distribution, corresponding to the three replicates from inbred line Lo32 and the three replicates from inbred line F618.

Statistical analyses of feeding bioassays: AFratio. Rigorously, we could use multinomial regression to test whether line or replicate change the behavioral distribution. However, the experimental set-up, with three replicates per inbred line lacks of power. Instead, we proposed to build quantitative statistics to measure consumption behavior by reducing the behaviors into two classes: *consumers* versus *haters*:

$$CHratio = log(\frac{\#\quad consumers}{\#\quad haters})$$

There are many possible combinations to group six classes (A to F) into two (consumer, hater). Among all possible combinations, we decided to choose the one that allowed for the best discrimination between the inbred lines. For each possible combination, we ran a linear model

$$CHratio_{ij} = \mu + Line_i + Rep_j + \epsilon_{ij} \quad \textbf{(1)}$$

and recorded the adjusted $R2$ for the model, as well as the heritability of the line effect

$$H^2 = \frac{MSL - MSE}{3}$$

where MSL is the mean square associated with the Line effect, MSE is the error mean square and 3 is the number of replicates.

Results are detailed in Supplementary Methods S2. The most discriminant combination was the AFratio, *i.e* the log-ratio of the proportion of A-type fast consumers over the proportion of F-type non-consumers:

$$AFratio_{ij} = log\left(\frac{Z_{ij}^A}{Z_{ij}^F}\right) \quad \textbf{(2)}$$

The anova model (eq 1) was used to compute the mean AFratio for each inbred line, as well as confidence interval. Comparison of inbred lines means were performed using Tukey Honest Significant Difference tests (71).

Pearson's correlation coefficient between AFratio and Flowering time (FFT) was also computed.

Correlations with plant metabolism and physiology. Thirteen inbred lines from our panel were characterized at two developmental stages for a set of physiological, enzymatic or metabolic data. We used the data available as Supplementary Dataset1 from (18) to compute the average value for each inbred line. In (18), the vegetative stage V was considered as plants with 7 to 8 visible collar leaves. It roughly corresponded to the ear initiation stage (GRO:0007013 from the Cereal Plant Development Ontology) and is comparable to the developmental stage used in the present study. As in the present study, samples from the sixth leaf were used for enzymatic, metabolomic and physiological analyses. The second developmental stage was 15 calendar days after silking (15DAS). It corresponded to the blister stage (GRO:0007030). This stage is initiated when significant starch accumulation begins, approximately 12-17 days after pollination.

Altogether, the dataset comprised enzymatic activity (Vmax) from 29 enzymes from central carbon metabolism and relative concentration ($nmol.mg^{-1} leaf\ FW$) from 155 metabolites at two developmental stages (V and 15DAS). It also comprised the measurement of Yield, kernel number and Thousand Kernel Weight at maturity, as well as dry matter content, C/N ratio, and the C, N and nitrates content at the two developmental stages (V and 15DAS).

Among the 383 traits measured, 228 were variable between the 13 lines of our panel. 89 of them showed a qualitative variation (presence/absence or no more than three different abundance values). Among those 89 traits, 27 were present or absent in a single inbred line and were subsequently discarded. 139 traits showed a quantitative variation within our panel.

The 62 remaining traits with presence/absence were treated as qualitative. Their relationship with AFratio was analyzed

with a linear model:

$$AFratio_{ik} = m_0 + \Delta_k + \epsilon_{ik} \quad \textbf{(3)}$$

where m_0 is the mean $AFratio$ among the inbred lines where the trait is absent, and Δ_k is the average effect of the presence of trait k.

For the 139 traits with quantitative variation within the panel, Pearson's correlation coefficient with AFratio and FFT was computed, as well as the corresponding pvalue. Traits with a $pvalue < 0.05$ were retained as associated. A Principal Component Analysis was run to explore the correlations between associated traits.

Results

We used an original high-throughput design for feeding bioassays (57) to measure genetic variation of maize leaf palatability to second instar larvae from the european corn-borer *Ostrinia nubilalis* Hbn within a maize inbred lines core-panel. The maize panel covered the main maize genetic groups that represent the diversity of maize varieties cultivated in Northern America and Europe (11). As shown in Table 1, the panel presented a wide range of variation for flowering time between ND36, that flowers 59 degree-days after sowing, and F64 that flowers 79 degree-days after sowing.

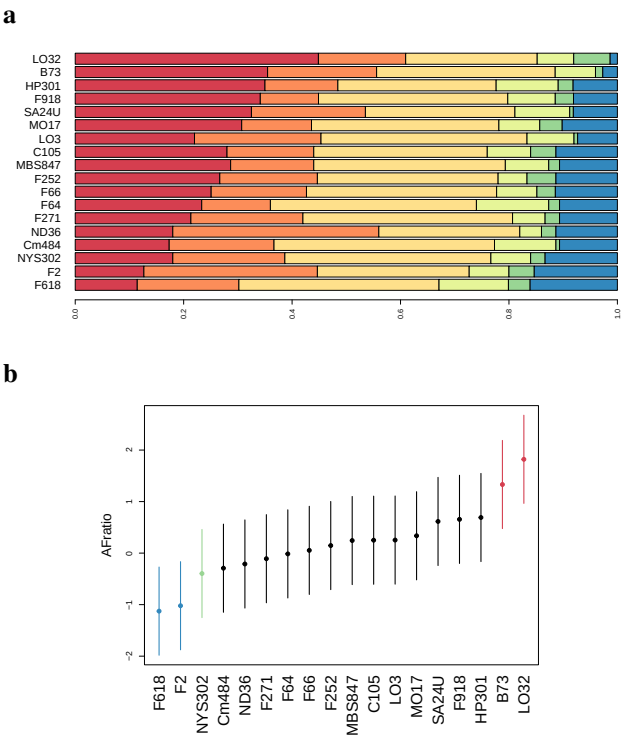

Fig. 2. Feeding bioassays a. Average proportion of each feeding behavior for each of the 18 inbred lines from the core-panel. Colors are the same as in Figure 1. **b** Range of variation for the AFratio. Dots indicate the mean. Lines the 95% confidence interval round the mean. Colours highlight groups of lines with significant differences.

Classifying feeding behaviors. Maize leaf palatability was assessed during the vegetative growth stage, when plants exhibit between 5 and 7 visible leaf collars. Delayed sowing dates were used to sample plants from the different inbred lines at the same developmental stage. Feeding bioassays consisted in measuring the consumption of leaf discs from the sixth leaf by second instar pyralids larvae. Instructions for building-up the feeding consumption bioassays device are freely available (56). Image analysis was performed using plugins embedded into the image software Icy (20). R scripts for statistical analyses were deposited in (55) Fig. 1 gives a general overview of the process. Clustering methods were used to classify individual consumption curves into six ordered behavioral profiles, names from A to F that captured both differences between leaf samples and behavioral differences between larvae. Fig. 1d, shows the percentage of intact leaf disc as a function of time for each behavioral profile. Clearly A types are *consumers* that feed fast and consume all the leaf disc, while F types are *haters* that hardly feed on the leaf disc. In between, B to E behavioral profiles are intermediate. B and C mainly differ from A by the existence of a lag-time: larvae wait before feeding. D and E mainly differ from F by the fact that at least part of the leaf disc is consumed at the end of the experiment. They differ from A, B or C by the consumption rate, which is always lower. The same range of variation of behavioral profiles were observed in (57), where larvae were confronted to leaf discs from a single maize variety treated with different concentrations of antifeedant molecular compounds. Here, **the variability of behavioral profiles reflects natural variations for palatability between sampled leaf discs**. Those differences may come either from growing conditions or from differences between the inbred lines.

Assessing genetic differences for maize leaf palatability. In order to assess for genetic differences between lines, the distribution of behavioral profiles was established for each inbred line and each replicate by counting-out the number of leaf discs exhibiting the different behavioral types. Fig. 1e show the results for the three replicates from inbred lines Lo32 and F618. Clearly, there were variations between replicates. However, the proportion of A behavioral type is always high in Lo32 and low in F618, while the proportion of E behavioral types is always low in Lo32 and high in F618. Fig. 2a shows the average proportion of each feeding behavior for each inbred line of the core-panel. The average proportion of A-type behaviors ranges from 12% in F618 to more than 40% in Lo32 and clearly exhibits a quantitative variation within the panel. The average proportion of F-type behaviors decreases with the average proportion of A-type behaviors. It ranges from 18% in F618 to 2% in Lo32. Hence, it seems that genetic differences between lines at least partly drive the observed differences between leaf discs.

In order to provide with a statistical test, AFratio was computed as the log-ratio of the number of leaf discs attributed to the A behavioral type to the number of leaf discs attributed to the F behavioral type (eq 2). The AFratio was shown to be the most discriminant log-ratio among all possible comparisons (Supplemental Methods S2). An analysis of variance tacking into account the inbred line and the replicate effects

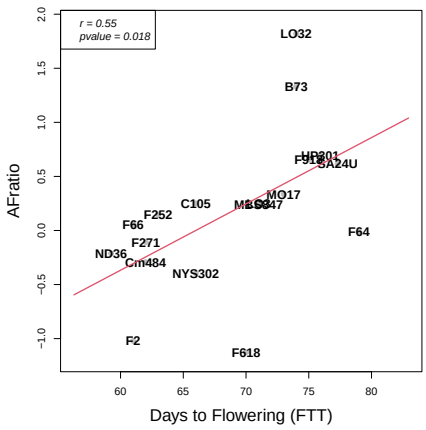

Fig. 3. Correlation between Flowering time and AFratio The scatterplot shows the relation between the average AFratio estimated for each inbred line and its average female flowering time FFT. The red lines corresponds to the regression line.

(eq 1) showed that both effects were significant. The pvalue associated to the replicate effect was 0.007. Indeed, replicates **r2** and **r3** tended to have a higher AFratio than replicate **r1** (Fig. 8 from Supplementary Methods S2). The pvalue associated with the line effect was 0.0015 and the between-line heritability was $H2 = 0.40$. Fig. 2b shows the mean AFratio and its confidence interval estimated for each inbred line of the panel. AF ratio ranged from -1 (excess of F-types in F618 and F2) to $+1$ to $+2$ (excess of A types in B73 and Lo32). Inbred line NYS302 was close to F618 and F2 and significantly different from B73. Despite the lack of power to detect significant differences, AFratio showed a continuous variation between lines that reflects genetics differences of maize leaf palatability to european corn-borer.

Fig. 3 shows the correlation between AFratio and flowering time (r=0.55, pvalue=0.017). The pattern is clearly nonlinear. However, except for the four extreme lines (F2, F618, B73, Lo32), there seem to be a positive relationship between AFratio and flowering time. Inbred lines that flower earlier seem to be less attractive to pyralids, with an excess of F behavioral types.

Correlation with metabolic and physiological traits. Thirteen inbreds lines of the panel were thoroughly characterized for a large set of enzymatic, metabolic and physiological traits (18) at two developmental stages : vegetative (V) and grain-filling (15DAS). The vegetative stage, around rapid stem elongation and ear initiation was the same as the one targeted in the present study for feeding bio-assays. We took the opportunity of the availability of the data to explore the relationships between maize leaf palatability measured by $AFratio$ and metabolic or physiological characteristics of the inbred lines.

Among the 62 qualitative traits that were either present or absent and where analyzed by anova using (eq 3), four metabolites and one amino-acid concentrations showed a significant effect on AFratio and were reported in Table 2. They all belong to the phenylpropanoid pathway (3). Notice that the average effect associated with the presence of the compound was important and corresponded to almost half of the differences in leaf palatability between the two most extreme lines. Presence/absence of the compound defines two groups of lines, one always comprising the less palatable line F2, and one always comprising the most palatable line B73. The presence of Tyrosine, Coumaroylquinate, and Tocopherol in the F2 group is associated with a decrease of leaf palatability. The presence of Caffeoylquinate.trans and Caffeoylquinate.cis in the B73 group is associated to an increase of leaf palatability. Notice that the number of maize lines composing the two groups changes depending on the compound. While the organic acid *Coumaroylquinate* was only present in the three less attractive inbred lines of the sub-panel (F2, ND36, NYS302), the effect of the presence of the other compounds seems to be less clearcut, suggesting that the modulation of leaf palatability has complex mechanisms.

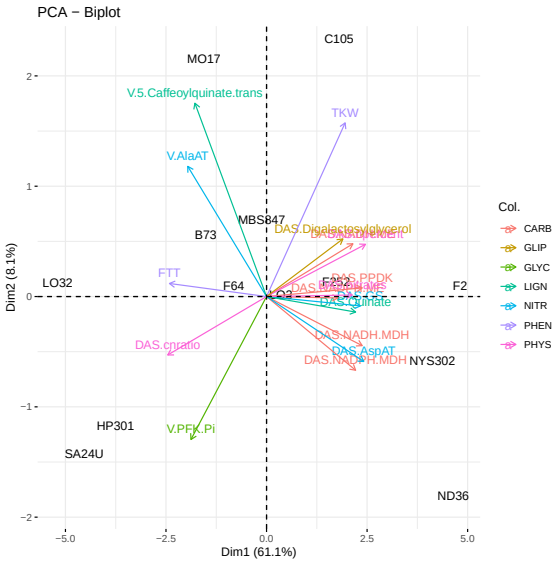

Fig. 4. Correlations between associated quantitative traits. Biplot representation of the Principal component analysis showing the position of the inbred lines in the 2-dimensional space engendered by the first two principal components, along with the corresponding correlation circle. The colors of the traits correspond to their attribute, including metabolic pathway : Carbon fixation (CARB), glycerolipids (GLIP), glycolysis/neoglucogenesis (GLYC), lignins and phenylpropanoids (LIGN), nitrogen assimilation (NITR), physiological (PHYS) or phenotypic (PHEN).

Among the 139 traits with a quantitative variation within the sub-panel, 17 were significantly correlated to AFratio, while 13 of them were also significantly correlated to flowering time (Table 3). The functional annotation of all metabolites from Table 2 and Table 3 was achieved using the MetaCyc (17) and KEGG (37) databases and presented in Supplementary Data S3. Fig. 4 shows the correlations between those 17 traits and flowering time in the form of the correlation circle of the principal components' analysis. The first PCA axis explains 57% of the total inertia and separates inbred lines according to their flowering time. It is mainly driven by differences between the late Lo32 and SA24U and the early F2, that were also the most extreme for leaf palatability. Traits correlated to PCA axis1 were mainly traits measured at a late developmental stage (15DAS) during grain filling. Early inbred lines are associated with higher activity of enzymes involved in carbon fixation and nitrogen assimilation 15 days

Trait	Δ $(pvalue)$	Lines
DAS.Tyrosine	-0.96 (0.011)	F2, F64, MBS847, ND36, NYS302
V.4.Caffeoylquinate.trans	0.85(0.045)	*F2, F252, F64*
V.3.Caffeoylquinate.cis	0.79(0.050)	B73, C105, Lo32, MBS847, MO17
DAS.Coumaroylquinate...	-1.11 (0.011)	F2, ND36, NYS302
DAS.Tocopherol	-1.61 (0.031)	Lo32, SA24

Table 2. Association between AFratio and qualitative traits Δ is the average effect of the presence of the trait on AFratio (eq 2). Pvalues are between brackets. When inbred line names are *emphasized*, this means that the compound is absent. Otherwise, list of the inbred lines where the compound is present. Abbreviated traits names are the same as in (18).

Trait	$r_{AFratio}(pval)$	$r_{FTT}(pval)$
V.PFK.Pi	0.64 (0.018)	0.39 (0.192)
V.AlaAT	0.75 (0.003)	0.65 (0.016)
V.5.Caffeoylquinate.trans	0.69(0.009)	0.46 (0.117)
V.Glycerate	-0.55 (0.049)	-0.42 (0.148)
DAS.NADPH.ME	-0.60 (0.030)	-0.62 (0.023)
DAS.NADH.MDH	-0.76 (0.003)	-0.78 (0.002)
DAS.NADPH.MDH	-0.66 (0.013)	-0.62 (0.024)
DAS.NADH.ME	-0.61 (0.026)	-0.57 (0.044)
DAS.PPDK	-0.58 (0.038)	-0.84 $(3e-04)$
DAS.AspAT	-0.68 (0.010)	-0.72 (0.005)
DAS.GS	-0.57 (0.041)	-0.64 (0.018)
DAS.cnratio	0.63 (0.022)	0.80 (0.001)
DAS.npercen	-0.63 (0.022)	-0.78 (0.002)
DAS.nitrates	-0.68 (0.011)	-0.70 (0.008)
DAS.Digalactosylglycerol	-0.75 (0.003)	-0.43 (0.141)
DAS.Quinate	-0.67 (0.012)	-0.74 (0.004)
TKW	-0.61 (0.026)	-0.64 (0.017)

Table 3. Correlation between AFratio, FTT and quantitative traits For each trait, Pearson pairwise correlation coefficient with AFratio (eq 2) and flowering time (FTT), respectively. Corresponding pvalues are given between brackets. Traits names were the same as in (18).

after silking (15DAS). This results in a higher percentage of nitrogen and nitrates, and a lower C/N ratio. For those traits, causal relationships with AFratio are difficult to disentangle from a pleiotropic effect of phenology. The second PCA axis explains 10% of the total inertia and separates lines that belong to the group with a high AFratio (C105, MO17, MBS847, B73) from lines that belong to the group with a lower AFratio (F252, HP301, ND36). Traits correlated to PCA axis 2 were mainly traits measured during the vegetative development (V). In particular, PCA axis 2 shows a strong positive correlation with *caffeoylquinate*, an organic acid involved in the biosynthesis of lignin (phenylpropanoids) that belong to plant secondary metabolism.

Fig.5 shows two examples of correlations between metabolic traits, AFratio and flowering time (FTT). The positive correlation between the activity of the enzyme *Alanine aminotransferase* (AlaAT) and AFratio during vegetative stage can be explained by flowering time differences. On the contrary, the concentration of *caffeoylquinate* during the vegetative growth stage is positively correlated to AFratio and independent of earliness.

Discussion

We used a new feeding consumption test (57) to evaluate maize leaf discs' palatability to European Corn Borer larvae within a core panel of 18 temperate maize inbred lines. The objectives were to assess the extent of genetic variability for palatability within the panel, and to link those variations to plant metabolism.

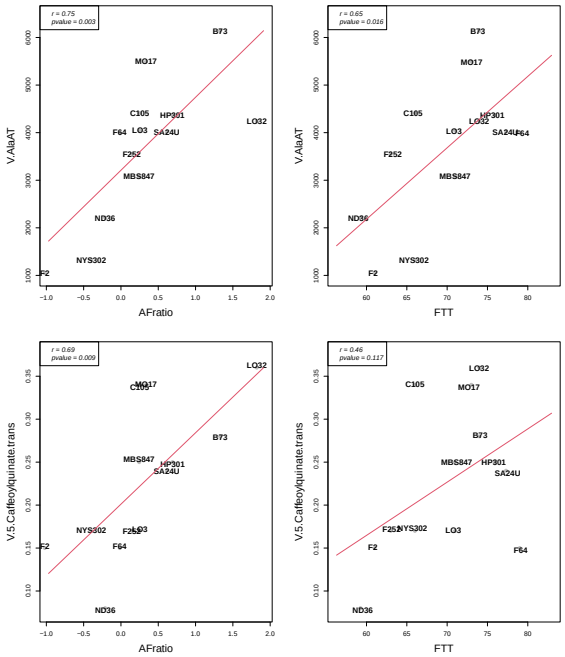

Fig. 5. Examples of correlations with quantitative variables. Scatterplot of the relation between quantitative traits and AFratio and flowering time (FTT).

Most consumption tests characterize leaf consumption through time by a single instant parameter like the time to consume half of the leaf disk (21, 45, 58). Those tests typically lack of power for two reasons. First, they fail to take into account the natural variability of individual larvae behaviors (6). Second, because larvae may change their behavior through time (35) and (Fig1d). Our experimental set-up bypasses both drawbacks. First, it allows for the observation of the feeding behavior of a large number of individual larvae (50) within each biological replicate. Second, instead of summarizing the behavior by an instant or average value, it proposes an original method to classify individual consumption curves into ordered feeding behavioral types, named by letters from A to F. In this study, we observed six main feeding behaviors that go from the immediate consumption of the whole leaf disc (A type) to the absence of consumption (F type). Intermediate behaviors correspond to lag-time before consumption (B and C types) or slower consumption rates (E type) with breaks (D type). In a previous study, we demonstrated the link between larvae behavioral type and leaf palatability by using antifeedant molecules (57). Here, we observe the same kind of variability for larvae be-

havior when confronted to different maize inbred lines. The proportion of the different feeding behavior types within a biological replicate takes into account natural variability between individual larvae and measures the average palatability of the sample. We propose here a quantitative measure of leaf palatability, named AFratio, and computed as the log-ratio of the two most extreme behaviors, A and F. **We found genetic variability for AFratio between inbred lines within the panel with a broad-sense heritability around $H2 = 0.40$ and confirmed the interest of the method for consumption tests**.

While our data clearly show genetic variation for leaf palatability to ECB, we only have indirect evidence concerning the link between leaf palatability and plant tolerance in the field. Classically, tolerance to ECB is assessed through the measurement of plant damages after artificial field infestation. Our panel comprised a few inbred lines for which tolerance/sensitivity to ECB have already been assessed in field experiments. The tolerant line $F618$ (3) is the less palatable from our panel while the sensitive line $B73$ (42, 70) is amongst the lines with the highest AFratio (Fig 2). $Mo17$ is reputed to be sensitive and stands in the top 6 inbred lines with the highest AFratio. In the same line, the relative ordering of the lines $B73$ (36% of A types and 3% of F types), $HP301$ (35% of A types and 8% of F types) and $Mo17$ (31% of A types and 11% of F types) is similar to the one obtained by measuring *S. frugiperda* Smith larvae growth rates on leaf disks (36). However, the link between leaf palatability and the amount of plant damages in the field stays complex. The inbred line $F918$ shows a moderately high palatability while it was derived from $F618$ and selected for tolerance. **Altogether, our new feeding consumption test allowed us to classify the panel inbred lines for leaf palatability. Lines $Lo32$ and $B73$ were the most palatable, and lines $F6128$, $F2$ and $NYS302$ the less palatables.**

Interestingly, the link between leaf palatability and earliness is not straightforward. There is a moderate positive linear correlation between earliness and AFratio ($r = 0.55$, $pvalue = 0.018$, Fig 3). Fast development tends to be associated with a higher level of defenses when measured during the vegetative plant stage, in contradiction with the *growth or defend* trade-off (32). However, Late lines exhibit a wider range of variation for leaf palatability and comprise both the tolerant poorly palatable $F618$ and the sensitive highly palatables $B73$ and $Lo32$. Such patterns can be explained by gain or losses of metabolic functions due to random genetic drift or selection history (remember that $F618$ have been selected for tolerance to ECB (3)). The relatively high palatability of early maize inbreds could be explained by local adaptation between plant and insect phenology: in environments favorable to the culture of early maize varieties, insect phenology leads to earlier attacks and resulted in the selection of plant lines able to mobilize their defenses at earlier developmental stages. This hypothesis could have been tested by setting-up leaf consumption tests at different plant developmental stages.

In maize, there is a long standing literature about genetic

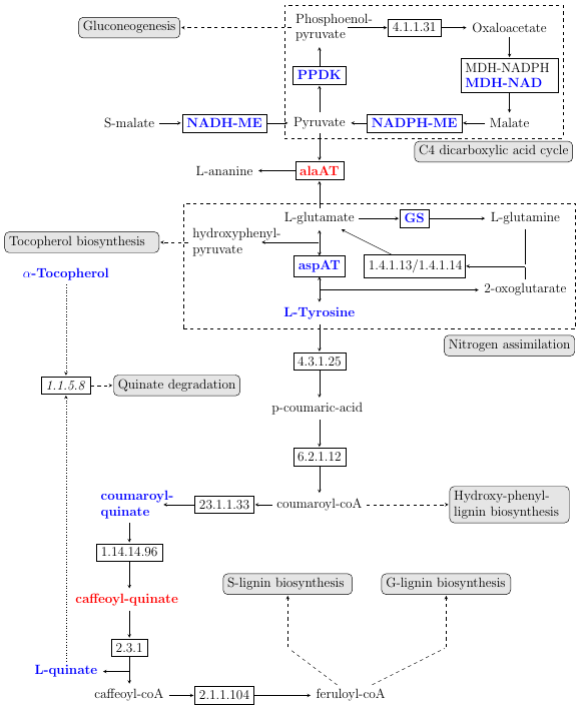

Fig. 6. Metabolic pathways associated to maize leaf palatability: phenylpropanoids. Enzymes (rectangles) are given their EC number or their abbreviated name. Straight lines indicate a direct relation between enzymes and substrates/products and arrows the main direction of the reaction. Dashed lines indicate the link to a pathway. Point lines indicate an hypothetical direct reaction. Colors indicate a significant positive (red) or negative (blue) association with maize leaf palatability.

variability for plant defenses against herbivores that concerns both induced and constitutive defenses. For example, maize inbred lines differ for the volatile compounds emissions induced by injection of *Sprodoptera littoralis* regurgitant (22). Genes involved in the phenylpropanoid pathway were shown to be polymorphic (2). Within the phenypropanoid pathway, QTLs were found for stem-wall hydroxycinnate contents like p-coumaric or ferulic acids (39), but also for resistance to lepodiptera *Spodoptera frugiperda* Smith and coleoptera *Sitophilus zeamais* Motsch (5). Using a MAGIC population of 408 recombinant inbred lines, (40) showed that a greater concentration of p-coumaric acid was associated to a higher resistance to corn-borers, measured by tunnel length in infested plants, and also a lower yield. Altogether, those studies evidence the metabolic complexity of plant defenses and pinnpoint the central role of phenylpropanoid pathway (59). Here, we benefited from the availability of the metabolomic and enzymatic characterization of 13 of our 18 core panel inbred lines (18) to investigate the metabolic bases of maize leaf palatability. Among the 201 variable metabolic traits, only 22 were found significantly associated to variations in maize leaf palatability. Interestingly, those 22 metabolic traits were clustered into a small number of metabolic pathway according to `https://metacyc.org`: chlorogenic acids pathways, chorismate-tyrosine pathway, malate metabolism, hydroxilated fatty-acids pathway, all involved in the establishment of plant defenses. Besides, maize leaf palatability is associated with a hich C:N ratio and a low

percentage of nitrogen and nitrates, as well as with a low yield. In tomato, C:N ratio was considered as a good indicator of secondary compounds concentrations, especially those involved in the chemical defenses (54).

Digalactosil-glycerol is a glycolipid specific from plant plasma membrane possibly associated to host-pathogens interactions (66). A high level of this compound or its precursor glycerate (Fig 9) is associated with a low palatability, while a high enzymatic activity of phosphofructokinase (PFK), which mediates carbon allocation to pentose-phosphates, sucrose or hydroxilated fatty acids is associated with higher palatability. Malate metabolism is at a crossroads between gluconeogenesis and the biosynthesis or aromatics amino-acids (16). We found five enzymes from C4 dicarboxilic acid cycle and nitrogen assimilation that lead to tyrosine biosynthesis and were associated to maize leaf palatability (Fig6). A high level of activity for those enzymes is associated with a lower leaf palatability and a higher tyrosine concentration, except for alanine-aminotransferase. Note that alanine-aminotrasferase is at a crossroads between tyrosine and alanine biosynthesis. Chorismate-tyrosine pathway is another regulatory hub that was shown to control vitamine E content in tomato (13). In our study, both tyrosine and trocopherol concentrations were found negatively correlated with maize leaf palatability. Trocopherol is an amphiphilic lipid with vitamine E activity. It protects membranes against oxidative stress with a special role associated with the protection of plant photo-system II (46). Trocopherol biosynthetic pathway modulates salicylic acid accumulation and affects basal resistance against *Pseudomonas siryngae* in the model plant *Arabidopsis thaliana* (61). Finally, we found three metabolites from the chlorogenic acids pathway which concentration was associated to leaf palatability : coumaroyl-quinate, caffeoyl-quinate and quinate. Those metabolites are substrate and products of two successive enzymatic reactions (Fig 6) and possibly linked to trocopherol biosynthesis through quinate degradation. The enzyme that transforms coumaroyl-quinate into caffeoyl-quinate have been identified as p-coumarate 3-hydroxylase (C3H) found in the *ref8* mutant in *Arabidopsis thaliana* (27). *ref8* plants deposit an unusual lignin enriched in p-hydroxyphenyl sub-units and are prone to fungal attacks. It was suggested that phenylpropanoid pathway products downstream of REF8 may be required for normal plant development and disease resistance. **Altogether, our results confirm the biological complexity of the metabolic response associated to plant defenses (59). However, all metabolic changes related to leaf palatability seem to be related to changes in membrane and cell-wall composition.**

Conclusions

The original consumption test used in this study allowed-us to highlight genetic variability of leaf palatability to European Corn Borer within a core-panel of maize inbred lines representative of the varieties cultivated in temperate areas. Our results are in accordance with existing data about tolerance/sensitivity of the inbred lines observed in the field. Correlation analyses between leaf palatability and the concentration of metabolites and enzymes points out candidate maize metabolic pathway that could be explored through functional analyses.

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Supplementary Data S1: Feeding bioassay experimental set-up

Flowering time and precocity groups For each inbred line, data about flowering time came either from (11) or from the yearly recordings of the INRAE field station at St Martin de Hinx, with 10 out of the 18 lines having records for both. In (11), female flowering time (FFLW) was recorded in sum of temperatures while data from St Martin de Hinx (FFT) were recorded in [à vérifier : dans le tableau plutôt des jours après semis] degree-days. A linear regression was performed on the set of common lines to predict the FFT from the lines that were not measured at St Martin de Hinx. Hence, the flowering time (FFT) presented in Table 1 results either from observation at the INRAE field station or from predictions. FFT data were used to group the maize lines of the panel into four sewing groups, labelled from A to D. All available information is summarized in Table S4

Line	FFLW	FFT^a	LFNB	FFT^b	sewing group
ND36	194	NA	13	59.25	A
F2	196	61	14	61	A
F66	NA	61	NA	61	A
F271	198	NA	15	62.02	A
Cm484	NA	62	NA	62	A
F252	199	63	16	63	A
ND283	204	NA	16	66.19	B
NYS302	207	66	15	66	B
C105	204	66	18	66	B
F618	NA	70	18	70	C
MBS847	210	71	18	71	C
Lo3	209	71	17	71	C
Mo17	217	73	17	73	C
B73	217	74	21	74	D
Lo32	215	74	17	74	D
F918	NA	75	20	75	D
HP301	218	NA	20	75.91	D
SA24U	220	NA	20	77.3	D
F64	218	79	19	79	D

Table 4. Flowering time informations.

Shifted sewing dates Because we wanted all plants to be sampled at comparable developmental stage for the feeding bioassays, lines from each sewing group were sewed at different dates. At each date, six seeds per lines for all lines belonging to the same sewing groups were sewed. Below are the different sewing dates and the sewing groups that were concerned.

Date	A	B	C	D
2019-10-01	X			
2019-10-04		X		
2019-10-07	X		X	
2019-10-11		X		X
2019-10-14	X		X	
2019-10-18		X		X
2019-10-21	X		X	
2019-10-25		X		X
2019-10-28			X	
2019-11-01				X

Experimental design for feeding bioassays Each replicate consisted in three batches of six maize inbred lines. Plants from the different lines were chosen to be at the same developmental stage, between five and seven visible leaf collars. Lines were randomly assigned to batches, that were launched every successive day, so that full data from one replicate were obtained in three days. For each batch, lines were randomly given a plate number (from a to f). A plate was filled with 50 leaf discs from the sixth leaf of the three plants from the same inbred line sewed at the same date. The developmental stage (number of visible leaf collars) and the number of days after sewing (DAS) was recorded.

The table below summarizes the experimental design.

	r1					r2					r3				
Line	stage	DAS	b1.1	b1.2	b1.3	stage	DAS	b2.1	b2.2	b2.3	stage	DAS	b3.1	b3.2	b3.3
F64[A]	V5	44		d		V6	45		c		V6	44	d		
SA24U[A]	V6	43	e			V6	44	a			V7	44	a		
HP301[A]	V6	43	f			6	44	d			V6	44	c		
F918[A]	V5	43	d			V6	44	f			V7	44	b		
B73[A]	V6	43	c			V6	44	b			V7	44	e		
Lo32[A]	V6	43	a			V6	44	e			V6	44	f		
M017[B]	V5	41		c		V5	42		e		V5	42			b
MBS847[B]	V5	41		b		V6	42		a		V6	41		e	
Lo3[B]	V5	41		e		V5	42		b		V5	41		d	
F618[B]	V5	41		a		V6	42		f		V5	41		c	
NYS302[C]	V5	38		f		V6	39		d		V6	41		b	
C105[C]	V6	37	b			V6	38	c			V6	39		f	
F252[D]	V5	35			d	V5	36			e	V5	39		a	
F271[D]	V5	35			b	V5	36			d	V5	36			d
Cm484[D]	V5	35			e	V5	36			c	V5	36			e
F2[D]	V5	35			c	V5	36			f	V5	36			a
F66[D]	V5	35			f	V5	36			b	V5	36			c
ND36[D]	V5	35			a	V5	36			a	V5	36			f

Supplementary Data S2: Choosing the most discriminant model for CHratio

Feeding bioassays allowed to classify larvae preferences into six ordered behavioral profiles, names from A to F. Clearly A types are *consumers* that feed fast and consume all the leaf disc, while F types are *haters* that hardly feed on the leaf disc. In between, B to D behavioral profiles are intermediate. B and C mainly differ from A by the existence of a lag-time. D and E mainly differ from F by the fact that at least part of the leaf disc is consumed at the end of the experiment, but at a lower pace than in A, B or C.

In order to find significant differences between behavioral profiles, the variable CHratio was used to transform the data and analyze them on a logarithmic scale

$$CHratio = log(\frac{\#\quad consumers}{\#\quad haters})$$

All possible grouping combination were explored and tested for the ability of the new variable CHratio to discriminate between inbred lines and replicates. For example,

$$CHratio_{ij}^{ABC/DEF} = log\left(\frac{Z_{ij}^{A} + Z_{ij}^{B} + Z_{ij}^{C}}{Z_{ij}^{D} + Z_{ij}^{E} + Z_{ij}^{F}}\right)$$

For each grouping combination, the following linear model was run:

$$CHratio_{ij} = \mu + Line_i + Rep_j + \epsilon_{ij}$$

where $Line_i$ is the inbred line effect, and Rep_j is the Replicate effect. Summary statistics were compiled and the graph of residuals versus fitted values was checked. Results are summarized in the table below

Model	pval(rep)	pval(line)	$\hat{\sigma}^2$	$R2$	$h2$
AB/CDEF	2e-09	0.0100	0.135	0.65	0.07
A/CDEF	3e-06	0.0028	0.278	0.57	0.19
A/BCDEF	1e-04	0.0014	0.264	0.54	0.20
AB/F	6e-04	0.0032	0.256	0.49	0.17
A/F	**0.007**	**0.0015**	**0.528**	**0.48**	**0.40**
A/EF	0.003	0.0074	0.505	0.43	0.28
AB/DEF	1e-04	0.0670	0.323	0.41	0.09
A/DEF	0.002	0.0301	0.557	0.38	0.21
AB/EF	0.005	0.0401	0.816	0.34	0.28
ABC/F	0.821	0.0180	1.222	0.28	0.54
ABC/EF	0.210	0.0811	0.869	0.21	0.22
ABC/DEF	0.061	0.2357	0.276	0.15	0.03

Table S2. Summary statistics. pval(rep) is the pvalue of the replicate effect. pval-line) is the pvalue of the inbred line effect. $\hat{\sigma}^2$ is teh residual variance. $R2$ is the adjusted model determination coefficient. $h2$ is the line heritability.

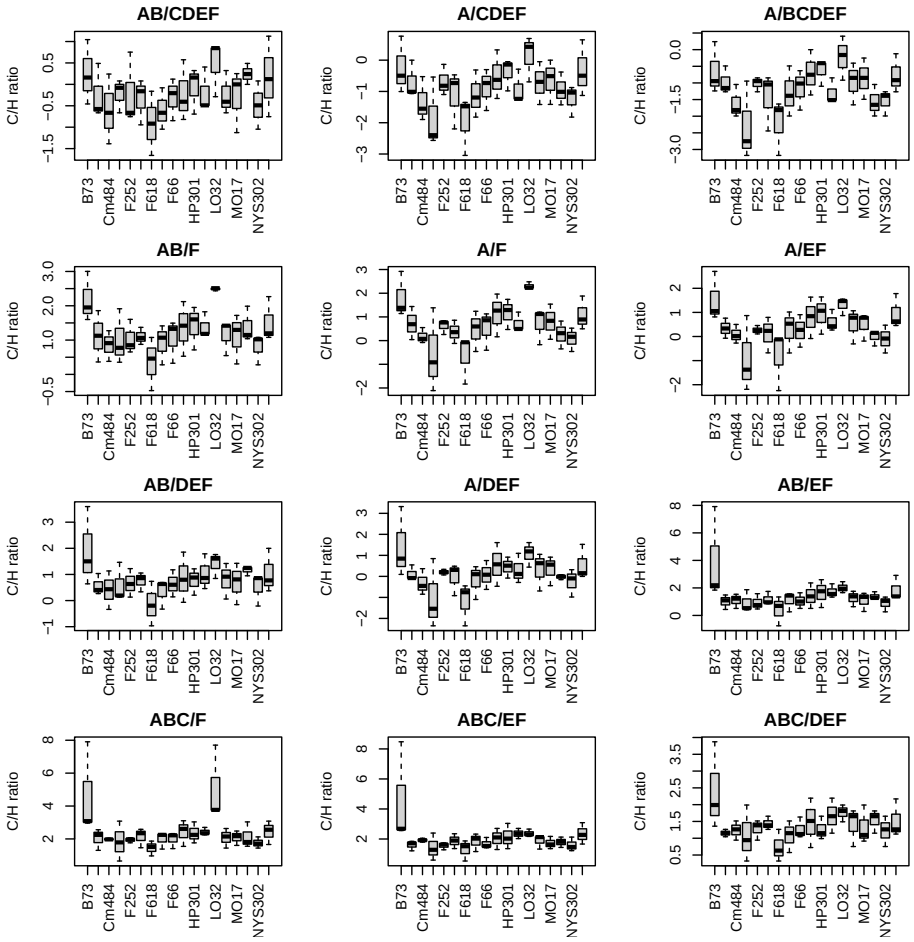

Fig. 7. Differences between lines according to the grouping choices Barplot representation of the variable CHratio for each maize inbred line. Grouping models have been range according to their anova R2 value.

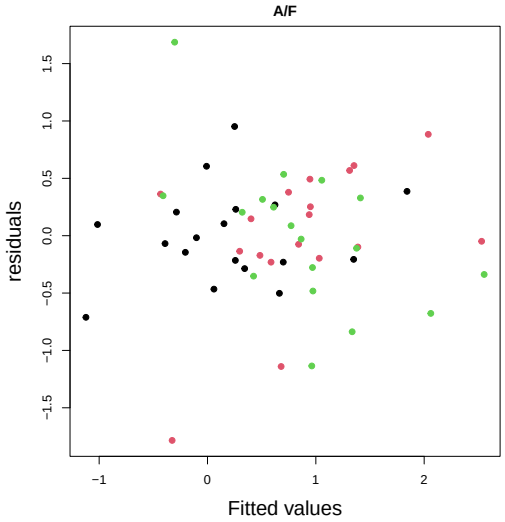

Fig. 8. Residuals versus fitted values. The residuals plot is shown for the A/F grouping model. Colors indicate the replicates.

Supplementary Data S3: Functional annotation of enzymes and metabolites associated with AFratio variations

We used the MetaCyc (17) and KEGG (37) databases to complete the annotation of the 15 enzymes and metabolites that were found associated to AFratio variations. Below are the names and identifiers of the molecular compounds in KEGG, PubChem and CHEBI

Abbreviation	Name	KEGG	PubChem	ChEBI
AlaAT	Alanine aminotransferase	2.6.1.2		
AspAT	Aspartate aminotransferase	2.6.1.1		
GS	Glutamine synthetase	6.3.1.2		
NADPH-ME	malate dehydrogenase (NADP+, decarboxylating)	1.1.1.40		
NADH-ME	malate dehydrogenase (oxaloacetate-decarboxylating)	1.1.1.38		
NADPH-MDH	Malate dehydrogenase (NADP+)	1.1.1.82		
NADH-MDH	NAD-L-malate dehydrogenase	1.1.1.37		
PPDK	Pyruvate, phosphate dikinase	2.7.9.1		
PFK-Pi	inorganic pyrophosphate-dependent phosphofructokinase	2.7.1.90		
Glycerate	D-Glycerate	C00258	439194	32398
Digalactosylglycerol	Digalactosyl-diacylglycerol	C06037		
Tyrosine	Tyrosine	C00082	6057	17895
5-Caffeoylquinate-trans	trans-5-O-caffeoyl-D-quinate	C00852	1794426	57644
3-Caffeoylquinate-cis	3-Caffeoylquinate-cis			
4-Caffeoylquinate-trans	4-Caffeoylquinate-trans			
3-Caffeoylquinate-trans	3-Caffeoylquinate-trans			
Coumaroylquinate 3061.9/345	trans-5-O-(4-coumaroyl)-D-quinate	C12208	40466964	57575
Quinate	Quinate	C00296	3590	17521
Tocopherol	α-Tocopherol	C02477	14985	18145

Metabolic pathway databases were also used to refine the functional categories. Altogether, the 15 compounds belonged to five different main pathways that were used on Figure 4.

Abbreviation	Class	Functional cat.	Pathway
AlaAT	Enzyme	Nitrogen assimilation	Nitrogen
AspAT	Enzyme	Nitrogen assimilation	Nitrogen
GS	Enzyme	Nitrogen assimilation	Nitrogen
NADPH-ME	Enzyme	Carbon fixation (mal > pyr)	Carbon
NADH-ME	Enzyme	Pyruvate metabolism (mal -> pyr)	Carbon
NADPH-MDH	Enzyme	Carbon fixation (mal -> oaa)	Carbon
NADH-MDH	Enzyme	Carbon fixation (mal -> oaa)	Carbon
PPDK	Enzyme	Carbon fixation (pyr -> pep)	Carbon
PFK-Pi	Enzyme	Glycolysis/Gluconeogenesis	Glycolysis/Gluconeogenesis
Glycerate	Carboxylic Acid	Glycerolipid metabolism	Glycerolipids
Digalactosylglycerol	Glycolipid	Glycerolipid metabolism	Glycerolipides
Tyrosine	Amino Acid	Phenylpropanoid biosynthesis	Phenylpropanoids
5-Caffeoylquinate-trans	Organic Acid	Phenylpropanoid biosynthesis	Phenylpropanoids
3-Caffeoylquinate-cis	Organic Acid		Phenylpropanoids
4-Caffeoylquinate-trans	Organic Acid		Phenylpropanoids
3-Caffeoylquinate-trans	Organic Acid		Phenylpropanoids
Coumaroylquinate 3061.9/345	Organic Acid	Phenylpropanoid biosynthesis	Phenylpropanoids
Quinate	Organic acid	Phenylpropanoid biosynthesis	Phenylpropanoids
Tocopherol	Quinone	Quinate degradation pathway I	Phenylpropanoids

Figure 6 shows the link between carbon fixation, nitrogen assimilation and the phenylpropanoid biosynthesis pathway. Thfigurere below shows the position of Glycerate, Digalactosilglycerol and the enzyme PFK-Pi in central carbon metabolism.

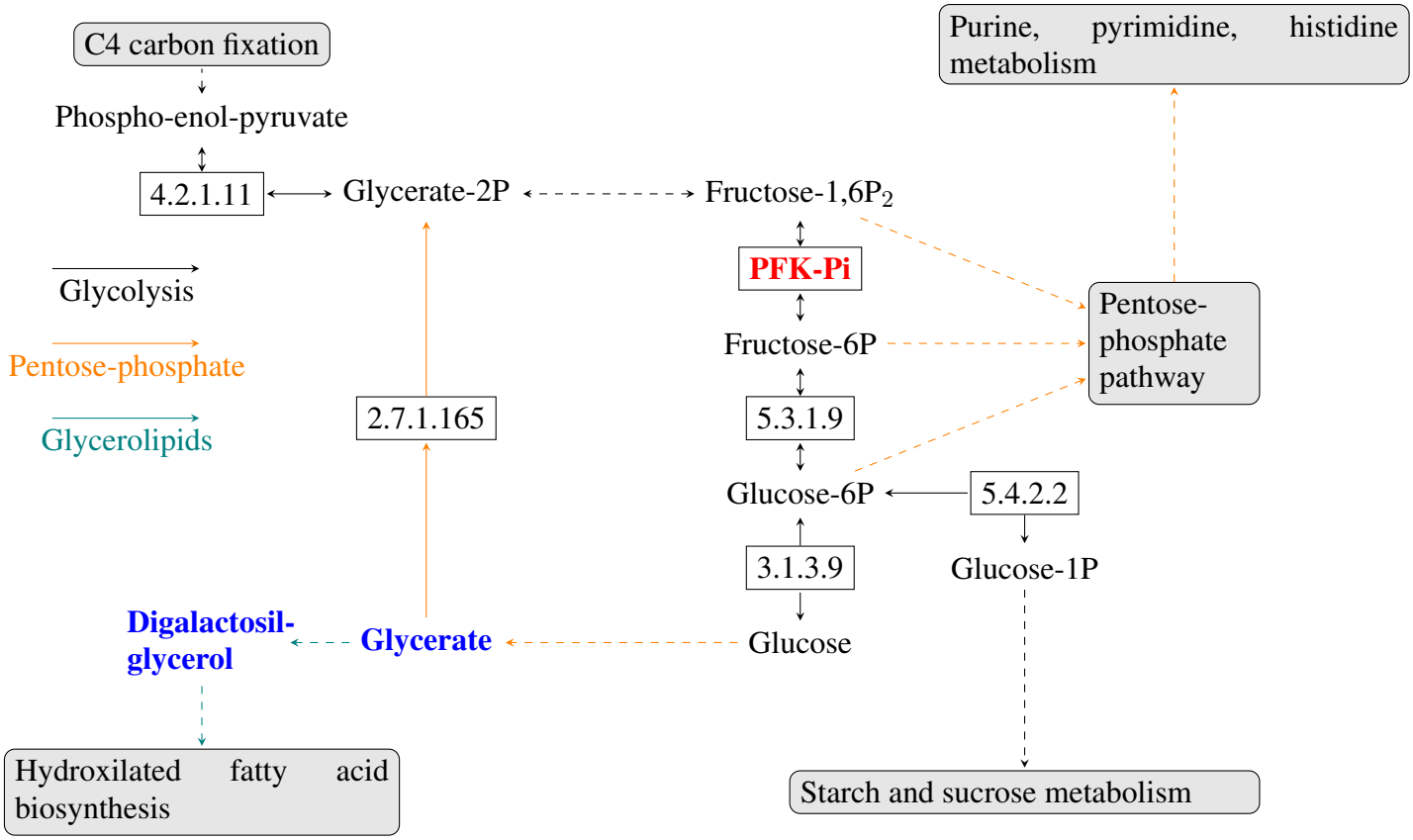

Fig. 9. Metabolic pathways associated to maize leaf palatability: central carbon metabolism. Enzymes (rectangles) are given their EC number or their abbreviated name. Straight lines indicate a direct relation between enzymes and substrates/products and arrows the main direction of the reaction. Dashed lines indicate the link to a pathway. Line colors correspond to a pathway among glycolysis (black), pentose-phosphate (orange) or glycerolipids (green). Enzyme/metabolites colors indicate a significant positive (red) or negative (blue) association with maize leaf palatability.