Breeding for freezing tolerance in plants

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Outline

• Where I am from
• Freezing tolerance in plants
• Reverse genetics in winter wheat
• Association mapping in perennial ryegrass
• PPP pre-breeding project
• Future targets
LITHUANIAN RESEARCH CENTRE FOR AGRICULTURE AND FORESTRY (LRCAF) was established in 2010 as a merger of three related research institutions:

- Institute of Agriculture
- Institute of Horticulture
- Institute of Forestry

Research staff: 183
PhD students: 58
Total staff: 636

Budget: 12 M€
HISTORY OF THE CENTRE

1911 Agricultural school was established in Dotnuva.

1919 Dotnuva Agricultural college was set up.

1922 Breeding Station was founded in Dotnuva.

1923 Dotnuva experimental station started operating.

1924 Academy of Agriculture was established in Dotnuva, transferred to Kaunas in 1947.

1927-1960 A network of experimental stations was established.

1956 Lithuanian Institute of Agriculture was established in Dotnuva.

2010 Lithuanian Research Centre for Agriculture and Forestry was formed.
Research programs at the Lithuanian Research Centre for Agriculture and Forestry

From single genes to ecosystems
LABORATORY OF GENETICS AND PHYSIOLOGY

Staff:
5 researchers
1 PhD and 2 Bsc students
3 technicians

Research:
DNA marker development
DH production in wheat
Freezing tolerance testing

Facilities:
DNA laboratory
Experimental greenhouse
Growth and freezing chambers

Collaborators:
Iowa State University
ETH Zurich
Aarhus University
Cultivation area in Lithuania, %

- Grasslands: 32%
- Winter wheat: 20%
- Spring wheat: 9%
- Spring barley: 10%
- Winter rye: 3%
- Winter triticale: 4%
- Winter rape: 4%
- Spring rape: 9%
- Maize: 2%
- Pulse crop: 2%
- Potato: 1%
- Sugar beet: 1%
- Oat: 3%
- Pulse crop: 2%
- Potato: 1%
- Sugar beet: 1%
- Maize: 2%
- Oat: 3%
Winter wheat

Spring wheat

Area under wheat in 2005-2014

Kha


Spring wheat
Plant responses to cold (and light)

• **Chilling** – low above zero temperatures
• **Freezing** - below zero temperatures
• **Cold acclimation** – acquisition of freezing tolerance (easy come easy go)
• **Vernalization** - acquisition of competence to flower (takes time and stays)
• **Winter hardiness** – ability to overwinter
Winter versus spring wheat
Norstar NILs were grown at 20°C and 16-h day from 7 to 42 days and then LT acclimated at 2°C for 28 days. a LT50 versus plant age. b Dissected shoot apices indicating stage of development at the start of LT acclimation.

Limin and Fowler, 2006
Near-isogenic lines (NILs)
Decoupling of Vrn1 and Fr1

Vágújfali et al., 2006
Wheat transcriptome response to cold

(a) Statistically significant two-fold or greater changes (P = 0.05) in transcript abundance in plants exposed to a 4 °C for 2 days. (b) Venn diagram showing the number of genes expressed in common between the three varieties after exposure to a ‘cold shock’.

Winfield et al., 2010
An early light-inducible protein (ELIP) showing a distinct response to a ‘cold shock’, but no response to a slow decline in temperature. C, crown; L, leaf; H, Harnesk; P, Paragon; S, Solstice.
Wheat transcriptome response to cold acclimation

(a) transcript abundance in plants exposed to a gradual decline in temperature, light intensity and day length; (b) Venn diagrams showing the number of two-fold or greater changes (P = 0.05) in transcript abundance. The values refer to genes that changed in expression during the period 21–63 days.

Winfield et al., 2010
Cold response in plant cells

ABRE, ABA response element
AFP, anti-freeze protein
An, annexins
CBF, C-repeat binding factor
CBP, calcium binding proteins
CCH, calcium channel
COR, cold-responsive genes
CRT, C-repeat elements
GLU, glutathione
ICE, inducer of CBF expression
KIN, kinases and phosphatases
LEA, late embryogenesis-abundant
PSI/PSII, photosystem I and II
RLK, receptor-like kinase
ROS, reactive oxygen species

Winfield et al., 2010
Chilling injury in chilling-sensitive plants

Theocharis et al., 2012
Cellular processes induced by cold acclimation

- Accumulation of cryoprotectants (sugars, proline, ...)
- Accumulation of ROS and activation of scavenging systems
- Changes in gene expression and protein synthesis
- Modification in plant membranes
- Photosynthetic acclimation
- Reduction of lower threshold temperature in acclimated plants
- Change in lipid composition
  - Increase in desaturated fatty acids
  - Increased fluidity of membranes

Theocharis et al., 2012
Identification and analysis of FT genes in winter wheat

Winter wheat lines ‘5899-16’ and ‘5450-1’

Cold acclimation 0, 2, 4, 6 weeks

cDNA-AFLP analysis

BLAST analysis

Mutagenesis TILLING population

Mutation detection (HRM analysis)

(qRT-PCR analysis)

Freezing tolerance testing
AFLP and cDNA-AFLP
cDNA-AFLP results

Leaf

Crown

Weeks

0               2                4              6

0                2                4               6

cDNA-
AFLP

results
Differentially expressed genes

2 weeks after the start of cold acclimation

Sucrose synthase 1
Heat-shock protein
Serine carboxypeptidase-like
Vacuolar-sorting receptor 1
Zinc finger AN1 domain

Phosphoglycerate kinase, chloroplastic
Signal recognition particle 54 kDa protein, chloroplastic-like
ATP-dependent Clp protease ATP-binding subunit ClpC homolog 1
Clp-P protease subunit

Sucrose + NDP ↔ NDP-glucose + fructose

Crown

Beta-3-tubulin
Phototropin-2-like
Digalactosyldiacylglycerol synthase 2, chloroplastic
TILLING
Targeting Induced Local Lesions in Genomes

Colbert et al., 2001

PCR amplification & heteroduplex formation followed by Cel I digestion of heteroduplexes

Li-cor gel images

96 - well plates

Fluorescence

Normal DNA
e.g. agctagctacgctagctacgctacg

Mutated DNA
e.g. agctagctacgctgtagctacgctacg

55°C  95°C
Mutagenesis and mutation screening

(0.4% - 1.0%)

EMS

‘5450-1’ ir ‘5899-16’
Winter wheat lines

M1 generation
(heterozygous and chimeric)

M2 seeds

M2 generation
(segregating)

M3 seeds
(stored)

DNA isolation

Data Analysis by HRM Software v3.0

Sample Melt

7500 Fast Real-Time PCR System
(Applied Biosystems)

HRM analysis

Exon X

2nd PCR
of amplicon with lengths <250 bp

1st PCR
region of interest

Creation of TILLING population

Mutagenesis and mutation screening

Gene X

Mutagenesis and mutation screening
Mutation discovery by HRM analysis

Aligned curves of fluorescence vs temperature showing wild type (blue) and mutant (red) samples.
2 mutations per 75.68 kb
Nonsense-mediated *Sucrose synthase 1* mRNA decay during cold acclimation in winter wheat

wt – wild type
M692 – missens mutation
M631 – nonsense mutation
A – leaf tissue
B – crown tissue
Reduced freezing tolerance of M631

Freezing tolerance of wild type ‘5899-16’ and M631 winter wheat acclimated at 5 °C for 0–4 weeks.
Perennial ryegrass (*Lolium perenne* L.)

- The main forage grass species in Denmark and further south and west in Europe
- Finland: 2%, Estonia: 13%, Norway: 13%, Lithuania: 30% of certified seed sale
- Superior feed quality and productivity under frequent cutting regimes
- Is expected to expand further north due to milder winters with shorter periods of snow cover
Main problems with perennial ryegrass

- Susceptibility to low-temperature fungi
- Inadequate growth cessation in autumn to allow for sufficient cold hardening
- Can de-harden too early with increased risk of frost injury in spring

*Festuca arundinacea* and *L. perenne*
PPP perennial ryegrass pre-breeding project

• Aim:
  – Identify and select new plant materials for development of cultivars with a suitable adaptation to future climates
  – Recombine exotic materials with existing germplasm to create new genetic resources
Biparental versus Asociation mapping
Linkage disequilibrium (LD)

\[ D_{ab} = (\pi_{AB} - \pi_A \pi_B) \]

\[ r^2 = \frac{(D_{ab})^2}{\pi_A \pi_a \pi_B \pi_b} \]

Flint-Garcia et al., 2003
LD in plants

- Mating type
- Population structure
- Genetic drift
- Selection intensity

Hamblin et al., 2011
Sample preparation for freezing tolerance test

Freezing test
Regrowth at 3 weeks after freezing

Control
-4°C
-6°C
-8°C
-10°C
-12°C
## Phenotypic variation for freezing tolerance traits

<table>
<thead>
<tr>
<th>Trait</th>
<th>Ecotypes</th>
<th></th>
<th></th>
<th></th>
<th>Cultivars</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>Minimum</td>
<td>Maximum</td>
<td>Sd^2</td>
<td>Mean</td>
<td>Minimum</td>
<td>Maximum</td>
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<tr>
<td>EL at -8°C [%]</td>
<td></td>
<td>14.27</td>
<td>6.09</td>
<td>25.80</td>
<td>5.23</td>
<td>15.83</td>
<td>7.82</td>
<td>25.06</td>
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<tr>
<td>EL at -12°C [%]</td>
<td></td>
<td>38.91</td>
<td>22.27</td>
<td>57.06</td>
<td>9.14</td>
<td>43.09</td>
<td>23.85</td>
<td>63.51</td>
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<tr>
<td>PTS at -8°C [%]</td>
<td></td>
<td>51.12</td>
<td>0.00</td>
<td>100.00</td>
<td>35.24</td>
<td>56.28</td>
<td>0.00</td>
<td>100.00</td>
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<tr>
<td>PTS at -12°C [%]</td>
<td></td>
<td>11.02</td>
<td>0.00</td>
<td>91.67</td>
<td>22.10</td>
<td>9.92</td>
<td>0.00</td>
<td>53.33</td>
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<tr>
<td>PC [μg g^{-1} DW]</td>
<td></td>
<td>2.558.86</td>
<td>473.34</td>
<td>6157.35</td>
<td>1366.34</td>
<td>2.521.75</td>
<td>519.68</td>
<td>5840.46</td>
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</tbody>
</table>

EL – electrolyte leakage  
PTS – plant tiller survival  
PC – proline content
Correlation between phenotypic traits

<table>
<thead>
<tr>
<th>Trait</th>
<th>EL at -8 °C</th>
<th>EL at -12 °C</th>
<th>PTS at -8 °C</th>
<th>PTS at -12 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>EL at -8 °C</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EL at -12 °C</td>
<td>0.43***</td>
<td>-</td>
<td></td>
<td></td>
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<tr>
<td>PTS at -8 °C</td>
<td>-0.40***</td>
<td>-0.21 ns</td>
<td>-</td>
<td></td>
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<tr>
<td>PTS at -12 °C</td>
<td>-0.14 ns</td>
<td>-0.49***</td>
<td>0.46***</td>
<td>-</td>
</tr>
<tr>
<td>PC</td>
<td>-0.06 ns</td>
<td>0.02 ns</td>
<td>-0.20 ns</td>
<td>0.03 ns</td>
</tr>
</tbody>
</table>

EL – electrolyte leakage  
PTS – plant tiller survival  
PC – proline content
**LpIRI1** – ice recrystalization inhibition

Griffith and Yaish, 2004
(a) ice crystal morphology in (b) water (c) dilute AFP (d-f) concentrate AFP solution. Scale bar = 10 µm. Recrystallization of ice (g-j) in nonacclimated (NA) and cold-acclimated (CA) winter rye extracts. Scale bar = 1.75 cm.

Griffith and Yaish, 2004
Survival rates and phenotypic appearance of control and transgenic Arabidopsis plants after freezing at -4°C and recovery. From left to right wild-type (Col-0); plants carrying an empty pMDC32 vector (EV-6); transgenic plants overexpressing LpIRI-b; transgenic plants overexpressing LpIRI-a.

Zhang et al., 2010
**Linkage disequilibrium in *LpIRI1***

- ORF 855 bp
- 52 SNPs (MAF >5)
- SNP density 1/16 bp
- 1 INDEL of 15 bp
Marker-trait associations in *LpIRI1*

(A) Marker-trait association in *LpIRI1*. The figure shows the gene structure with ATG and TAA codons. The significance of associations is marked with different p-values: $2.40E-02^a$, $7.26E-03^b$, and $3.48E-02^c$.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of accessions</th>
<th>Locus 322</th>
<th>Locus 369</th>
<th>Locus 726</th>
<th>EL at-8°C [%]</th>
<th>EL at-12°C [%]</th>
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<tbody>
<tr>
<td>Wt</td>
<td>52</td>
<td>T:T</td>
<td>T:T</td>
<td>C:C</td>
<td>14.02</td>
<td>39.45</td>
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<tr>
<td>Rec-1</td>
<td>5</td>
<td>A:T</td>
<td>G:T</td>
<td>C:C</td>
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<td>46.94</td>
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<td>T:T</td>
<td>T:T</td>
<td>C:T</td>
<td>16.82</td>
<td>42.41</td>
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<tr>
<td>Rec-3</td>
<td>6</td>
<td>A:T</td>
<td>T:T</td>
<td>C:T</td>
<td>14.83</td>
<td>34.86</td>
</tr>
<tr>
<td>Rec-4</td>
<td>8</td>
<td>A:T</td>
<td>G:T</td>
<td>C:T</td>
<td>19.78</td>
<td>50.32</td>
</tr>
</tbody>
</table>

(B) Graph showing electrolyte leakage (EL) for different genotypes and temperatures. The genotypes are Wt (wild type) and Rec (recombinant). EL is measured at -8°C and -12°C. The graphs indicate a significant increase in EL at lower temperatures for all loci.

*Wt* – wild type  
*Rec* – recombinant  
*EL* – electrolyte leakage
Genotyping by Sequencing (GBS)

**Step 1**
Construct reduced representation libraries (RRLs) by digesting each DNA sample with a restriction enzyme (ApeKI)

**Step 2**
Ligate custom ‘barcoded’ adaptors to sticky ends of restriction site. Each sample has its own unique barcode sequence

**Step 3**
Pool digested and barcoded DNA into a single tube. Perform PCR amplification, library preparation, and sequencing on Illumina platform

**Step 4**
Use barcodes to assign sequences to samples. Produce a file of DNA sequence data for each sample

Elshire et al., 2011
Genotyping of 380 PPP populations

4 libraries, each with 88 populations, 1 library with 28 populations
Evaluation of $LT_{50}$ value in perennial ryegrass for PPP (workflow)

1. The collection
   - 20 seed per population;
   - 3 replicates;
   - soilless substrate;
   - 30,000 plants in total.

2. Cold-acclimation
   - At $+2 \, ^\circ C$ for 15 days,
   - 12/12 h photoperiod.

3. Sample preparation
   - Leaves will be trimmed and the tillers will be counted prior the freezing test.

4. Freezing test
   - 6 freezing temperatures;
   - temperature probes in each cell pack.

5. Regrowth
   - After freezing plants will be moved to phytotron;
   - regrowth will be evaluated after 21 days.

6. $LT_{50}$ calculation
   - $LT_{50}$ values will be calculated for each population.
GWAFF and Genomic Selection

Heffner et al., 2009
Acknowledgements