

Zea Lip Base: An atlas of glycerolipid species across development in maize B73, CML312 inbreds and Palomero Toluqueño landrace.

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Abstract

Glycerolipids are the predominant building block of non-plastidic membranes thus essential for plant growth and development. To better understand how a specific genome sequence results in certain lipid phenotypes we need to explore the temporal and spatial patterns of these metabolites. In this study we present leaf lipid profiles of three different genotypes (B73, Palomero Toluqueño and CML312) at different developmental stages and along three different leaf zones.

Keywords Glycerolipids, natural variation, developmental stages,

Introduction

In *Zea mays*, and monocots in general, the leaf differentiation occurs basipetally, having the less differentiated cells in the tip and the most differentiated ones in the base (Sharman 1942; Nelson and Dengler 1997). This continuous developmental gradient represents a good model to investigate the lipid differentiation among leaves in different developmental stage and different zones within the leaves. In previous studies of maize global RNA sequencing it has been reported that there is a very contrasting expression pattern between tip and base of maize leaves in same or different developmental stages (Sekhon et al. 2011; Stelpflug et al. 2016). Moreover, Li and collaborators found that based on morphological, anatomical and physiological characteristics maize leaf blade can be divided into four zones, which are representative samples for the study of photosynthetic differentiation in maize leaves (Li et al. 2010). Based on those two different approaches we defined three different developmental zones within maize leaves Figure 1B.

Materials and Methods

Plant material and growth conditions

The plant material used in this study consisted of Mexi 5 Palomero Toluqueño (mexican highland landrace), B73 and CML312, the latter being a tropical line developed by CIMMYT. All seeds were weighed before planting. These varieties of maize were planted in a greenhouse in four different blocks, each block with 10 days of difference in between sowing dates and each block had two replicates of each genotype. A randomized block design was employed. The plants were watered with 500 mL of tap water every third day. After germination, the growth was daily measured in terms of number of emerging leaves and fully expanded leaves by plant (Figure A). The vegetative stage used describes the number of leaf counting the coleoptiles as the first one (Sharman 1942).

All blocks were harvested 50 days after germination (dag) of block 1, harvested tissue was immediately frozen in liquid nitrogen and stored at -80°C until extraction.

From block 1, leaves were sampled in three different zones: base (B), center (C) and tip (T), see Figure X. From each zone 50 mg of fresh blade tissue were collected for lipid extraction, excluding the mid rib. A list of all tissues sampled is listed in Table 1.

** Table 1 **

Genotype	Sampled leaf	DAG	Developmental Stage	Replicates
B73	11	57	V11	3
B73	11	57	V12	3
B73	11	49	V9	2

Genotype	Sampled leaf	DAG	Developmental Stage	Replicates
B73	13	57	V11	3
B73	13	57	V12	3
B73	13	49	V9	2
B73	4	49	V10	1
B73	4	27	V4	2
B73	4	40	V6	2
B73	4	49	V9	1
B73	5	57	V11	1
B73	5	57	V12	3
B73	5	27	V4	2
B73	5	40	V6	2
B73	5	49	V9	2
B73	7	57	V11	3
B73	7	57	V12	3
B73	7	40	V6	2
B73	7	49	V9	2
B73	9	57	V11	3
B73	9	57	V12	3
B73	9	49	V9	2
CML312	11	49	V10	2
CML312	11	57	V11	3
CML312	11	57	V14	3
CML312	13	49	V10	1
CML312	13	57	V11	4
CML312	13	57	V14	3
CML312	4	49	V10	1
CML312	4	49	V11	1
CML312	4	26	V5	1
CML312	4	26	V6	1
CML312	4	40	V8	2
CML312	5	49	V10	1
CML312	5	49	V11	1
CML312	5	57	V14	3
CML312	5	26	V5	1
CML312	5	26	V6	1
CML312	5	40	V8	1
CML312	7	49	V10	1
CML312	7	57	V11	4
CML312	7	57	V14	3
CML312	7	26	V5	1
CML312	7	26	V6	1
CML312	7	40	V8	2
CML312	9	49	V10	1
CML312	9	57	V11	4
CML312	9	57	V14	3
CML312	9	40	V8	2
PT	11	49	V12	1
PT	11	57	V14	6
PT	11	49	V8	1
PT	13	57	V14	6
PT	13	49	V8	1
PT	4	49	V12	1

Genotype	Sampled leaf	DAG	Developmental Stage	Replicates
PT	4	28	V4	1
PT	4	27	V5	1
PT	4	40	V7	1
PT	4	49	V8	2
PT	5	49	V12	1
PT	5	28	V4	1
PT	5	27	V5	1
PT	5	40	V7	1
PT	5	49	V8	2
PT	7	49	V12	1
PT	7	57	V14	6
PT	7	27	V5	1
PT	7	40	V7	1
PT	7	49	V8	2
PT	9	49	V12	1
PT	9	57	V14	6
PT	9	40	V7	1
PT	9	49	V8	2

Field samples were collected from plants grown in a CIMMYT experimental field located in Metepec, Mexico State, (19°13'28.7"N 99°32'51.6"W) within the Trans-Mexican Volcanic Belt. The experimental field is 2610 masl, the range of average monthly temperatures along the year go from 5 °C to 21.5 °C with an average annual of 13.6 °C. Its average annual precipitation is around 809 mm. Plants were planted in on April 5th, 2016 and were sampled for lipid analysis by June 9th 2016, when the plants were around the V5 developmental stage. Samples were taken from the tip of the S2 leaf (two leaves younger of the last leaf with a fully developed collar). Eight plants per genotype were sampled. The average GDD for Metepec was 5.52 and the total GDD (over the length of the growing season) was 364.25. On the other hand, during this period the average daily precipitation (mm) was 1.6 while the total precipitation was 96.5 mm.

Lipid extraction

Leaf tissue was collected when the plant was, in average, in the V3 - V6 stage. We tested using 50 mg wet weight leaf tissue for lipidome profiling, as this amount was deemed appropriate for Arabidopsis (Degenkolbe et al. 2012). Frozen material was homogenized in a tissue grinder Retsch (Haan, Germany) during 40 seconds at 30 1/s. After grinding all tubes were stored in liquid nitrogen.

Lipid extraction used was reported by Matyash and collaborators (Matyash et al. 2008). First 225 L of cold methanol (MeOH), previously prepared with a Quality Control (QC) mix, was added to each sample, always keeping MeOH on ice during the extraction. Each sample was vortexed for 10 seconds, keeping the rest of materials on ice. Then 750 L of cold methyl tert-butyl ether (MTBE) were added, keeping MTBE on ice along all the process. Again each sample had to be vortexed for 10 seconds, followed by shaking each for 6 minutes at 4°C in the orbital mixer. After that, 188 L of LC/MS grade water at room temperature (RT) were added, vortexing all the samples for 20 seconds.

In order to separate the phases, all samples were centrifuged for 2 min at 14, 000 rcf (12, 300 rpm). The upper phase is the organic one and it was possible to remove around 700 L of supernatant, so it was split into two aliquots of 350 L, one serving as back up and for the preparation of pools. Finally, samples were dried using a speed vacuum concentration system and stored at room temperature (RT) but protected against light.

Lipid Profiling

Dry samples were resuspended in 110 μ L of MeOH-Toluene 90:10 (with CUDA 50 ng/mL). The rack of samples was vortexed at low speed for 20 seconds and then all tubes were sonicated at RT for 5 min. Aliquots of 50 μ L per sample were transferred into an insert within an amber glass vial.

Before start running samples a new column was set, it is recommended to change the column (Acquity UPLC C18 1.7 m 2.1x100 mm Column) (Waters; Milford, MA, USA) every 1000 samples. After the column change, the new one must be purged for 5 min to take air out. The UPLC column is coupled to a VanGuard pre-column (Acquity UPLC C18 1.7 m 2.1x5 mm Pre-column Waters), which is replaced after ~330 sample injections. Six “no sample injections” were injected at the beginning of each run to condition the column, followed by ten samples, one pool (made out of the mix of the second aliquot of all the samples contained per UPLC plate) and one blank.

According to the diversity and amount of lipid species identified under ESI (+) and (-) during an optimization of lipid extraction and lipid analysis, we selected ESI positive mode on Agilent® Ultra-Performance Liquid Chromatography and Quadrupole Time of Flight Mass Spectrometry (UPLC-QTOF MS/MS). The UPLC-QTOF MS/MS utilized was Agilent 6530 ESI (+) injects into UPLC-QTOF MS/MS 1.67 μ L per sample, the running time per sample is 15 min.

Results

In total we analyzed 150 samples from three genotypes at 10 different developmental stages from V4 to V14. From these tissue was collected from leaves of 6 different ages. Most of the samples were collected along the leaf, but in a subset of them samples were collected at defined locations of the leave (base, mid and leaf tip) See Figure 1. In total around 2500 data points were collected and analyzed. We also analyzed set of 24 plants (8 per genotype) grown in the field and sampled at around V5 developmental stage.

A total of 171 lipid species were identified with lipid profiling. The lipid classes found are: cholesterol esters (CE), diacylglycerols (DG), non-hydroxyacyl sphingosines (CerNS), digalactosyldiacylglycerols (DGDG), glycosphingolipids (GlcCerNS), lysophosphatidylcholines (LPC), monogalactosyldiacylglycerols (MGDG), phosphatidylcholines (PC), phosphoethanolamines (PE), phosphatidylglycerols (PG), plasmeyl phosphatidylcholines (plasmeylPC), sphingomyelins (SM), sulfoquinovosyl diacylglycerols (SQDG) and triacylglycerides (TG). See Table 1.

Table 1 Table S. Number of lipid species identified, within each lipid class, using UPLC-QTOF MS/MS positive mode ESI.

Lipid classes	Number of species
Cholesterol esters	1
Diacylglycerols	7
Non-hydroxyacyl sphingosine	2
Digalactosyldiacylglycerols	21
Glycosphingolipids	4
Lysophosphatidylcholines	6
Monogalactosyldiacylglycerol	13
Phosphatidylcholines	26
Phosphoethanolamines	11
Phosphatidylglycerols	8
Plasmeyl phosphatidylcholines	3
Sphingomyelins	5
Sulfoquinovosyl diacylglycerols	7
Triacylglycerides	52

95 The average coefficient of variation of the sample pool, analyzed every 10 samples, was in average 9.7% and
96 it ranged between 1.7 and 29.6 %. In the samples analyzed we observed a coefficient of variation 63.9% with
97 a range between 22.7 and 222.8% across all tissues. By genotype, coefficients of variation were 51.9% in the
98 case of B73 with a 14.6-192.8% range, 57.7 with 13.1-223.6% range in CML312 and 58.6% with a 14.0-137.1%
99 range in PT. In the set of plants that were grown in the field coefficients of variation were 25.0% in the case
100 of B73 with a 7.5-60.9% range, 30.6% with a 10.2-76.6% range in CML312 and 28.3 with a 9.1-156.2%.