NATIONAL AGRICULTURAL AND FOOD CENTRE
RESEARCH INSTITUTE OF PLANT PRODUCTION

2nd Symposium on Innovation
Cooperation in Technology and International Transfer of Technology China + 16 CEEC Format
Nitra, Sept 23, 2015

Plant Biotechnology Innovations in Plant Production

(cases from our practice)

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Piešťany, Slovak Republic

Topics:

1. Assisted plant breeding
    - Healthier grains, grains as medicine
2. Plant molecular breeding
3. Gene transfer
    - Wheat quality improvement by new proteins
    - Cereals producing polyunsaturated fatty acids in grains
    - GM plants for agriculture

Plant production & Biotechnology

Plant production - cultivation of plants (and other life forms) for **food**, **feed**, **fiber**, **raw materials**, **energy** (biofuel), **drugs** and **other** products used to sustain and improve the human life (International Labour Organization)

Biotechnology - the **use** of **living systems** and **organisms** to **develop** or make **products**, or "any technological application that uses biological systems, living organisms or derivatives thereof, to make or modify products or processes for specific use" (UN Convention on Biological Diversity, Art. 2)

1. Assisted Plant Breeding

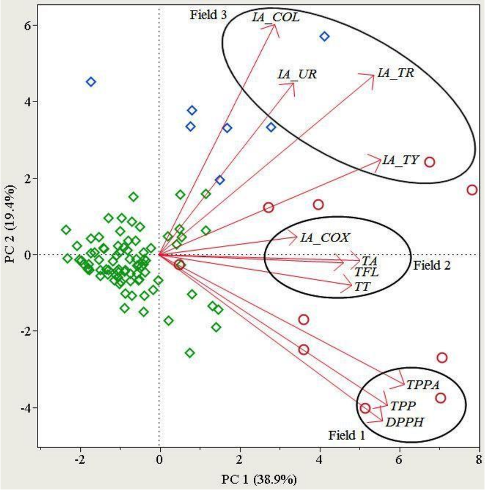
Biological activity of cereal grains

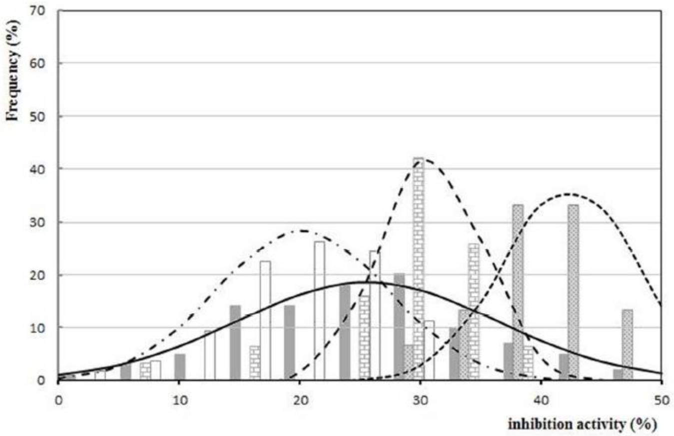
- „**Colored**" wheat – new compounds in grain – **anthocyanins** - glycosylated cyanidins, delphinidins, malvinidins, pelargonidins, petunidins, and peonidins accumulated in **aleurone** layer or pericarp - **natural compounds** - **purple**, **blue**, and **red** colored wheat seeds

- Improvement of staple foods and feeds consumed daily by **health-promoting** and **disease-preventing** agents
- Special wheat cv. **PS Kalkulka** (2014) – **purple** grains – functional foods production

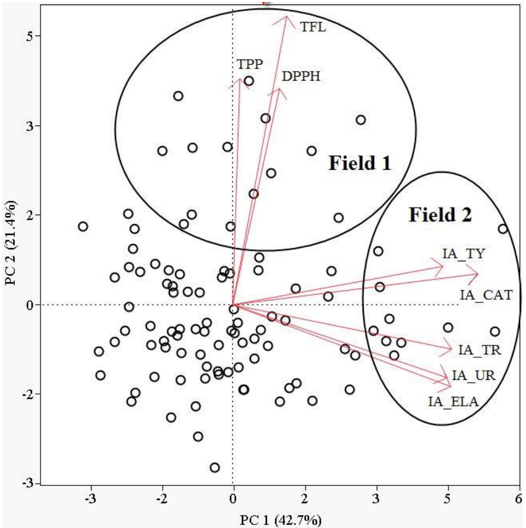
Biological activity of cereal grains

- Extracts from seeds - wheat, barley, oat
- Total **polyphenols**
- Total **flavonoids**
- **Antioxidant** activity (DPPH)
- **Inhibitory** activities to **proteases** - TRY, THR, URO, CAT, ELA, COX





Frequency distribution of IA_ELA of oat extracts



2. Plant Molecular Breeding

Wheat improvement – **Breeding** + **MAS**

Plant molecular breeding approach:

- **Hybridization** Donor x Acceptor

- **Selection** by **molecular markers**
- Line(s) development, multiplication, testing, registration, marketing

Hybridizations
P_1xP_2, P_1xP_3, P_1xP_4, ...
↓
F_1 progenies
Self-pollination ↓
F_2 progenies
Self-pollination ↓
MAS
↓
Selected F_3 lines
↓
Development of new cultivar

Wheat improvement – **resistance**

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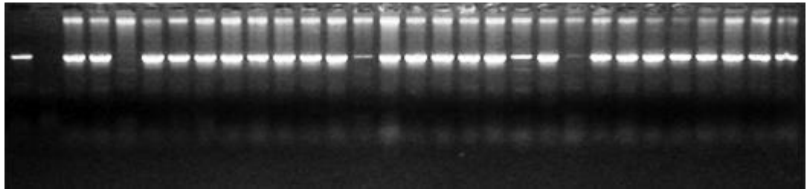
- Improvement of **leaf rust resistance**
- Effective ***Lr*** gene(s) – *Lr19*, *Lr24*, *Lr35*, *Lr19* + *Lr24*, ...
- Effective (DNA) marker system - PCR-based

P1 **NIL** P2

R S

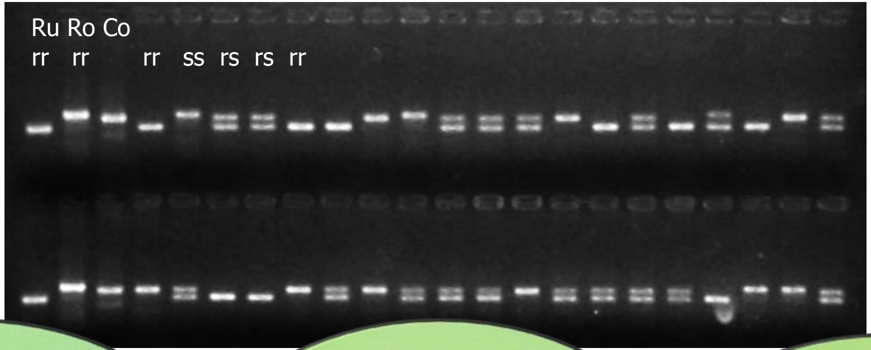


NIL
P1
P2

Hybridizations
P_1xP_2, P_1xP_3, P_1xP_4, ...
↓
F_1 progenies
Self-pollination ↓
F_2 progenies
Self-pollination ↓
MAS
↓
Selected F_3 lines
↓
Development of new cultivar

Barley improvement – **resistance**

- Example – **virus resistance** (BaYMV + BaMMV)
- Effective ***rym*** gene(s) – *rym4*, *rym11*, *rym4 +* rym11
- Effective (DNA) marker system - PCR-based



Hybridizations
P_1xP_2, P_1xP_3, P_1xP_4, ...
↓
F_1 progenies
Self-pollination ↓
F_2 progenies
Self-pollination ↓
MAS
↓
Selected F_3 lines
↓
Development of new cultivar

3. Gene transfer

Wheat quality improvement by new proteins

Wheat improvement – **proteins**

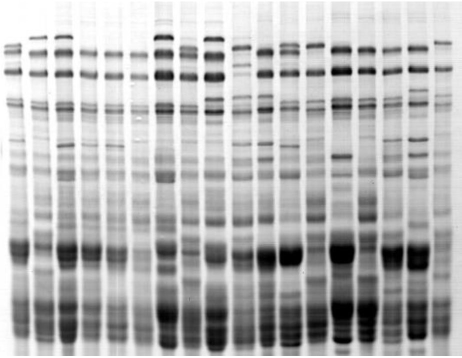
- Wheat **quality** - influenced by seed storage **proteins**
- Gliadins & **Glutenins** - LMW-GS & **HMW-GS**
- HMW-GS - **x-** or **y-type** of subunit - ***Glu-1* loci** → hexaploid wheat contains theoretically ≤ 6 different HMW-GS
- HMW-GSs contain signal sequence + domain + N- and C- terminal domains consists of amino acids (including **Cys**)
- Cysteine residues - important role in intermolecular **S-S bonds in dough** influencing final **bread-making quality**

Where are **„new and better" genes** & **proteins ???**

Genetic resources - protein & gene sources

- **Where ?** – national Genebank – maintained wheat **genetic resources** collection contains ~5000 wheat accessions

- **How ?** – extensive **screening** in seed storage **protein patterns**



Protein mining in genetic resources

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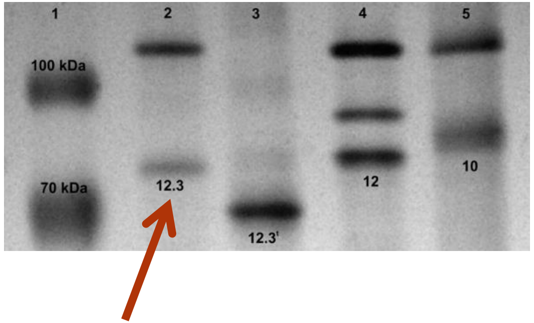
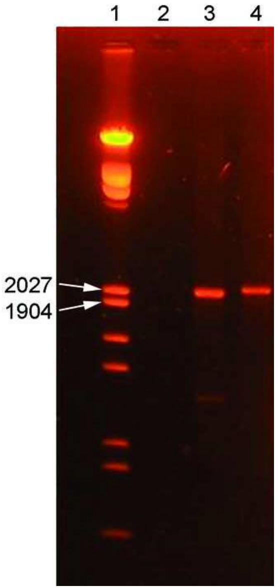
Interest to:

- Identify – **well-known** & highly **frequented** HMW-GS improving quality (1, 2, 2*, 7+8, 7+9, 5+10 ...)
- Identify **low frequented** HMW-GS increasing quality (15+16, 17+18 ...)
- Find **new** HMW-GS with **unknown** effect in **obsolete cultivars** or **landraces** of *T. aestivum* originated from similar environment and agricultural systems
- Look for **new** HMW-GS with **unknown** effect in **„exotic“ germplasm** of *T. aestivum* or other *Triticum* species

From protein to gene (story of cv. Noe)

- *T. aestivum* L. cv. **Noe** – French **obsolete cultivar**, genetic background of Russian winter wheat Odyssea, released in 19th century – possess **new** HMW-GS **1Dy12.3**
- **Amplification** with primers derived from 1Dy12 (X03041) and 1Dy10 (X12929)





Cloning
+
sequencing

Protein and **gene characterization** (story of cv. Noe)

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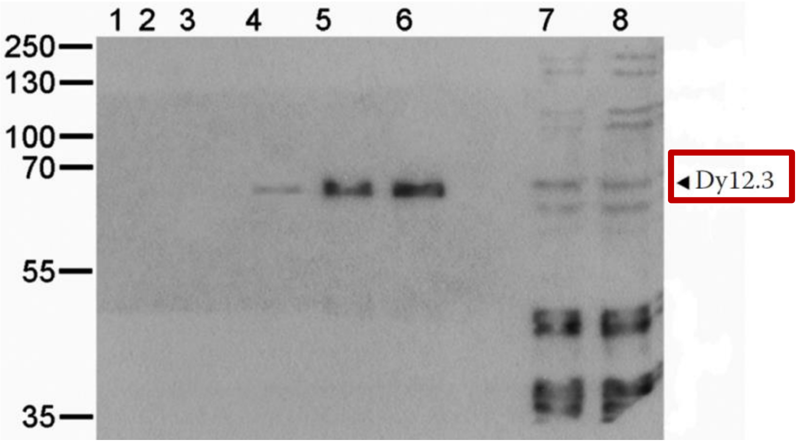
- **1Dy12.3** – different **amino acid** composition

Table 3. Comparison of essential amino acid content in selected HMW-GS 1Dy subunits of genus *Triticum* L. and *Ae. tauschii*

Essential amino acids	Subunits							
	1Dy12.3	1Dy10	1Dy12	1Dy10.1	$1D^ty10$	$1Dy12^t$	$1Dy12.1^t$	$1Dy13^t$
Phe	3	3	3	3	3	3	3	3
His	12	13	13	13	14	14	13	13
Ile	7	5	8	8	7	6	6	5
Lys	9	8	9	8	7	8	9	8
Leu	28	28	29	27	28	29	29	29
Met	6	4	5	4	4	4	4	4
Thr	25	26	25	26	27	26	26	27
Val	19	20	19	19	19	18	18	18
Trp	6	6	6	6	7	6	6	5

Heterologous expression of 1Dy12.3 (story of cv. Noe)

- Western blott analyses of cell lysates of *E. coli* transformed with 1Dy12.3



3. Gene transfer

Cereals producing polyunsaturated fatty acids in grains

Polyunsaturated fatty acids (PUFAs)

- **Unique** structural and functional **characteristics** of PUFAs:
  - Regulation of architecture, dynamics, phase transition, **permeability of membranes** – modulating behavior of membrane-bound proteins
  - **Controlling** of certain **genes expression**, affecting processes including FA biosynthesis and cholesterol transport
  - **Precursors** of many **metabolites** (prostaglandins, leukotrienes, hydroxy-fatty acids) regulating critical biological functions
  - PUFAs are **required** in **every organ** in body to function normally
  - **Insufficient consumption** of PUFAs leads to **abnormalities** in skin, diabetes, cardiovascular, endocrine, nervous, immune, inflammatory, respiratory, reproductive systems, etc.
  - **Mammals** are not able to synthesize **PUFAs** → must be **supplied** in diet

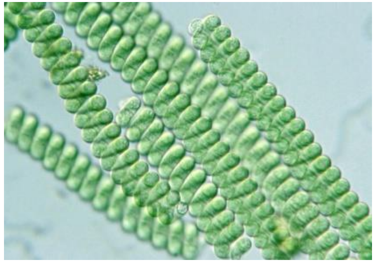
PUFAs of interest

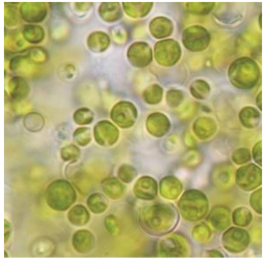
- PUFAs of interests – **γ-linolenic acid** (18:3 n-6; GLA), dihomo-γ-linolenic acid (20:3 n-6; DGLA), arachidonic acid (20:4 n-6; AA), eicosapentaenoic acid (20:5 n-3; EPA), docosapentaenoic acid (22:5 n-3; DPA), docosahexaenoic acid (22:6 n-3; DHA)
- PUFAs are **insufficient** in **natural sources** of food and feed
- Strategies for food and feed improvement by PUFAs:
  - ✓ **enrichment** by PUFAs produced elsewhere (external)
  - ✓ **production** by own (internal)
- **Cereal** grains are **low** in fatty acids content
- Linoleic acid – major fatty acid of n-6 family in grains, α-linolenic acid (n-3 family) in very small quantities

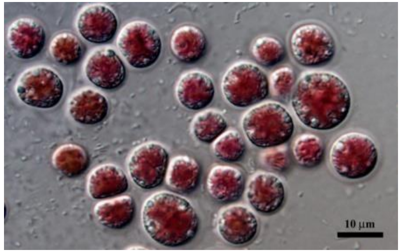
Sources of **PUFAs**

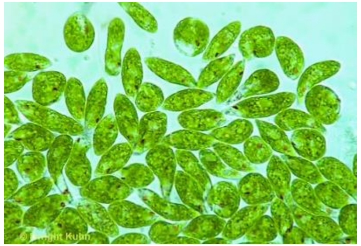
- Beneficial sources of PUFAs – **fishes** (tuna, herring, menhaden, sardine, salmon) or **shellfishes** (blue crab, oyster, lobster, mussels) – oils (C20, C22, n-3) and fatty acids – EPA, DPA, DHA

- **Alternative sources** – marine bacteria, heterotrophic and phototrophic microalgae (*Spirulina platensis, Chlorela vulgaris, Porphyridium cruentum, Sargassum salicifolium, Euglena gracilis*), mosses



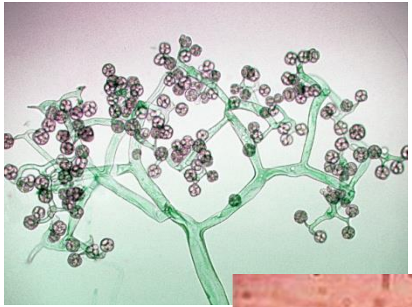


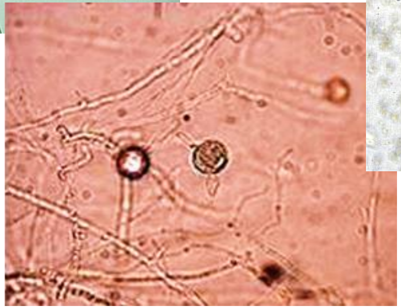


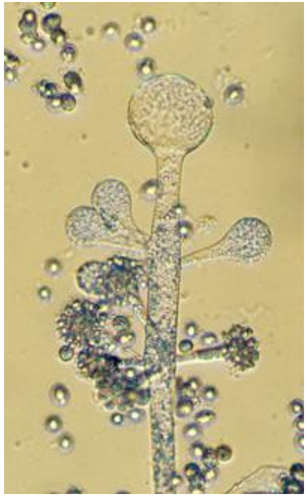


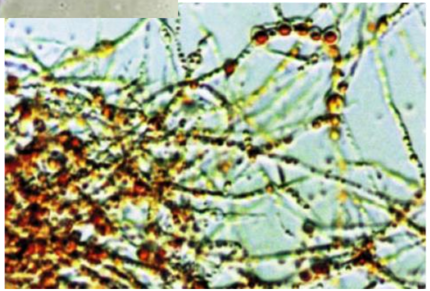
Sources of **PUFAs**

- **Alternative sources** – **oleaginous fungi** – fungi ***Zygomycetes*** (*Thamnidium elegans, Pythium irregulare, Rhizopus arrhizus, Cunninghamella echinulata, Mucor circinelloides, Mortierella alpina*)
- Particularly active in synthesis of PUFAs – donors of **genes** involved in PUFAs biosynthesis









Plant sources of **PUFAs**

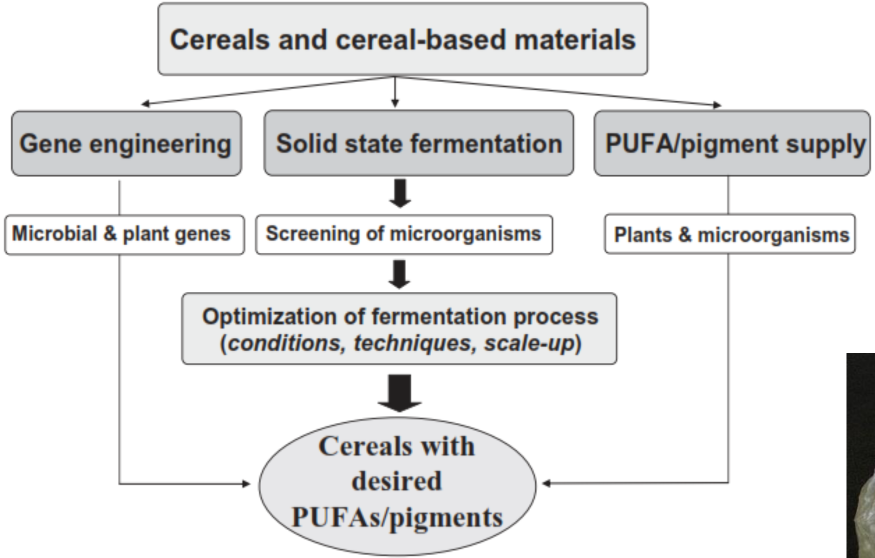
- Main sources of C18 PUFAs – seeds with dominant LA (18:2, n-6) and ALA (18:3, n-3) but occurrence of significant levels of essential **GLA in plants** is **rare**
- Most commercially important sources of GLA – seeds of **evening primrose** (8–10%), **borage seeds** (24–25%), **black currant seeds** (16–17%)



- PUFAs **above C18 cannot** be synthesized **by higher plants** in any significant amounts owing to a **lack** of requisite **enzymes**

PUFAs in **cereals ?**

- Challenge for enrichment of cereals with PUFAs:
  - **Solid state fermentations** – oleaginous fungi (*Zygomycetes*, *Thamnidium* sp., *Cunninghamella* sp., *Mucor* sp., *Mortierella* sp.) on crop residues
  - **Genetic engineering** of plants → „producers" of FA



Gene transfer & **PUFAs** production **in plants**

- Some plant species were modified for GLA production:

Transformed Plant	**Source of *D6D* gene**	**Content of GLA (% of FA)**	**Authors, year**
Tobacco (*Nicotiniana tabacum*)	*Cyanobacterium*	~1,2 %	Reddy, Thomas, 1996
Tobacco (*Nicotiniana tabacum*)	Borage (*Borago officinalis*)	~13 %	Sayanova et al., 1997
Canola (*Brassica na*pus L.)	*Mortierella alpina*	43 %	Liu et al., 2001
Mustard greens (*Brassica juncea*)	*Pythium irregulare*	25-40 %	Hong et al., 2002
Soybean (*Glycine max*)	Borage (Borago officinalis)	31 %	Sato et al., 2004
Tobacco (*Nicotiniana tabacum*)	*Phaeodactylum tricornutum*	29,3 %	Abbadi et al., 2004
Linseed (*Linum usitatssimum*)	*Phaeodactylum tricornutum*	16,8 %	Abbadi et al., 2004
Mustard greens (*Brassica juncea*)	*Pythium irregulare*	27-29 %	Wu et al., 2005
Safflower (*Carthamus tinctorius*)	*Saprolegia diclina*	70 %	Nykiforuk et al., 2012
Safflower (*Carthamus tinctorius*)	*Mortierella alpina*	50 %	Nykiforuk et al., 2012

Barley improvement with **PUFAs**

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- Barley (*Hordeum vulgare* L.) – **absence** of n-3 and n-6 PUFAs
- **Cereals** do **not produce** essential **PUFAs** but produce metabolites that are substrates for enzymes catalyzing formation of n-6 PUFAs – **Δ-6-desaturase** - catalyses conversion of LA (C18:2, n-6) to GLA (C18:3, n-6)
- Changes in composition of fatty acids in cereal grains are **not feasible** by **conventional breeding**

How to **produce GLA** in **barley ???**

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Barley improvement with **PUFAs**

- **Δ-6-desaturase** – catalyses conversion of LA (C18:2, n-6) to GLA (C18:3, n-6)

Cereals **terminate** fatty acid **synthesis** at this level due to **absence** of **D6D**

Linolenic acid (LA)	18:2n-6		18:3n-3	α-Linolenic acid (ALA)
	↓	Δ6-desaturase	↓	
γ-Linolenic acid (GLA)	18:3n-6		18:4n-3	Stearidonic acid (SDA)
	↓		↓	
Dihomo-γ-linolenic acid (DGLA)	20:3n-6		20:4n-3	
	↓	Δ5-desaturase	↓	
Arachidonic acid (AA)	20:4n-6		20:5n-3	Eicosapentaeonic acid (EPA)
	↓		↓	
Docosatetraeonic acid (DPA)	22:4n-6		22:5n-3	
	↓	Δ4-desaturase	↓	
	22:5n-6		22:6n-3	Docosahexaeonic acid (DHA)

Barley improvement with **PUFAs**

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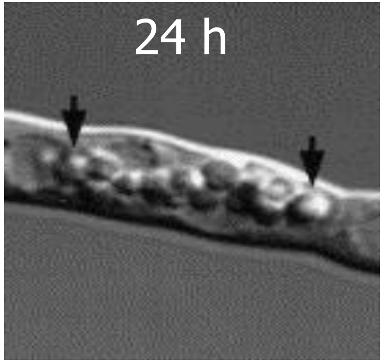
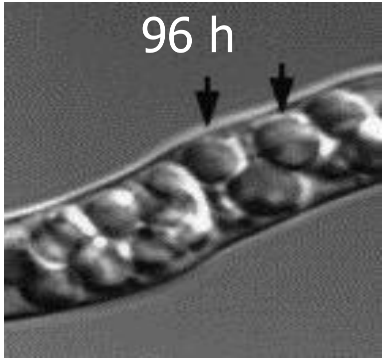
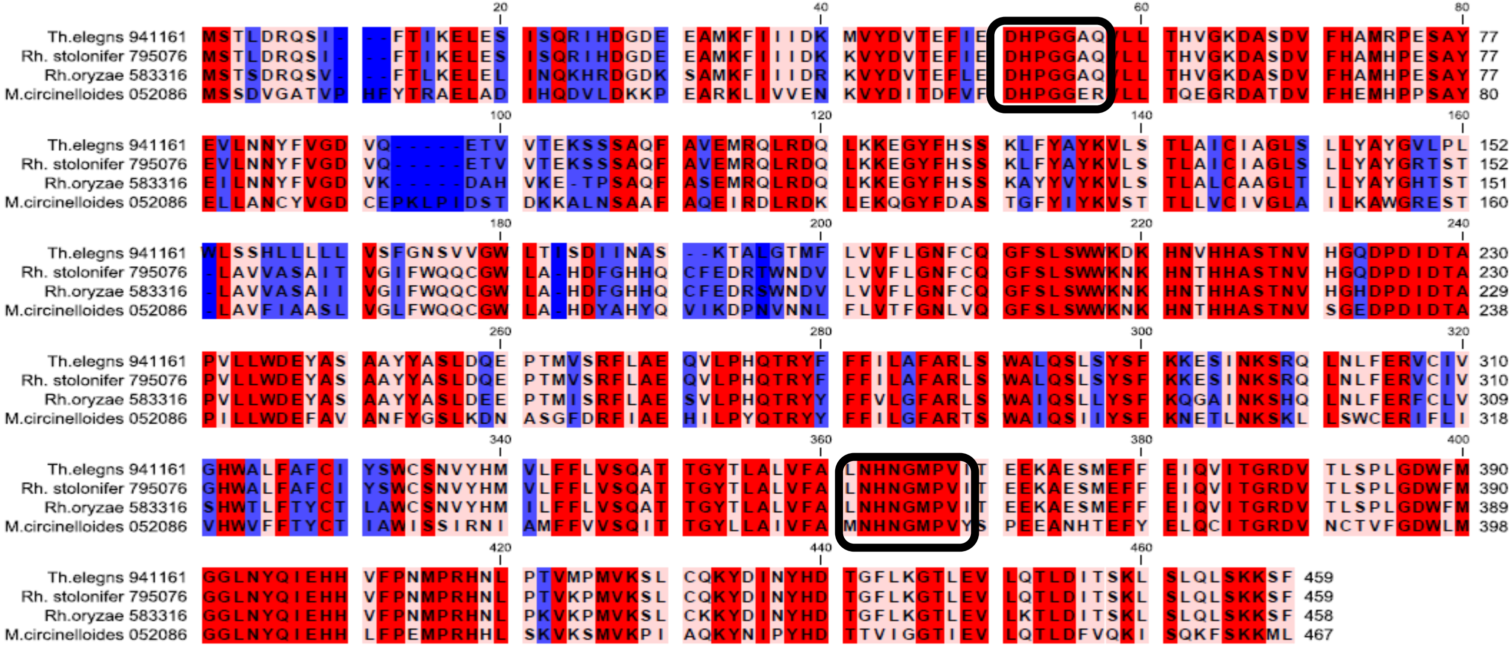
- **Source** of **Δ-6-desaturase gene** for genetic transformation of barley – oleaginous fungus ***Thamnidium elegans*** CC1456 producing:
  - **20%** of **fats** in dry mass
  - **15-30%** of **GLA**





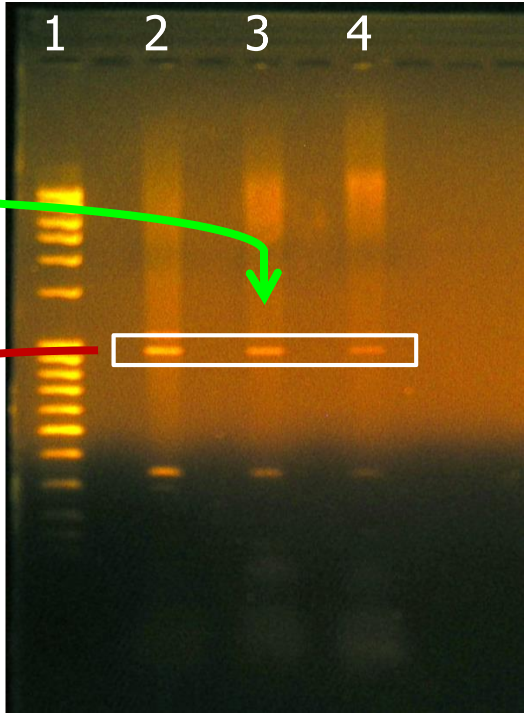
Barley improvement with **PUFAs**

- ***D6D* gene** isolation from *Thamnidium elegans* CC1456:
  - **RNA** isolation and **cDNA** synthesis
  - PCR – design of degenerated primers for **D6D** cDNA **amplification** by: primers 5‘-GAYCAYCCYGGWGGWCT-3’ and 5’-ACRGGCATWCCGTTRTGGTT-3’:



Barley improvement with **PUFAs**

- Amplification of D6D gene from *T. elegans* cDNA:
  - 1 – 100 bp ladder
  - 2-4 – fragment from PCR cca 930 bp



- **Cloning** and **sequencing**

Barley improvement with **PUFAs**

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- ***Thamnidium elegans*** – sequence of **D6D cDNA** – Genbank accession **HM856138**:

Thamnidium elegans strain CCF 1456 delta-6 desaturase mRNA, partial cds

GenBank: HM856138.1

FASTA Graphics

Go to:

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LOCUS       HM856138                 932 bp    mRNA    linear   PLN 09-OCT-2010
DEFINITION  Thamnidium elegans strain CCF 1456 delta-6 desaturase mRNA, partial
            cds.
ACCESSION   HM856138
VERSION     HM856138.1  GI:308096429
KEYWORDS    .
SOURCE      Thamnidium elegans
  ORGANISM  Thamnidium elegans
            Eukaryota; Fungi; Fungi incertae sedis; Early diverging fungal
            lineages; Mucoromycotina; Mucorales; Mucorineae; Mucoraceae;
            Thamnidium.
REFERENCE   1  (bases 1 to 932)
  AUTHORS   Mihalik,D. and Klempova,T.
  TITLE     Partial cDNA of delta-6-desaturase from Thamnidium elegans CCF 1456
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 932)
  AUTHORS   Mihalik,D. and Klempova,T.
  TITLE     Direct Submission
  JOURNAL   Submitted (21-JUN-2010) Dept. of Plant Biotechnology, Research
            Centre of Plant Production, Bratislavska Cesta 122, Piestany,
            Piestany 92168, Slovakia
FEATURES             Location/Qualifiers
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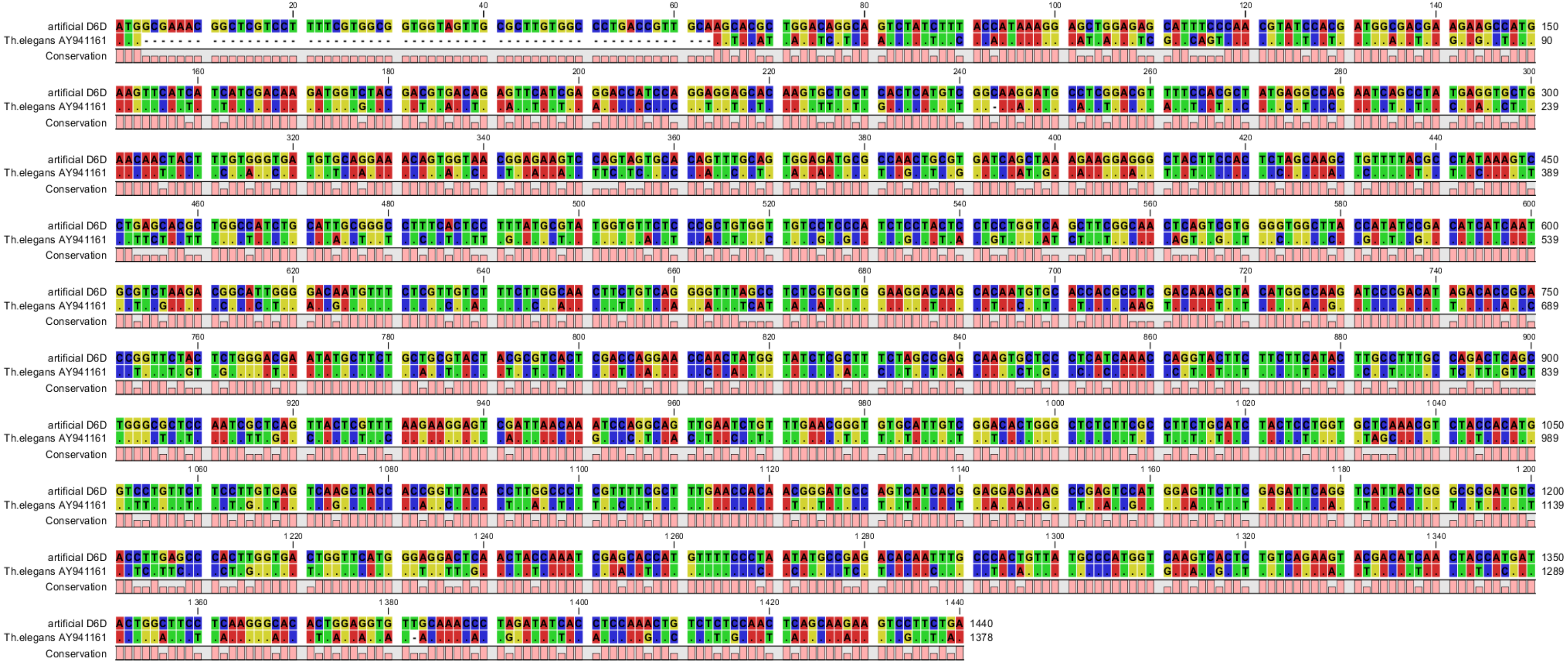
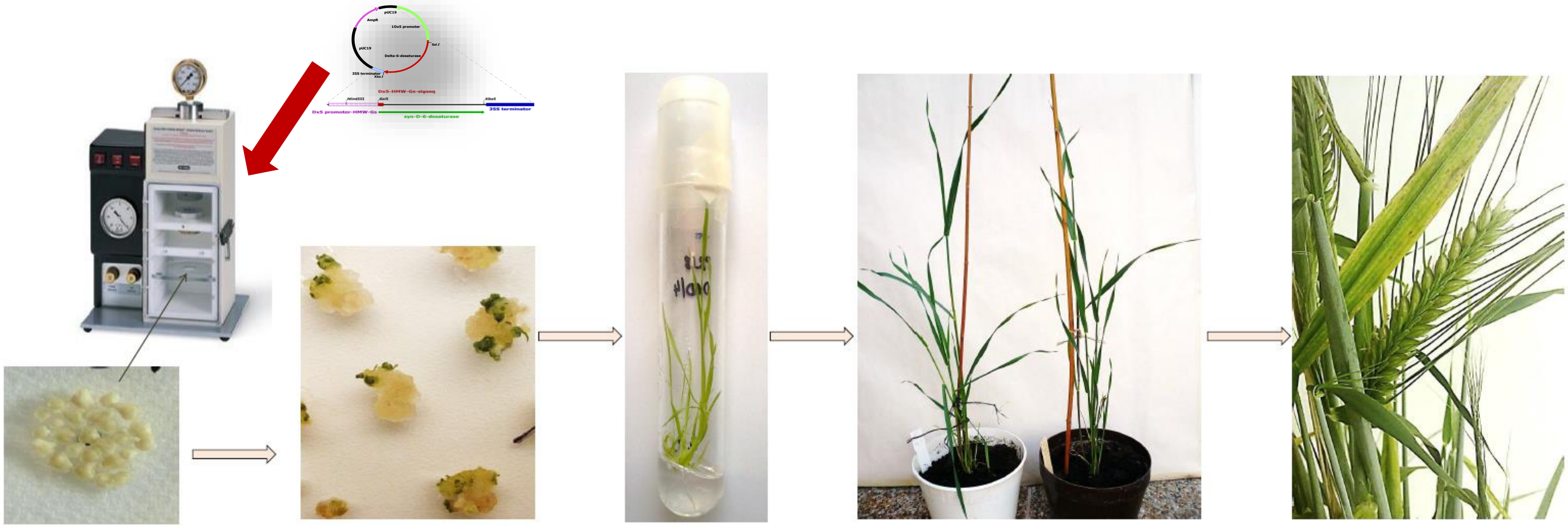
Barley improvement with **PUFAs**

Synthetic biology approach – artificial **gene synthesis** of **gene** – based on *T. elegans* (AY941161) sequence :



Barley improvement with **PUFAs**

- **D6D gene transfer** to barley:



Immature scutellum | Plant regeneration | Rooted plants | Regenerated transgenic plants (right) | Fertile transgenic plants

Barley nutritional quality **improvement**

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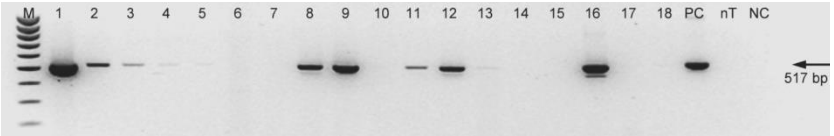
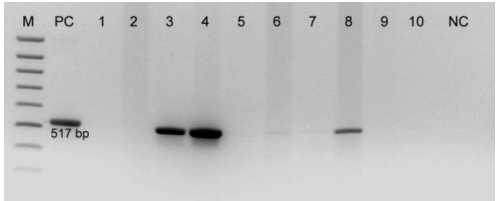
- Presence and **transcription** of Δ6D transgene in T_0 barleys
- **Expression** of GLA and SDA in **mature** barley **seeds**

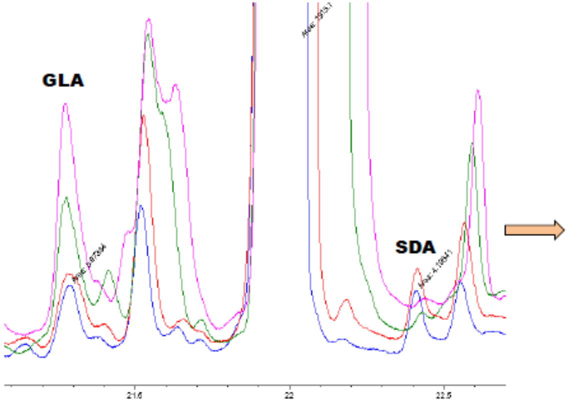


DNA

RNA

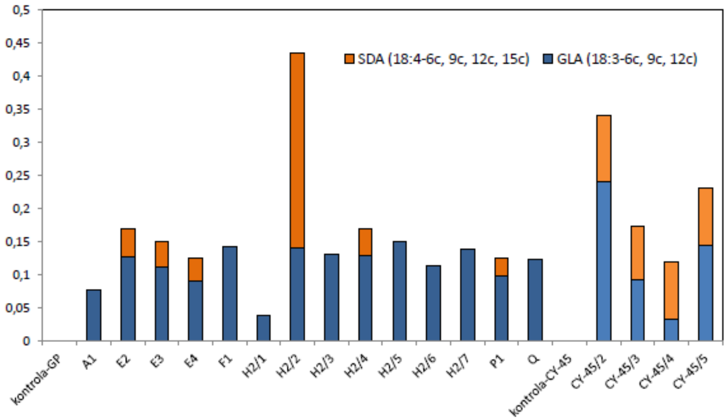


Fatty acids



Analýza spektra mastných kyselín

Obsah polynenasýtených mastných kyselín GLA a SDA v transgénnych rastlinách jačmeňa siateho, genotyp Golden Promise (A1 – Q) a pšenice letnej, genotyp CY-45.

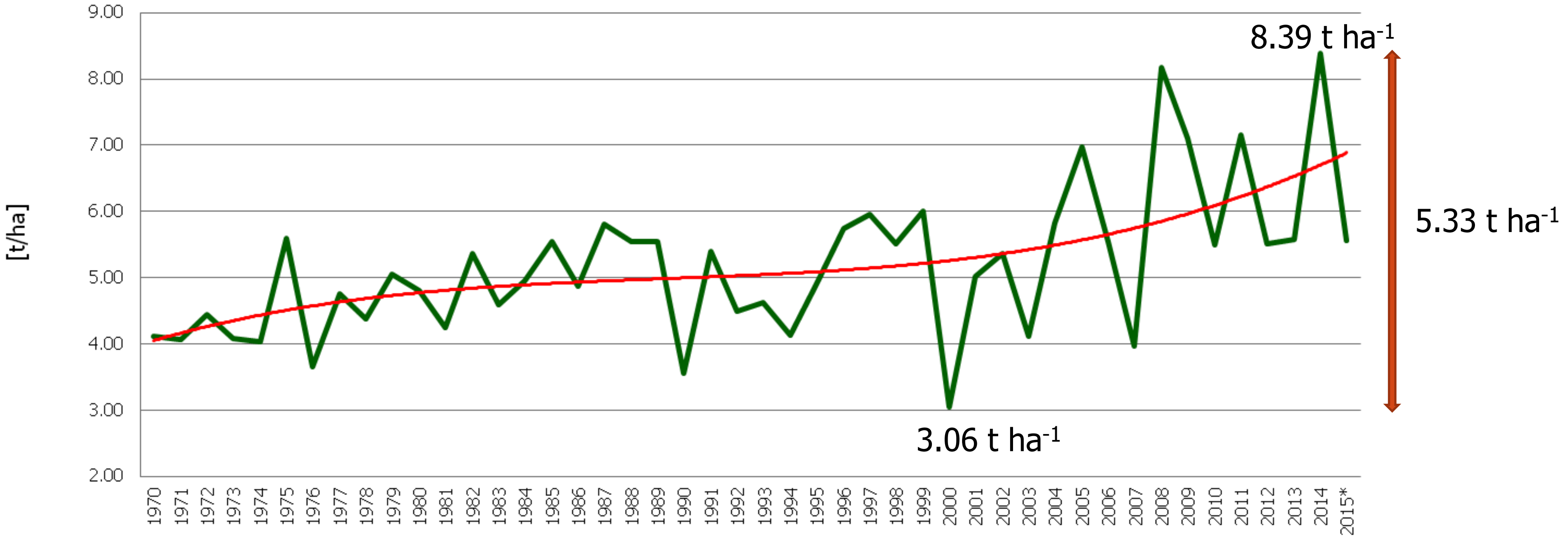


3. Gene transfer

GM plants for agriculture

Long-term **trend** in **maize production** in Slovakia

- Average yield of grain maize in Slovakia 1970-2015*:



- Average yield (2000-2015): **5.80 t ha^{-1}**

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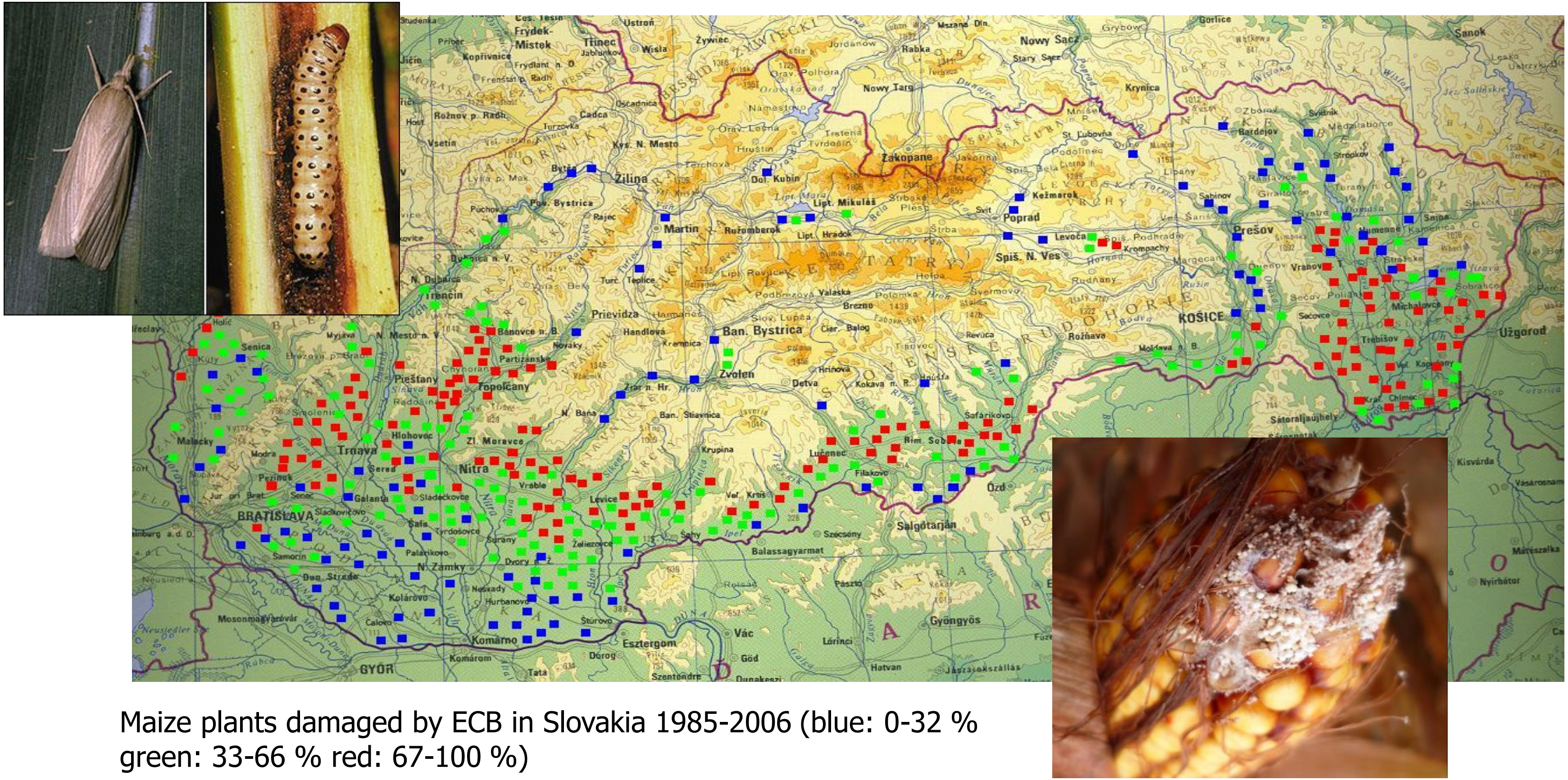
Potention of ECB resistant GM maize in Slovakia



Maize plants damaged by ECB in Slovakia 1985-2006 (blue: 0-32 % green: 33-66 % red: 67-100 %)

Cagáň, 2007

Resistance of ECB resistant MON 810 to in Slovakia

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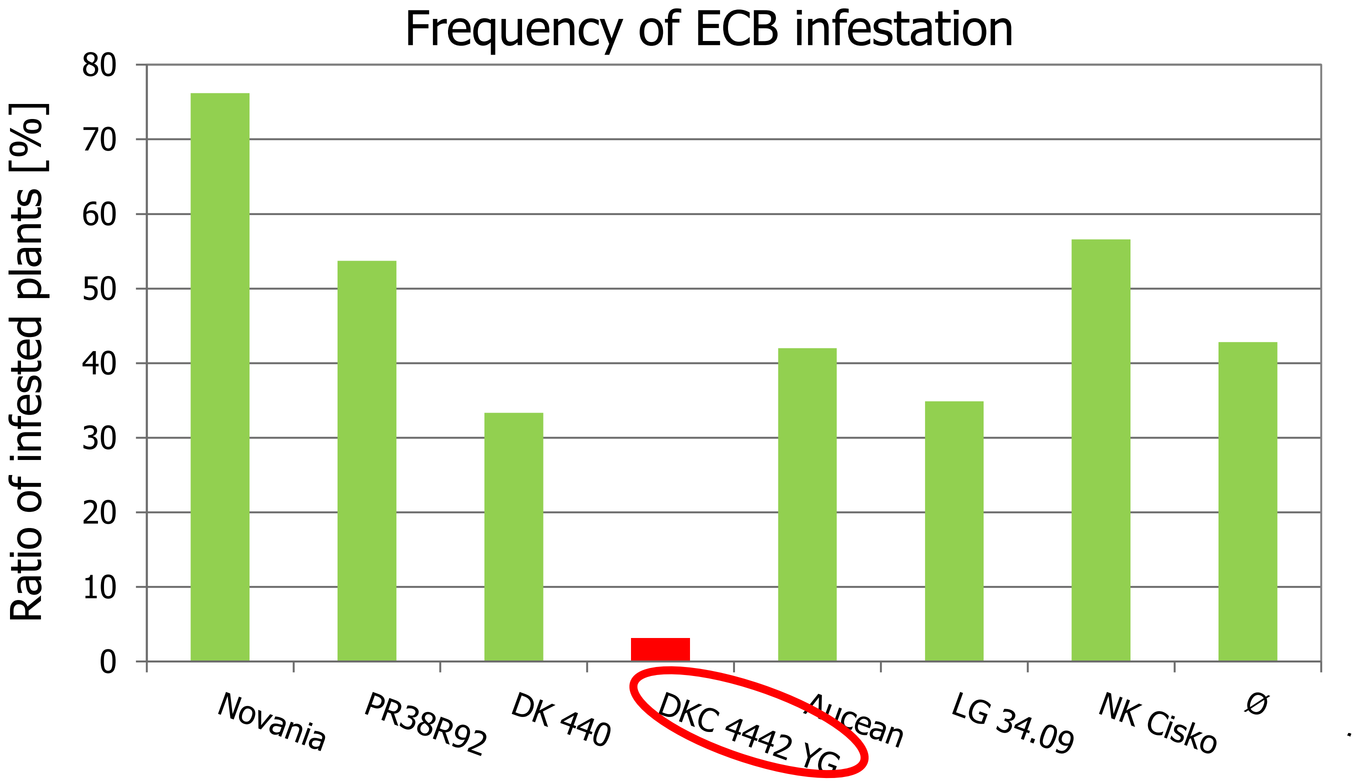
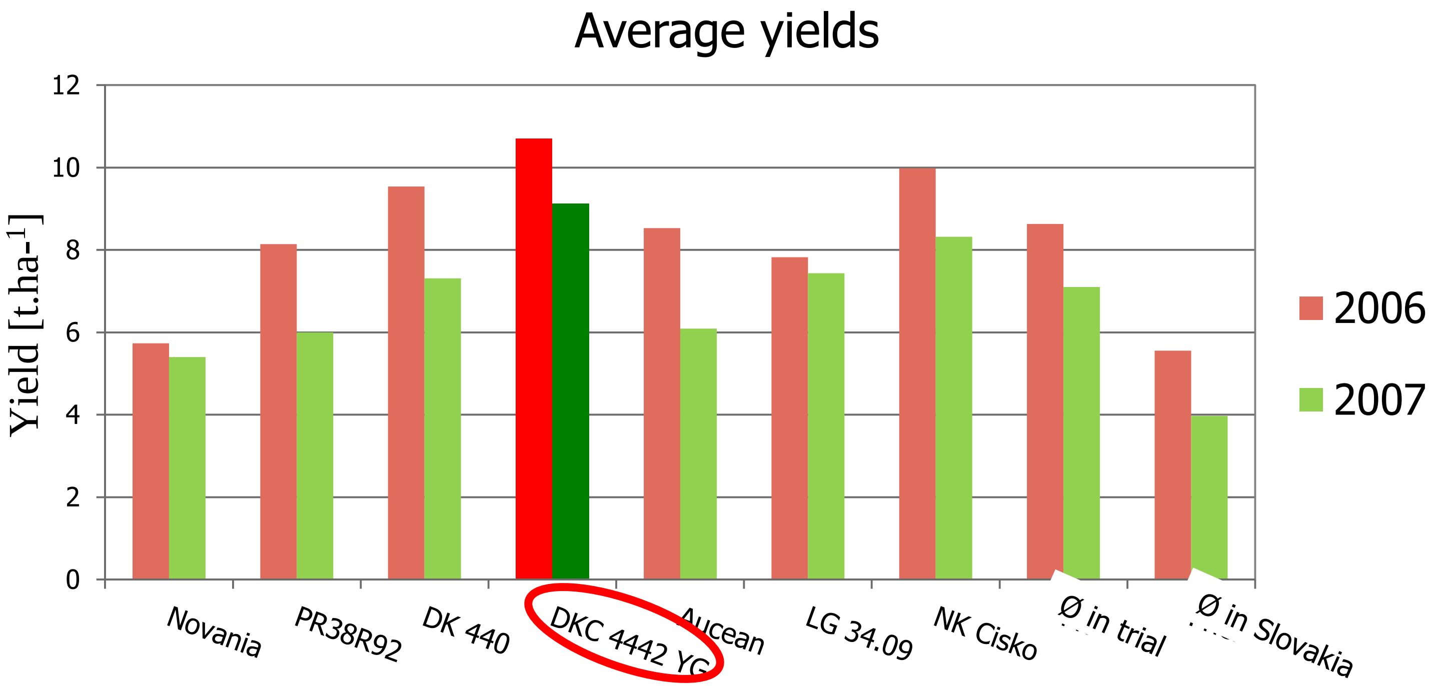


Benefit of **ECB resistant** MON 810 to in Slovakia in climatically different years (2006, 2007)



Story of MON 810 in Slovakia (2006-2015)

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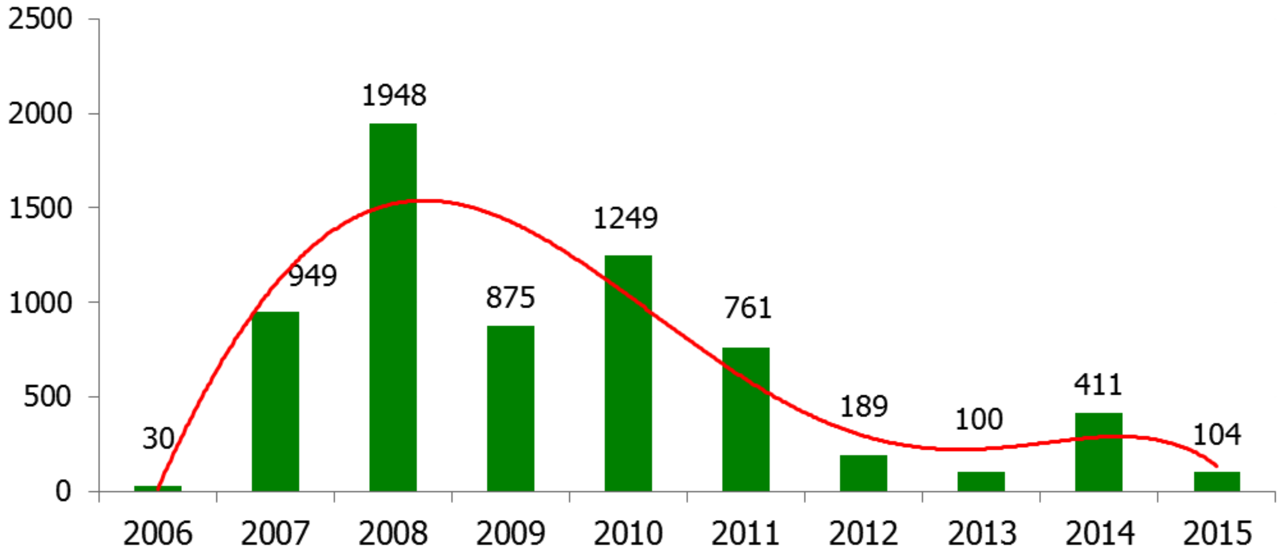

- Average (2008-2012) maize production: 1.15 mil tons
- **Overproduction** by ≈ 45 %
- **Starch processing** (Amylum) – 350 000 tones (700 000 tones)
- **Bioethanol production** (Enviral) – 300 000 tones

Expected economical **benefit** of **MON 810** growing in Slovakia

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	E	F	D	CZ	P	PL	SK
Ø yield Bt vs. conventional maize (%)	+1-15 %	+5-24 %	+14-15 %	+9-10 %	+12 %	?	+10-14%
Ø impact on profitability (%)	+12	+16-21 %	+12-14 %	+15	+22	?	+8-18%
Impact on content of mycotoxins	Signif. decreas.	Signif. decreas.	Signif. decreas.	Signif. decreas.	?	Signif. decreas.	?

(Brookes, G.: The benefits of adopting genetically modified, insect resistant (Bt) maize in the European Union (EU): first results from 1998-2006 plantings)

Observed economical **benefit** of **MON 810** growing in Slovakia

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Locality	Yield of control* [t/ha]	Yield of YieldGard® [t/ha]	Yield increasing [t/ha]	Yield increasing [%]	Income increasing [€/ha]
1	5,13	6,37	1,34	**+24**	**+119.50**
2	9,13	10,47	1,24	**+14**	**+132.78**
3	10,31	11,34	1,03	**+10**	**+89.62**

Mean: +16 % +113.97 €/ha

*control = isogenic hybrid withouth insecticide resistance

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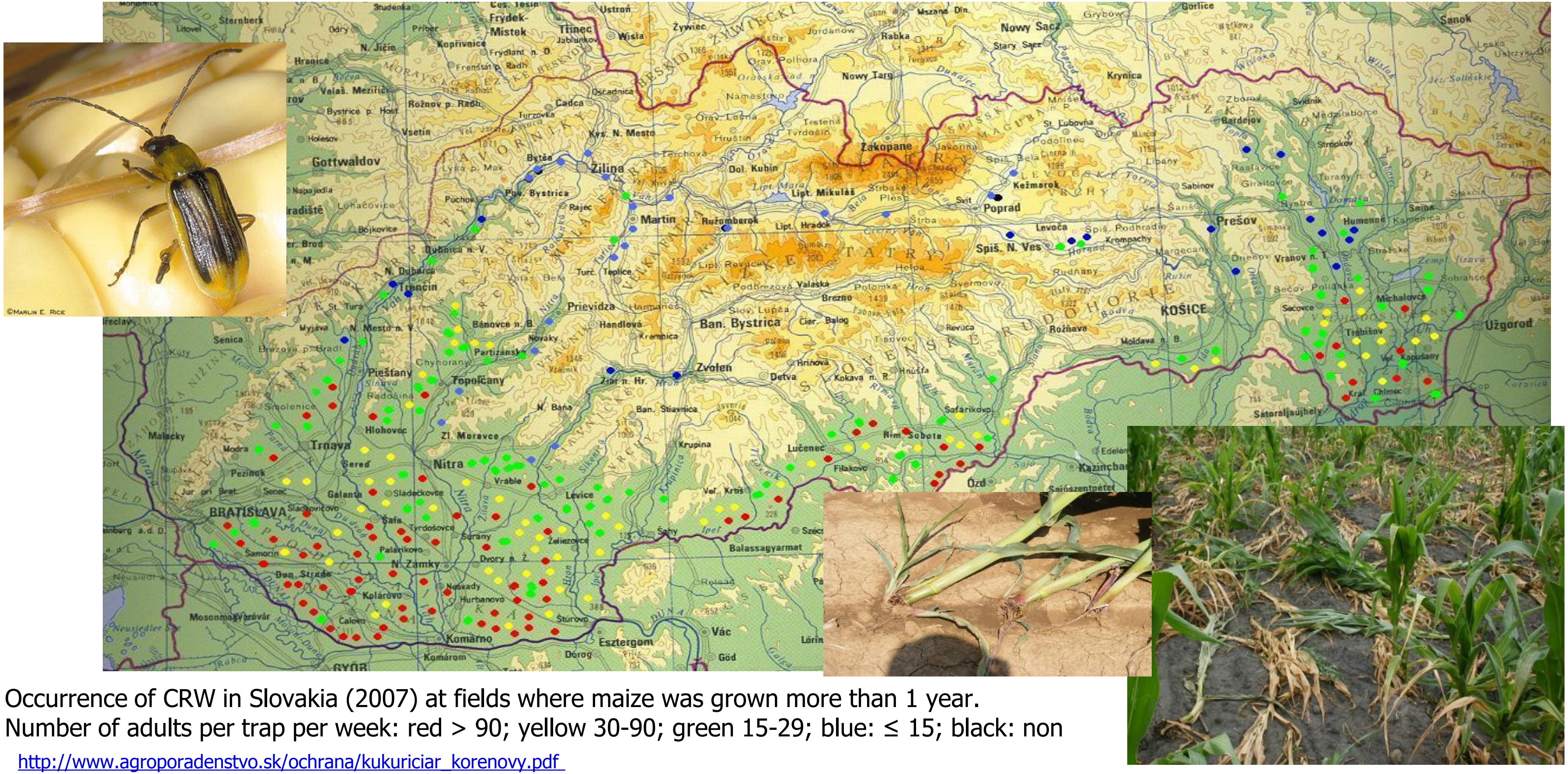
Potention of CRW resistant GM maize in Slovakia



Occurrence of CRW in Slovakia (2007) at fields where maize was grown more than 1 year.
Number of adults per trap per week: red > 90; yellow 30-90; green 15-29; blue: ≤ 15; black: non

http://www.agroporadenstvo.sk/ochrana/kukuriciar_korenovy.pdf

GM sugar-beet H7-1 in field trials in Slovakia

GM sugar-beet H7-1 in field trials in Slovakia

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Conventional sugar-beet			
Herbicide	Application l/ha kg/ha %	Cost per l, kg (€)	Cost per ha (€)
T1 Goltix Top (l)	1.3	42.00	54.60
Kontakttwin (l)	1.8	22.63	40.73
T2 Mix double FL 2 (l)	1.0	32.08	32.08
Safari 50 WG (kg)	0.03	1386.32	41.59
0,05% Trend 90 (l)	0.1	9.60	0.96
Agil 100 EC (l)	0.5	44.16	22.08
T3 Betanal Expert (l)	1.0	51.50	51.50
Goltix Top (l)	0.5	42.00	21.00
Garland Forte (l)	0.8	44.19	35.35
Cost			**299.90 €**

Glyphosate resistant **GM sugar-beet**			
Herbicide	Application l/ha kg/ha %	Cost per l, kg (€)	Cost per ha (€)
Roundup Rapid (l)	2.4	11.58	27.79
Roundup Rapid (l)	2.4	11.58	27.79
Roundup Rapid (l)	2.4	11.58	27.79
Cost			**83.37 €**

Diference betwen conventional and GM sugar-beet (€/ha) **-216.52 €**

Conclusions:

We are able to provide know-how for reciprocally profitable co-operation in topics:

- Breeding of new plant cultivars possessing novel and very specific traits
- Gene transfer technology for introduction of new traits into main cereals
- Long-term experiences in field testing of GM crops
- DNA profiling assays of plant genomes
- Development of protein and DNA-based diagnostic assays of plant pathogens