The role of phospholipids in maize adaptation to Mexican highlands

Background

The ancestor of maize, Balsas teosinte (*Zea mays ssp. parviglumis*), is native from low to mid elevation regions of southwest Mexico. A second subspecies, Chalco teosinte (*Zea mays ssp. mexicana*), is distributed in the cooler, drier highlands of central Mexico, over 1800 meters. Maize (*Zea mays ssp. mays*) was domesticated from *parviglumis* about 9,000 years ago in the Balsas valley (Matsuoka et al., 2002). After domestication, maize colonized the Mexican highlands, possibly aided by significant introgression of adaptive variation from *mexicana* (Hufford et al., 2013). In the Mexican highlands, maize was exposed to higher levels of radiation, lower air pressure, temperature and precipitation; furthermore, owing to the volcanic nature of the Mexican highlands, maize had to adapt to Andosol soils, characterized by low bio-availability of phosphorus (Krasilnikov and Carmen Gutiérrez-Castorena, 2013). Therefore, maize colonization of these highland areas represents a great model to study plant local adaptation. While several studies using evolutionary and population genomics approaches have described the patterns of genetic differentation associated with maize adaptation to the Mexican highlands, studies to functionally validate the underlaying metabolic changes involved in these processes are still largely missing.

Plants growing in phosphorus limiting soils develop a series of metabolic strategies, including recyling of P containing compounds such as RNA and phospholipids. Phospholipids constitute around 1/3 of the available phosphorus in the plant and are the major component of membranes, specially in chloroplasts (Veneklaas et al., 2012). In addition to their structural role, some of the most abundant phospholipids such as phosphatidylcholine also have interesting signaling functions and are involved in multiple regulatory processes associated with environmental stimuli (Xue et al., 2009). Plants challenged with low temperatures tend to increase the proportion of phospholipids in the composition of membranes with respect to other polar lipids such as galactolipids and sulfolipids (Degenkolbe et al., 2012), probably as a mechanism to modulate membrane fluidity. On the other hand, plants under phosphorus deficiency recycle phospholipids -to free up phosphate- and substitute them with galactolipids and sulfolipids (Veneklaas et al., 2012). The Mexican highland transvolcanic belt that maize colonized is characterized by both lower temperatures and phosphorus bio-availability. Therefore during the process of adaptation to Mexican highlands we hypothesize that maize phospholipid metabolic pathways were under a high selective pressure. Although these pathways are relatively well known (see figureure 1A), recent results suggest that the diversity of polar lipids involved may be greater than we expected (Okazaki et al., 2013). Okazaki and colleagues discovered that Arabidopsis plants challenged with low concentrations of phosphorus were able to synthesize glucuronosyldiacylglycerol using the sulfoquinovosyldiacylglycerol pathway enzymes (Okazaki et al., 2013).

There are several studies that point to an important role of phospholipids in maize highland adaptation. Takuno and colleagues identified genes showing signals of selection between lowland and highland maize populations in Mesoamerica and South America (Takuno et al., 2015). Approximately 90 genes were identified in common between Meso American and South American populations, over a third of which belonged to Gene Ontology groups related to the synthesis of lipids / phospholipids. Interestingly, one of these genes is described as a CONSTANS interacting protein. CONSTANS is a transcription factor that is upstream of the FLOWERING LOCUS T (FT) transcription factor, which controls flowering time by integrating daylength signals.

Using a maize diversity panel, lipid profiles have been shown to be good predictors of flowering time (Riedelsheimer et al., 2012). In *Arabidopsis thaliana*, FT binds to di18:1-PC, a species of phosphatidylcholine (PC) -the most abundant phospholipid in plants- *in vitro* (Nakamura et al., 2014). When the proportion of PC with respect relative to phosphoethanolamine (PE) is genetically reduced () flowering time is reduced, probably because the FT complex with PC favors the union of FT to the promoter

of FT target genes. Additionally, both PC and other intermediate metabolites of PC synthesis are essential for proper embryo development (Lin et al., 2015).

In this project, we will systematically study the role of phospholipid metabolism in maize adaptation to highland conditions. Using high throughout liquid chromatography coupled to mass spectrometry and lipid mass spectra databases to quantify lipids involved phospholipid metabolism. We will use maize genotypes adapted to highland and lowland conditions and a combination of growth chamber experiments simulating highland (low phosphorus and temperature) and lowland (high phosphorus and temperature) and common garden experiments in Mexican highland and lowland locations.
In particular, the maize genotypes that will use are:

- Palomero Toluqueño (landrace adapted to highland conditions) and B73 (US inbred adapted to lowland conditions) maize lines and mutants of genes involved in PC biosynthesis grown in growth chamber with a factorial experiment combining low and high temperature and low and high phosphorus concentrations simulating highland and lowland conditions. In this plant material we will also collect tissue to study the expression of genes involved in the PC pathway.
- A mapping population composed of 100 Palomero Toluqueño (PT, a Mexican highland landrace) x B73 (sequenced US inbred line) Recombinant Inbred Lines grown in the same highland and lowland locations.
- A diversity panel of 30 highland and 30 lowland Mexican maize landraces grown in common gardens experiments in highland (Metepec, 2500 masl) and lowland (Nayarit, 25 m masl) field sites.
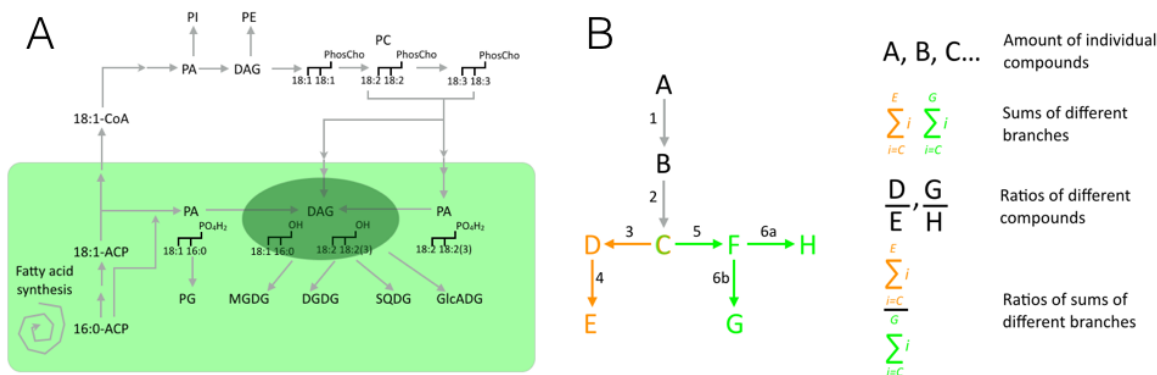
figureure 1 A) Biosynthetic pathway of plant glycerolipids. Adapted from Okazaki and cols(Okazaki et al., 2013). Abbreviations: B) Different metabolic variables that can be used in association or linkage mapping studies. Letters represent metabolites and numbers enzymes of an hypothetical pathways.

Hypothesis

We hypothesize that phospholipid metabolism reorganization played a key role in the adaptation of maize to the highlands of Mexico.

Relevance to the UC-Mexus program goals

The current project brings together a young research group in Langebio, México (Rubén Rellán-Álvarez) interested in understanding the biochemical basis of maize local adaptation and a well stablished group (Oliver Fiehn) that has developed methods and databases for lipid analysis in the Genome Center at UC Davis. Rellán-Álvarez has a proven record on the study of how abiotic stresses shape

different plant metabolic pathways and is starting an ambitious research program to understand at the biochemical level how maize has adapted to highland elevations in México. Oliver Fiehn is a world expert in the development and application of metabolomics techniques. His lab has developed analytical methods and data pipelines to identify a great variety of lipids (Kind et al., 2013). This project will enable precise, high throughput biochemical analysis in the UC Davis Genome Center of plants grown in different field locations in México. Rubén Rellán-Álvarez and Oliver Fiehn have experience in carrying out this type of collaborative research. During his PhD, Rubén Rellán-Álvarez visited the lab of Oliver Fiehn several times to perform similar types of work and worked on the logistics of preparing, sending and analyzing the samples in collaboration. Now that Rellán-Álvarez has started his own lab in México this project would provide a fantastic opportunity to establish new collaborations trough common visits and stays of Mexican students that are starting their Masters program in the laboratory of Rellán-Alvarez. In particular, the work of two students, Karla Juarez and Estefany Sánchez are expected to benefit from this collaboration.

Furthermore, is expected that the techniques developed in the Fiehn lab will be transferred to Langebio´s metabolomics lab.

General Aim

The general aim of this proposal is to systematically characterize the changes in phospholipid profiles and expression of genes involved in phospholipid synthesis to shed light on the possible role of phospholipids in Maize highland adaptation.

Specific Aims and relevant methodology

Aim 1. Characterize the profile of phospholipids and other polar lipids, as well as the expression patterns of genes involved in the synthesis of those lipids in B73 and Palomero Toluqueño

Rationale, design and potential problems

In this aim we will get a first characterization of the effect of phosphorus deficiency and cold the phospholipid profiles and the expression of genes involved in PC synthesis in two well characterized genetic backgrounds: B73 and Palomero Toluqueño (a highland adapted landrace). Plants will be grown in a growth chamber simulating highland and lowland weather conditions by regulating the chamber's temperature. The growth chamber's conditions for highlands will be 20°C during the daylight and 10°C during the night time. While for lowlands daylight temperature will be 30°C and 20°C during the night. For both experiments the photoperiod will be 14 hours of light and 10 h of darkness. At each of the temperature conditions half of the plants will be grown under low phosphorus and half under control phosphorus concentrations. So each genotype will be grown under two different phosphorus and temperature conditions. Before this experiment we will optimize lipid extraction procedures to adapt it to maize leaf tissues but once this is optimized we don´t expect too much complications as the rest of the procedures are already performed regularly in both labs. This experiment will set the basis for future experiments using B73xPT F1s to study Allele Specific Expression (ASE) using targeted RNA-Seq in collaboration with the laboratory of Daniel Runcie at the department of Plant Sciences UC Davis. Samples will also be collected to study methylation patterns of phospholipid pathway genes in collaboration with the laboratory of Luis Herrera-Estrella that have recently found interesting methylation pattens of genes involved in lipid recycling upon phosphorus deficiency in *Arabidopsis* plants (Yong-Villalobos et al., 2015).

Methodology

Plants will be grown for a period of 2-3 weeks. Samples will be collected at V3 stage from the youngest full developed leaf. Maize samples will be extracted according to the method of Matyash et al. (Matyash et al., 2008). Phospholipid analysis will be performed at the West Coast Metabolomics Center in UC Davis. Separation and identification of lipids will be accomplished using a method that has been optimized, in the laboratory of Oliver Fiehn, with UPLC-QTOFMS electrospray on modes negative and positive. The method allows the identification of about 500 types of lipids including phospholipids, galactolipids, and sulfolipids. Further identification will be done using the LipidBlast data base (Kind et al., 2013) developed in the laboratory of Oliver Fiehn. Samples will also be collected from the opposite side of the leaf midrib for RT-qPCR analysis of the genes involved in phosphatidylcholine biosynthesis pathway See figureure 1.

Aim 2. Characterize the natural variation and genetic architecture of phospholipid metabolism and identify regions of the genome (QTLs) controlling the re-organization of polar lipids in response to highland conditions

Rationale, design and potential problems

In this aim we will study the natural variation of lipid profiles from a collection of 30 highland and 30 lowland adapted Mexican landraces. This landraces (15 plants each) will be planted in March 2017 in a common garden experiment in both lowland (Nayarit field site) and highland conditions (Metepec field site). Alongside these landraces we will grow a collection of BC1S5 (Backcross with B73 and then selfed during 5 generations) 100 Recombinant Inbred Lines of B73xPT. Samples will be collected at V3-V5 (we expect different rates of development between the different landraces) from 3-5 plants per landrace and RILS in the field, stored in dry ice and transported to the laboratory in Langebio for extraction. Extracted samples will then be shipped to the UC Davis Genome Center for analysis by the laboratory of Oliver Fiehn. Lipid analysis will be performed as described in the previous aim. Phospholipid profiling data from the landraces will give us a very valuable information about genetic highland/lowland genetic variability in the synthesis of these compounds and on the environmental effect of highland and lowland growth conditions. Given the genetic diversity of our panel and the fact that previous data point to an important role of phospholipid metabolism since highland conditions imposed a high selective pressure in these pathways we expect to find great biochemical diversity and even new compounds as recently shown in the model plant *Arabidopsis* (Okazaki et al., 2013).

Since, metabolites are the end products of the metabolism they provide a good proxie to the quantify the activities of the enzymes that synthesize them. When combined with tools like linkage or association mapping of populations showing differences to the environmental perturbation being studied, metabolic phenotyping offers a very powerful tool to identify loci and ideally the underlying genes or allelic differences explaining metabolite synthesis and function (Wen et al., 2014). If the pathway of interest is know, metabolite quantification can provide not only information about the amount of different metabolites but also the ratio of different metabolites, pathway branches etc., as indicated in figure 1B. The LC-MS methods developed in the lab of Oliver Fiehn can identify around 400 different species of lipids including those described in figure 1A that are relevant for this project. Using this data we will be able to ask if a certain pathway is enhanced in certain highland or lowland genotypes and by analyzing samples of plants grown in highland and lowland conditions we will be able to tell if certain pathways can be considered adaptive to highland conditions. To do this we will calculate the different set of biochemical phenotypes described in figure 1B and also the ratios between highland and lowland accessions pairs and between highland and lowland conditions.

These biochemical phenotypes obtained from the RILS will be used to run QTL analysis (Broman, 2015). The use of ratios between two metabolite (or sums of metabolites) concentrations as phenotypes in GWAS and QTL analysis has been shown to provide more information than the corresponding metabolite concentrations alone, to identify the allele differences that explain changes in metabolite concentrations (Petersen et al., 2012). The rationale is that ratios are a good proxie to enzymatic

activities and that allelic differences lead to different enzymatic activities. When combined with experiments done in conditions where certain pathways could be considered adaptive, this effect known as *p-gain* becomes very powerful to identify the genetic architecture of

Once QTLs are identified we will use these QTLs to run association mapping on the set of 60 landraces (for which there is already genotypic (GBS) data from the Cimmyt Seeds of Discovery project).

The problems that we expect here are related with the nuances of field sampling experiments. However we have already grown and collected samples from both field sites in collaboration with the laboratory of Ruairidh Sawers at Langebio.
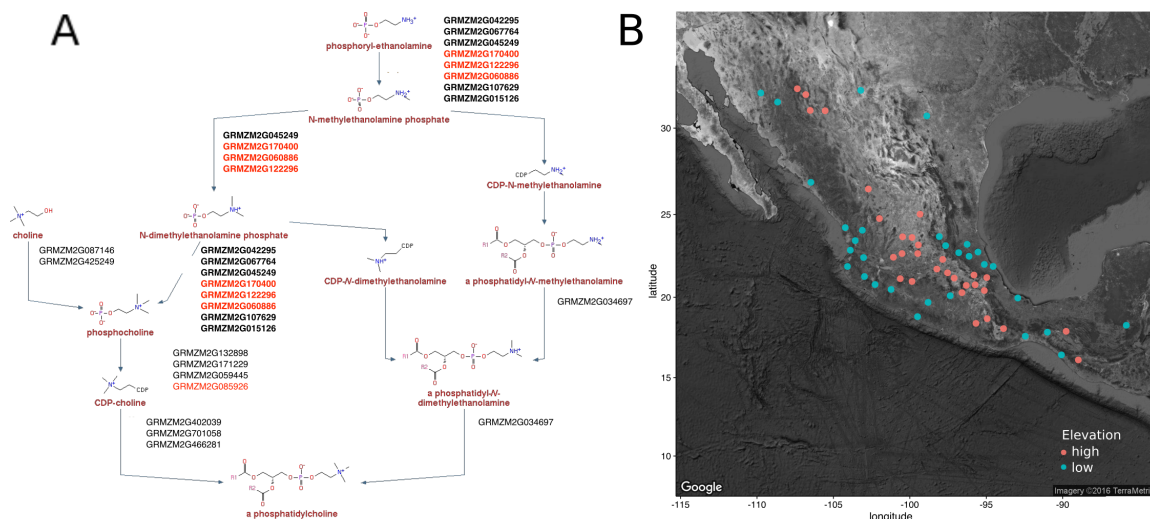
figureure 2 A) Phosphatidylcholine biosynthesis super pathway indicating the different pathways by which PC can be synthesized in maize. Predicted genes are indicated. (In bold those that can perform different steps of the pathway, in red those for which we are already homozygous single insertional mutants). Data was obtained from the Plant Metabolic Network, CornCyc webpage. B) Localization of the 30 highland and 30 lowland maize accessions that will be analyzed in this project. Accessions were selected and ordered from the Cimmyt Seed database as follows: 1) accessions were divided in high elevation (>2000 masl) or low elevation (< 1000 masl) classes; 2) 30 high elevation accessions were randomly chosen with the restriction that they cannot be closer than 50 km from each other; 3) for each a highland line a lowland `pair` was chosen within one degree of latitude band. These lines were selected by Daniel Runcie (UC Davis). As part of another project, they will be test-crossed with B73 the F1s used for RNA-Seq Allele Specific Expression (ASE) in highland and lowland conditions. Combination of lipidomics and transcriptomics data will aid into a better understanding of how this mechanisms are regulated. (See letter of support by Dan Runcie).

Aim 3. Identify the effect of mutations of genes involved in the Phosphatydilcholine biosynthesis pathway

Rationale, design and potential problems

In collaboration with the laboratory of Ruairidh Sawers, the lab of Rubén Rellán-Álvarez is developing different lines carrying insertional mutations in key genes of the Phosphatydilcholine biosynthesis. We have mutants of different alleles homologues to the *Arabidopsis* gene xipotl (Cruz-Ramirez et al., 2004), that encodes an enzyme with phospho-ethanolamine N-methyltransferase activity. In *Arabidopsis*, three different enzymes are needed to complete three consecutive N-methylation steps that are carried out on phospho-bases, phosphoethanolamine, phospho-N-methylethanolamine, and

phospho-N-dimethylethanolamine to produce PC, but in maize, all three steps can be carried out by at least 4 different *xipotl* alleles: GRMZM2G045249, GRMZM2G170400, GRMZM2G122296 and GRMZM2G060886. We have UniformMu or Ds insertional mutant stocks for the last three of them and we are in the process of finding homozygous lines for all of them. We also have a UniformMu insertional mutants of GRMZM2G085926 that encodes an enzyme in the Kennedy pathway that synthesizes PCl *de novo* from choline. Using the same experimental design as in Aim 1 all these mutants will be grown in high/low temperature and phosphorus conditions. Samples will be taken for phospholipid analysis to study the effect of the mutations in phospholipid profiles. Samples will also be collected for RT-qPCR of the genes involved in the PC super pathway. These data will allow us to study if: - we can modify PC concentrations in maize plants and in the future explore the effect of altering this concentrations in flowering time and more - compensation mechanisms between the different branches of the PC pathway exist and what is the importance of each of these pathways in Morphological traits of seedling will also be scored to identify effects in leaf and root development.

In the future this information will be used to design field studies where mutants of these genes will be evaluated in highland and lowland conditions and directly test the effect of the genes in plant fitness in contrasting growing environments. To this end, we have already started making crosses between these insertional mutants and selected RILS of the PTxB73 mapping population with extreme flowering times to test if changes in PC concentrations can indeed modify flowering times in field conditions (Nakamura et al., 2014).

Crosses and genotyping of all the mutants is well underway. Field experiments won't be possible within the timeframe of the UC-Mexus project but data generated in the growth chambers will guide theses experiments in the future.

Summary

In summary we will use precise biochemical phenotyping of phospholipids and other polar lipids to test the hypothesis that changes in phosphoplipid metabolism is an adaptive trait to maize highland adaptation in México. We will carry out a combination of growth chamber and field common garden experiments using maize lines that are adapted to highland and lowland conditions.

Work Group Contributions

RRA group, will carry out growth chamber and field experiments, perform lipid and DNA isolation and make maize crosses and genotyping to generate homozygous mutants of PC synthesis OF group will perform HPLC-MS analysis of samples to generate lipid profile data and train visiting students from RRA lab in data analysis. RRA and OF will work jointly on data analysis of data. RRA group will work closely with the metabolic services at Langebio to implement lipid profile analysis in Langebio.

Potential for Future Funding

Several parts of this project will be done in collaboration with the labs of Ruairidh Sawers and Daniel Runcie (see letters of support), that are already working on maize highland adaptation (not sure if it will be good here to mention the NSF project). Data generated in this project would benefit from data generated by these two labs and may constitute the basis for future grant applications. The lab of Rellán-Álvarez has also submitted an application to the Conacyt Fronteras de la Ciencia (awaiting decision) that would build on the results that could be obtained in this project.

Timeline

Aim 1
Feb - March (2016): Grow B73 and PT in growth chamber under highland and lowland conditions and low and high phosphorus.

March - May (2016): Optimize lipid extraction. UPCL-QTOFMS Lipid profile analysis.

June - July (2016): RT-qPCR analysis and gene methylation patterns from leaf samples taken.

Aim 2
March - April (2017): Sowing of 30 highland and 30 lowland landraces, in Nayarit and Metepec fields respectively. Grow 100 B73xPT RILs, collect V3 leaf samples.

April - May (2017): Lipid extraction and lipid profile analysis.

June - July (2017): QTL identification.

Aim 3
June - August (2016): Generation of insertional mutants on PtdCho synthesis genes. Study mutants for xipotl alleles.

September - October (2016): Find homozygous lines for maize xipotl alleles.

October - November (2016): Grow mutants in growth chamber under Aim 1 conditions. Take V3 leaf samples.

November - December (2016): Lipid extraction. UPCL-QTOFMS Lipid profile analysis. RT-qPCR analysis and gene methylation patterns of leaf samples.

Future Aims
- Crosses and genotyping of PtdCho maize mutants. - Grow mutants in highland and lowland fields.
- Analyze flowering time and root development phenotyping.