D1: Breeding maize for cold tolerance
Good morning, I’m going to explain you the state of maize breeding for cold tolerance and the contribution of my research group to this issue

D2: Maize is susceptible to cold conditions
Maize is a tropical cereal with high susceptibility to cold temperatures, widely cultivated throughout the World, with great ability for adaptation to new environmental conditions and climatic changes.

Maize adaptation to temperate and cold regions can be reached through escape due to selection for earliness or directly by increasing cold tolerance. However, maize has not achieved a noteworthy advance in cold tolerance.

D3: Why breeding for cold tolerance
There are several reasons for breeding maize for cold tolerance: enlarge growing area, increase growth period, advance sowings in order to avoid hot temperatures at sensible stages, avoid peaks of diseases, escape large impacts of pests, increase yield and prevent future needs facing climate change

D4: Variability for cold tolerance
The variability for cold tolerance in maize is quite narrow. The actual lower limits for maize growth are around 8 °C at germination and 15 °C at flowering. The optimum temperature is between 25 °C and 30 °C. The minimum requirements are 10 °C. This is important to us because temperate areas have temperatures lower than those

D5: Symptoms and effects of cold temperature
Next, I’ll explain the symptoms and effects of cold tolerance

D6: Symptoms of cold damage
At temperatures below 10 °C, seedlings suffer physical and physiological damage. Some damages are reversible depending on the intensity and duration of the stress. At temperature below 0 °C, seedlings die in few hours. The most evident symptom of cold stress is light green color or even albinism

D7: Effects of cold temperature
Germination is the most sensible stage to cold conditions and, unfortunately, cold conditions are more frequent at sowing than later. The main process affected by low temperatures is photosynthesis.

The main effects of cold temperature are reduced plant germination, cell membrane instability, reduced growth, reduced leaf area, decreased chlorophyll content or chlorosis, limit the potential photochemical efficiency of photosystem II, or sterility. Cold stress is often accompanied by relatively high light intensity, leading to photoinhibition and secondary damage of the photosystem

D8: Effects of cold temperature on maize germination
During seed imbibition, there is exudation of amino acids and carbohydrates from the kernels due to membrane disorders. Seedlings lose their ability to expend dry matter on the development of the root in favor of the shoot. And the shoot weight/root weight ratio increases

D9: Effects of cold temperatures on maize growth
Reduction of growth depends on several factors. During the process of imbibition, enzymatic breakdown of stored kernel reserves, cellular division, and cellular extension

D10: Other susceptible stages
In the leaf, there are reductions on number and elongation and modifications of shape.
At flowering, there happens abnormal development of flowers and failure to set seed or fruits.
At reproduction there are alterations on meiosis, pollen tube growth, early embryogenesis, and embryo competition

**D11: Physiological and biochemical responses**
At the physiological and biochemical levels, low temperatures induce oxidative stress, that’s why the antioxidant enzymes become crucial. Cold temperature partially or completely suppress other metabolic processes, affects the stability of the photosynthetic apparatus and the pigment ratios. Two of the enzymes more involved in cold susceptibility are RuBP carboxylase and NADP malate dehydrogenase. There are alterations in membrane lipid composition and increments of levels of soluble proteins, antioxidant enzymes, amino acids, such as proline, and also alters the levels of abscisic acid and carbohydrates

**D12: Other aspects related to cold tolerance**
There are other processes related to cold tolerance, particularly frost tolerance, which is absent in maize and independent from cold tolerance. Also acclimatation to moderate stress and recovery after moderate damage

**D13: Breeding**
Next I’m going into the breeding issue

**D14: Breeding perspective**
Breeding for cold tolerance is a difficult task that has shown limited success for one century with only a few inbred lines with limited cold tolerance. The main reason for that is that cold tolerance is a complex trait with polygenic inheritance: additive, dominance and maternal effects with many genes with low contributions and low heritability

**D15: Limits**
Cold susceptibility reduces crop on time and space. Selection has a roof that has not been broken by conventional breeding approaches. Small increases in cold tolerance could enlarge growth area and cope with climatic changes that can threaten our future agriculture

**D16: Genetic resources**
As for any other breeding objective, the genetic resources available are elite cultivars, landraces, related species and unrelated species

**D17: Sources of resistance**
The difficulty of transferring cold tolerance from a source to a target genotype increases with the genetic distance between them. That’s why the first resource for our breeding programs is primarily elite cultivars of the same species and, secondarily, landraces and different crops within the species. Other alternative sources are compatible species or even incompatible species, although this requires transgenesis. Finally, there is the option of generating variability through mutagenesis.

**D18: Table**
There have been a number of reports of researchers looking for cold tolerant genotypes in many crops. Here you can see a number of them in maize

**D19: Table**
And even some additional examples. Unfortunately the results were not very encouraging.
D20: Evaluation constrains
Evaluations for cold tolerance face a number of problems. First, stress produces large experimental errors, the methods are deficiently defined and the seed origin is a major source of variability that requires the production of seed in several environments. The solutions to these problems are the use of small experimental units with many replications in several environments and, of course, the use of various seed origins.

D21: Effects of environments on evaluation
Field breeding programs suffer environmental variations that limit or impede progress. The solutions are costly, e.g. marking plants or using many environments: years and locations. Laboratory programs are limited by the correlation between field and laboratory performance. Here also there are several options such as controlling temperatures, light intensity, moisture… and the evaluation under cold vs. control conditions. Finally, the best strategy is to combine laboratory and field trials.

D22: Evaluation conditions
Evaluation under controlled conditions is more precise than field experiments, has confident conditions and reduce genotype x environment interaction, although has some technical handicaps and poor correlation with field evaluation. On the other hand, field conditions are real and objective, but also unpredictable subjected to confounding factors and with large genotype x environment interaction.

D23: Laboratory evaluations
Under controlled conditions, we have many options:
- Temperature: each developmental stage different temperature requirements, namely, for evaluation for cold tolerance in maize is generally around 10 °C, although some authors use 9.5 °C, cycles of 14 °C and 8 °C or of 14 °C and 10 °C
- Substrates: Different authors use rolled paper towels, soaked filter paper, sterilized peat, sterilized sand, or hydroponic solutions
- Light: evaluations can be made with or without light or with cycles day / night

D24: Growth chambers
There is a wide assortment of growth chambers depending on the equipment. The main handicap of growth chambers is heterogeneity across space. Growth chambers are best for germination and early growth and preliminary selection and are complementary to field trials. Growth chambers cannot replace field trials.

D25: Cold chamber
As I said, cold chambers are best for small plants; they can manage thousands of entries and are repeatable. On the other hand, they are unreliable for whole cycle because they have poor performance and whole plants are hard to manage.

D26: Field trials
Again, filed trials are real and objective, but unpredictable, with confounding factors and large genotype x environment interaction.

D27: Inheritance of cold tolerance
Cold tolerance is a complex trait with polygenic inheritance. However, sometimes there are only a few genes involved. Normally there are small effects of multiple genes. The genic effects are diverse, but mostly they are additive, often dominance effects are significant and sometimes there are even epistatic effects. There are also maternal effects at first stages of development.
Besides, there are significant environmental effects and genotype x environment interaction
Another important consideration is that the genetics for diverse traits is independent.

**D28: Recommended breeding methods**
The best methods used for breeding for cold tolerance are reciprocal recurrent selection and genealogical selection. The most common selection criteria are color, biomass or yield under cold conditions. And the ways to apply these methods are sequential selection, selection index or independent cutting levels.

**D29: Objective**
The general objective of breeding for cold tolerance is to obtain genotypes with the ability to emerge from the soil and grow vigorously in cold soil and air temperatures.

**D30: Singular objectives**
There are a number of singular objectives, such as germination, competition against weeds, earlier canopy development, rapid growth, pollination prior to the hot days of summer, early maturity, normal physiological maturity in short seasons, and post-harvest conservation.

**D31: Breeding methods**
Along history, breeding methods have changed. Beginning by farmers’ selection that allowed the large crop expansion. Classical breeding methods have had limited results so far. As I said before, the most common are pedigree selection and recurrent selection. Finally, today with have modern technologies which results are still coming, particularly marker assisted selection, mutagenesis or transgenesis.

**D32: Classical breeding**
Classical breeding involves screening large collections of genotypes from all over the World, using selected populations and inbreds to make base breeding populations and the breeding methods already mentioned, namely recurrent selection, pedigree or mass selection. So far these methods have had limited success on their achievements on cold tolerance and unfavorable indirect effects on agronomic performance.

**D33: Approaches**
The approaches followed by different authors have been to choose those genotypes with higher germination or growth at low temperatures, to estimate the ratio of performance cold conditions/optimum conditions as the inverse of the ratio germination time under cold stress/germination time under control, or as the ratio of shoot dry weight under cold stress/shoot dry weight under control conditions. The best predicted results for all traits were given by a rank summation index, a multiplicative, and a base index.

**D34: Selection criteria**
The selection criteria reported in the literature are percentage germination or emergence, rate of emergence: \( RE = \sum p x d / T p \), dry matter accumulation on root or shoot biomass, shoot length / root length, vigor as a subjective scale, color, chlorophyll content, efficiency of photosystem II or spikelet sterility.

**D35: Relationships between traits**
The use of several traits has some conditions. Some of them are that the ability to germinate and survive under cold conditions may be necessary, but do not ensure early vigor, germination and seedling growth are under the control of different genetic factors, low-temperature tolerance is different at the vegetative and reproductive
growth stages, chilling stress at successive leaf-stages is similar, and the relationships between cold tolerance and yield is inconsistent. 

D36: Strategies for organizing criteria

In order to face those constrains, seed survival or germination is the first trait observed and limits the evaluation of subsequent traits. Obviously, dry matter accumulation does not allow estimation of further traits. Improving the cold tolerance at different stages in a combined fashion might be possible by using selection protocols that include all critical stages. When the traits selected behave independently, the most obvious procedure is sequential selection.

D37: Selection indexes

Several authors have published diverse selection indexes: such as a selection index including percentage emergence and seedling dry weight, multiple trait selection index based on 15 traits, a model combining several traits for screening sugarcane for low temperature resistance, freezing tolerance selected either as truncation or index selection, or an index combining data from stress and non-stress environments when heritability under non-stress is much higher than under stress.

D38: Emergence and emergence score

Some of the traits have peculiar methods for calculation, particularly emergence score, which requires counting plants every two days and calculation a rate of emergence per time: \(100 \times \Sigma(N_i / \text{time from planting}) / \text{time from planting to end of emergence}\)

D39: Effects of selection

Natural selection for adaptation to cold environments has been historically effective. However, improvements in chilling tolerance have been associated with lower fertility, moderate improvements had non-significant effects on other agronomic traits, breeding for northern Europe has released compact low stover hybrids. In Canada, most yield improvement is attributable to increased stress tolerance. There is even a report that shows that divergent selection has been effective in both directions.

D40: Selection constrains

Grain/stover yield antagonism is most severe in cooler climates because assimilates are diverted from grain filling to plant maintenance when temperatures drop. Selection under stress does not respond when evaluated under optimum conditions. Selection in controlled conditions is not necessarily related to field performance and GE interactions is large under stress conditions.

D41: Direct selection

Mass selection with early planting has limited success. Pedigree selection has been widely used with variable results and recurrent selection is sometimes efficient, depending on the species, breeders use full sibs, half sibs or S1-families. Sample size and selection intensity is crucial to avoid inbreeding depression.

D42: Direct vs. indirect selection

Selection for cold tolerance entails some problems of evaluation because of the unpredictable climatic variation of field trials and the inconsistent correlation between controlled environment and field performance. Reproducing field conditions in the laboratory is difficult and expensive, the heritability measured under stressful conditions generally decreases and stressful environments raise the GE variance. Given those facts, indirect selection is preferable to direct selection when the correlation of the selected traits compensates the reduced heritability.
D43: Indirect selection
Historical natural selection for yield under cold environments has increased cold
tolerance. It’s interesting to note that some stresses are genetically or physiologically
related, so that breeding for one stress produces improvements in other stresses, so
that associations between different stresses allow the indirect improvement of cold
tolerance when the breeding program focuses on another stress.

D44: Indirect criteria: characteristics
Indirect selection criteria can be anything from a component of stress tolerance to a
molecular marker associated with stress-related loci. A component of productivity
under stress can be useful if it is highly correlated to productivity under stress and
amenable to selection and economically assessable. Therefore, an indirect criterion
must have high heritability and high genetic correlation with productivity under stress.

D45: Indirect criteria: examples
Many indirect criteria have been identified as selection criteria for cold tolerance. Some
are physiological traits such as electrolyte leakage or chlorophyll fluorescence; some
are morphological traits, such as canopy size, stover color due to anthocyanin or
delayed maturity in soybean.

D46: Chlorophyll fluorescence
One of the most typical criteria for evaluating cold tolerance is chlorophyll
fluorescence, which is the ability of tolerant plants to maintain a higher efficiency of
excitation energy capture by open photosystem II. This trait is low-temperature
specific. Quenching analysis using the saturation pulse technique has been used in
responses to stress and this is the procedure:
- Leaf is exposed to light after a dark period, fluorescence increases to a level called F0
  or the initial fluorescence when all PSII reaction centers are open; soon, fluorescence
  increases up to a level Fm or the maximum fluorescence when all reaction centers are
  closed; the increase in fluorescence is called variable fluorescence (Fv), the ratio Fv/Fm
  is a measure of the intrinsic efficiency of PSII, and the maximum rate of the induced
  rise in chlorophyll fluorescence at cold temperatures, Fv, has also been used as cold-
  related trait.

D47: Chemical criteria in other species
Cold tolerance has been associated to changes in mitochondrial properties: a correlated
response in the interaction between membrane lipids and cytochrome C oxidase
content, isozyme variability, some proteins, as dehydrins that may play a critical role in
stabilizing cell functions or in acclimatation, particularly proline, ABA and putrescine,
fatty acid composition, soluble sugars and oligosaccharides combined with proteins
and hydroxyproline.

D48: Chemical criteria in maize
Cold tolerance has been associated in vitro to proline and ABA induce cold tolerance in
callus, cultured maize cells or diverse mechanisms of induction.

D49: QTLs
Conventional QTL identification has been carried out with only few QTLs with low
effects that are involved mostly in chlorophyll content, with significant genotype x
environment interaction and variable across genotypes.
Another strategy consists on mutational approaches that allow to locate genes and
subsequent identification through chromosome walking. The next step will be
studying gene expression.
D50: Examples of QTLs in other species
Some genes related to cold tolerance have been identified in other crops, for example the gene $T/t$, which controls pubescence color in soybean, abundance of interior spruce nuclear ribosomal RNA in seedlings, the probe $Xwg644$ as a marker for cold hardiness in wheat, the $Fcor1$ transcript accumulation for freezing tolerance in strawberry, or a RAPD marker for radiation frost injury in lentil

D51: Examples of QTLs in maize
There are also some reports showing QTLs, such as QTLs related to photosystem II activity and pigment composition in leaves, QTLs for development of root and shoot at low temperatures, QTLs for specific leaf area and for photosynthesis-related traits at low temperatures, QTL for leaf greenness, QTL related to the efficiency of photosystem II activity, or QTL for color

D52: Candidate genes for cold tolerance
These QTLs have allowed the identification of candidate genes as golden plant2 which mutant produces yellow or golden leaves, defective crown 1053A which mutant produces green striped leaves and luteus11, which alters leaf and seedling color

D53: Gametophytic selection
Other group of approaches to carry out breeding programs for cold tolerance is gametophytic selection. This is based on the fact that natural selection occurs during pollen germination and pollen tube growth. Pollen competitive ability correlates with sporophytic traits in maize. Most genes expressed in the sporophyte are also expressed in the gametophyte. Pollen selection allows the screening of large numbers of individuals without the confounding effects of dominance and epistasis. Thus, pollen can be selected for pollen germination or pollen tube growth since cold reactions are controlled by different sets of genes in the two phases. Combining sporophytic and gametophytic selection or repeated pollen selection during the inbreeding process

D54: Marker assisted selection
Other strategy is MAS, which requires an appropriate base population with high yield and variability for cold tolerance, the construction of a fine genetic map, a method to appropriately manage genotype × environment interaction of seed origin in order to obtain reliable QTLs. However, so far, no practical results have been reported for MAS

D55: Transgenic strategies
A possible solution investigated by several authors is transgenesis, which depends on the identification of major genes controlling cold tolerance. Once a major gene has been identifies, the gene is transferred to other organisms. However, the multigenic nature of cold tolerance is a deterrent because of the difficulty of transferring large numbers of genes. Some authors affirm that cold tolerance under the regulation of a few major genes. If this is true, the key for improving cold tolerance in chilling-sensitive species depends on the primary regulation of endemic genes rather than on the introgression of exotic structural genes. Most genetic engineering for cold tolerance has been focused on genes whose role in cold tolerance is not well known

D56: Transgenic maize
A number of reports have shown successful modification of maize with genes for increasing cold tolerance since 1999. Some of these examples are: Overproduction of Manganese superoxide dismutase (MnSOD) in maize chloroplasts increases the antioxidant capacity of the leaves
Expression of the *Nicotiana* PK1 gene enhances freezing tolerance in transgenic maize plants that are normally frost sensitive. The *betA* gene from *Escherichia coli* encoding choline dehydrogenase was transferred into maize via Agrobacterium-mediated transformation. A cold-tolerant Pyruvate or orthophosphate dikinase (PPDK) cDNA isolated from *Flaveria brownii* were introduced into maize by Agrobacterium-mediated transformation. The carboxy-terminal region of PPDK has been modified to mimic the amino acid sequence of the cold-tolerant PPDK of *Flaveria brownii*.

**D57: Transgenic considerations**

However, the enhancement of cold tolerance achieved by the genetic engineering of plants with cold or dehydration-responsive genes has been limited, the complexity of cold tolerance implies that the over-expression of a single gene results only in small or even undetectable increases in cold tolerance.

A deeper knowledge on regulator genes could provide the tools necessary for successfully transferring freezing tolerance to chilling-sensitive plants. A promising strategy may be to confer tolerance to cellular dehydration. Nonetheless, genetically modified maize is having a strong social rejection in Europe and we prefer not to use transgenesis.

**D58: In the following section I’ll explain the breeding program for cold tolerance we are carrying out in the Misión Biológica de Galicia since 1995**

**D59: State of the question**

The European Atlantic coast has cold and humid springs and short growth cycles. Breeders consider that European Flints are potential sources for cold tolerance but low yielders. Contrarily, Corn Belt Dents are higher yielders but poorly adapted to short growth cycles. Besides, summers are warm with some drought stress and corn borer attacks. Therefore, we need cold tolerant maize for advancing sowing, enlarging growth cycle and yield, and avoiding summer drought and pests.

**D60: Maize germplasm at MBG**

These is the collection of populations and inbred lines maintained at our bank of germplasm.

**D61: Indirect selection for cold tolerance in Europe**

Historically, adaptation to local conditions has involved indirect selection for cold tolerance in Europe along four centuries. Scientific maize breeding begun in the MBG in 1921. Actually our program is the first maize breeding program in Europe. Routinely we are carrying out genealogical selection of inbred lines focusing on early vigor, besides agronomic performance. As a consequence, new variability was generated due to recombination and selection for decades.

The heterotic pattern European Flint x Corn Belt Dent is the most frequent in Europe, along with Reid x Lancaster. European Flints had high heterosis with Reid and secondarily with Lancaster. In this combination, European Flints provide adaptation and Corn Belt Dents provide yield.

**D62: Direct selection for cold tolerance**

Breeding for cold tolerance begun in MBG in 1995 screening the germplasm collection for cold tolerance. Both populations and inbreds were screened for cold tolerance in a growth chamber. The 3 inbreds with more cold tolerance were EA2087, F7 and Z77016 but had poor agronomic performance. On the other hand, several inbreds from the humid Spain have high early vigor. Some other conclusions we reached are that
populations from cold areas are not necessarily more cold tolerant and that selection for cold tolerance releases genotypes with poor agronomic performance. Nowadays, combination of alleles for cold tolerance and agronomic performance are being carried out.

**D63: Evaluations under controlled conditions**

The methods we use consist on cycle day – night, where cold evaluations are made with light during 14 h provided by very high-output fluorescent lamps with a photosynthetic photon flux 228 μmol/m²/s at 14 °C and 10 h without light at 8 °C. The standard conditions consist on 25 °C and 18 °C in light and dark, respectively. Trials are carried out in plastic multi-cell seed trays for 30 days under cold and 15 days under standard conditions. Besides we conduct early sown field trials for combined analyses.

**D64: Cold tolerance Traits**

We use a number of traits namely proportion of emergence, number of survival plants, early vigor, chlorophyll content, anthocyanin content, activity of photosystem II, and dry matter accumulation.

**D65: Strategies and procedures**

Our program consists on screening of germplasm, genetic studies of genic effects, heritability, correlations, and identifying favorable alleles. Our breeding program involves mass selection, genealogical selection and recurrent selection. Further, we make QTL analyses and mutant analyses.

**D66: Germplasm screening**

Normally we do not publish our screening of germplasm, except this one: Rodríguez VM, MC Romay, A Ordás, P Revilla. 2010. Evaluation of the European Maize (Zea mays L.) germplasm under cold conditions. Gen Res Crop Evol 57:329-335. In this work we made a preliminary screening of 95 populations from the European Union Maize Landraces Core Collection (EUMLCC) followed by a more consistent evaluation of 11 populations with best germination and early growth under cold conditions in a cold chamber and in early field sowings. We concluded that there were some cold tolerant populations from the EUMLCC. However, populations from northern latitudes did not overcome the cold tolerant populations from southern latitudes.

**D67: Genetic studies: inheritance**

We have also made some genetic studies such as this one: Revilla P, RA Malvar, ME Cartea, A Butrón, A Ordás. 2000. Inheritance of cold tolerance at emergence and during early season growth in maize. Crop Sci 40:1579-1585. This work consisted on a diallel and a mean generation analysis and we found that cold-tolerant inbreds produced cold-tolerant hybrids, the inbred F7 may contribute cold tolerance at emergence, EA2087 contributed cold tolerance for both emergence and seedling growth, percent emergence was not related to other traits, and the genetic regulation of cold-tolerance traits conformed to an additive-dominance model. Therefore, we concluded that it should be possible to combine both high percent emergence and vigorous seedling growth.

**D68: Genetic studies: favorable alleles**

Using Dudley’s methods of identifying favorable alleles in populations for improving cold tolerance, we made this study:

Here we found that none of the maize inbred lines or populations was entirely cold tolerant, none of the inbred × population combinations fulfills all requirements for early sowings, EP80 x northwestern Spanish populations are the most promising base germplasm for further breeding programs for cold tolerance, EP80 × Puenteareas showed the largest yield and good performance at first stages of development under cold conditions, EP80 × Rebordanes had better performance at first stages of development under cold conditions, EP80 and Puenteareas showed the most favorable GCA for most traits, and early vigor the most suitable trait to select maize genotypes with superior cold tolerance during emergence and post-emergence stages.

D69: Breeding programs

Our genealogical selection program is still under way and here we make crosses between cold tolerant inbred lines x high yielders, ear – to – row selection of inbred lines, and evaluation of cold tolerance under cold conditions. This program has released some improved inbred lines.

We also carried out recurrent selection of cold tolerant populations by using selfpollinated families that were evaluated under cold conditions and the best ones were recombined. However, the results of this program were disappointing.

D70: QTL analyses

We have also published a QTL analysis:


IBM population derived from B73 x Mo17

Where we have found 2 QTLs for leaf color at low temperature in the short arm of chromosome 3 and the long arm of chromosome 6. The final fit for QTLs detected in our study explained 14.2% of phenotypic variance and 28.2% of genetic variance. Cross-validation analysis: QTLs explained 3.7% of the genetic variance. Therefore, MAS is not advisable because the proportion of variability explained was too low.

However, were found as candidate gene the locus luteus11 for the QTL in chromosome 6, while the QTL in chromosome 3 corresponds to an unknown gene.

D71: Mutant analyses

Nowadays we are performing a genetic screen using an EMS-mutated population on B73 background, developed by Professor Cliff Weil from Purdue University. We evaluated 708 mutant lines in a cold chamber and identified 18 putative mutants that were multiplied in the field. Seven of them showed a stable and heritable mutation:

Mutant       Phenotype at low temperature
058A7        High germination; High early vigor; Dark green
167G2        High germination
061A3        Green stem
066B3        Low germination
063G2        Green stem
061A3        Green stem
066B3        Low germination

D72: Current research on cold tolerance at Misión Biológica de Galicia

The main research activities we are currently carrying out on this program are:
D73: Chlorophyll regulation (Rodríguez et al. in redaction)
A study of chlorophyll regulation that is in redaction.
Chlorophyll is the primary pigment responsible of light harvesting. Chlorophyll biosynthesis is a branch of the tetrapyrrole pathway, which results in the synthesis of heme-related compounds and chlorophylls. Protoporphyrin IX is the intermediate of chlorophyll or heme biosynthesis by the chelation of magnesium or ferrous ions, respectively. Chlorophyll biosynthetic pathway is important for photosynthesis and for avoiding the accumulation of photo-toxic chlorophyll-intermediates. Expression of genes involved on chlorophyll biosynthesis depends on environmental conditions: temperature. Chlorophyll-less plants are useful tools to identify genes involved in the regulation of the chlorophyll biosynthetic pathway

D74: Chlorophyll regulation. Experimental conditions
The study is as follows:
The genotype is the inbred line A661 from the Maize Genetic Cooperation Stock Center (Urbana, IL). Seeds are grown on sterilized peat in phytotron. Fluorescent light (228 μmol m-2 s-1) in a 14 h light/10 h dark light regime and watered as needed. Control temperature 25 ºC and cold temperature 15 ºC. Recovery experiments are made at 15 ºC 3 weeks and 25 ºC 1 week. After four weeks of growing the third leaf showed two well defined sections: distal (chlorophyll-less) and proximal (chlorophyll-containing) sections

D75: Chlorophyll regulation in A661: cold effects and recovery
Here you can see the color of B73 and A661 at 25 ºC and 14 ºC and the recovery

D76: Recovery in A661 histological sections
And in this pictures you can see how the anatomy changes and the accumulation of chlorophyll in the tissue from the distal to the proximal sections of the leaf

D77: Chlorophyll regulation. Gene analyses
For studying the gene involved in this phenotype, A661 was crossed to EP42 and to EP74, plants were self-pollinated for two generations. A linkage map was constructed using 393 F2:3 families, that were genotyped with 98 polymorphic SSR markers. The QTL analysis was made with LOD 3. Total anthocyanin was extracted and analyzed with HPLC. Moreover, chlorophyll precursors were extracted from etiolated tissue, transverse sections of leaf observed at microscope, RNA was extracted for microarray hybridization and genes differentially expressed are identified

D78: CornFed project
The other major action is included in an international breeding project called “From diversity to energy: integration of advanced mapping and phenotyping methods to identify key alleles for building maize energy ideotypes” involving 9 research institutes and 4 private companies from Germany, France and Spain.

D79: CornFed: Objectives
The general objectives of this project are sharing a large collection of European germplasm, genetic analysis of a large set of maize genotypes, analyzing the genetics of traits related to yield and quality for feed and bioenergy and establishing a European maize resource database.

D80: CornFed: work packages
The project involves four working packages:
- Wp1: the assembling and creation of a set of original and complementary resources for LD and linkage mapping and near isogenic materials for further characterization of QTL effects
- Wp2: the evaluation of these materials for the traits of interest: cold tolerance and biomass production
- Wp3: polymorphism discovery efforts based on new approaches and specific materials to complement information already existing in this field, use and/or development of genotyping resources
- Wp4: organization and statistical analysis of data

D81: CornFed: cold tolerance
The following materials are developed and evaluated under cold conditions and genotyped with SNPs:
Two highly diverse LD mapping populations: consisting on 300 flint lines panel and 300 dent line panel. And two European NAM designs connected with the US NAM: the European Flint Connexion and the early dent Connexion.
In the picture you can see the genetic diversity of flint and dent inbreds from those panels.

D82: CornFed: Phenotyping of LD Mapping Populations
These are the field trials in this project across the three countries. We evaluate hybrids of the inbreds from the panels x complementary testers in five single replicates trials, with an appropriate block design to manage precocity. We measure some common traits for all trials, particularly male and female flowering, plant height, dry matter proportion, and dry matter yield.
Besides, there are some specific traits for special trials. For cold tolerance the evaluations are made in the field, cold greenhouse, and growth chamber.

D83: European Flint NAM
The European Flint NAM consist on 12 flint inbreds crossed to a common flint parent

D84: European Dent NAM
And the European Dent NAM consist on 16 dent inbreds crossed to a common dent parent

D85: Construction of NAMs
For the Flint NAM, we used 12 flint inbreds crosses with a central flint parent with B73 as connection to US NAM and UH007 as connection to the European Dent NAM. From those crosses, 12 RIL populations released by DH
For the Dent NAM, 16 dent inbreds crosses with a central dent parent with B73 as connection to US NAM and F353 as connection to European Flint NAM. From those crosses, 16 RIL populations released by DH

D86: Analyzing the DH lines
The more than 2000 DH lines from the 12 DH RILs from the European Flint NAM and the 16 DH RILs from the European Dent NAM are genotyped with SNP array from Illumina under cold tolerance
They were also evaluated in crosses: The 12 Flint DH RILs x UH007 and the 16 Dent DH RILs x F353

D87: Conclusions
1. There is enough variability for breeding programs
2. A combination of laboratory and field evaluations should increase the possibilities of success and the rate of improvement in breeding programs for cold tolerance

3. The most efficient indirect selection criteria are electrolyte leakage and chlorophyll fluorescence

4. Genetic regulation of cold tolerance is multigenic and mostly additive

**D88: Conclusions: breeding**

1. Simple programs, such as mass selection, can be successful, although recurrent selection programs are recommended

2. Selection criteria should be clearly defined at each growth stage, considered as independent traits, and improved using a multiple trait selection procedure

3. Alternative methods *(in vitro culture)* has had limited results

4. Marker assisted selection has been very limited

5. Genetic engineering is still at theoretical levels

**D89: Future perspectives**

- Genetic transformation is a promising methodology with social problems
- Further improvements require a better knowledge of regulatory genes
- Mutagenesis is a promising approach for identifying genes
- Genetic resources essential for future breeding

There is a long way to reach cold tolerant maize

**D90: Thanks for your attention**