

# Development of HOLL winter rapeseed genotypes accompanied by MAS

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GCIRC Technical Meeting, Sweden, 9.05.2017

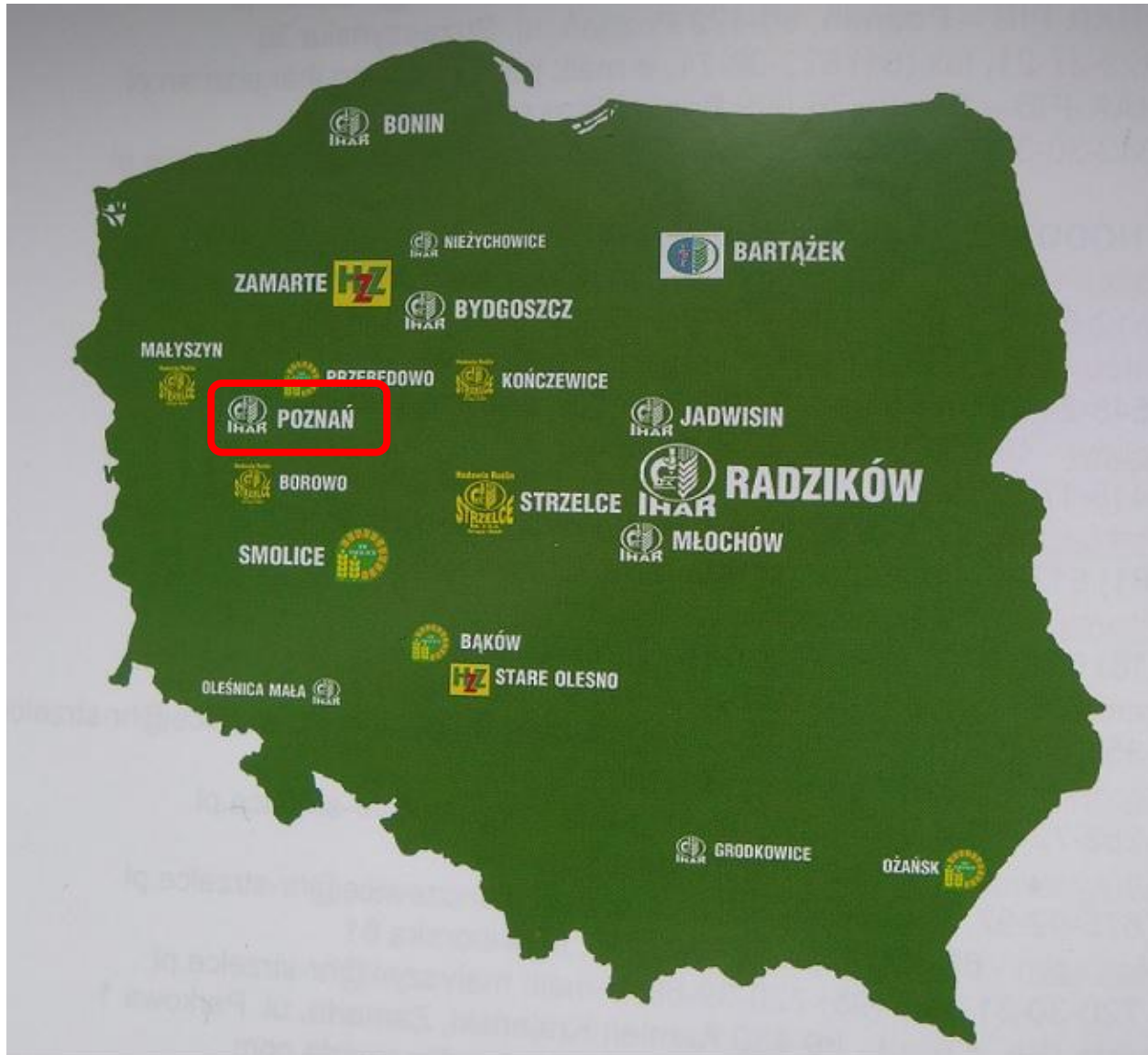
## **Development of HOLL winter rapeseed genotypes accompanied by MAS**

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High oleic and low linolenic (HOLL) lines of rapeseed (*Brassica napus* L.) are of increasing economic value due to their possible application for deep frying and also as a raw material for biofuel production. In Poland, winter rapeseed is the main oil crop and an important source of protein for animal feeding. In 2015, the acreage of winter rapeseed was over 880 000 ha, constituting about 96% of arable land used for oil crops. High oleic and low linolenic mutant lines were obtained through chemical mutagenesis using ethyl methylsulfonate (EMS), as described earlier. Moreover, allele-specific functional markers were developed for precise monitoring of the low-linolenic genotypes. Characterized by poor agronomic value and some morphological and phenological abnormalities, the mutant lines were improved by recombining with high-yielding canola varieties and following backcrosses and inbreeding. As a result, one of the world's first non-GM high oleic winter rapeseed cultivar, 'Polka', was registered in Poland last year. It is characterized by its content of about 80% of oleic acid in seed oil in addition to a zero erucic acid content, as well as about 15 – 20  $\mu\text{moles g}^{-1}$  of glucosinolates in seed meal. Furthermore, an analysis of the main effects in field trials revealed a strong influence of environmental conditions on the linolenic acid content in seed oil of low-linolenic mutant lines and their recombinants. Hence, a marker-assisted selection was necessary for an effective selection of low-linolenic mutant genotypes. The improved HO and LL mutant lines were crossed to obtain stable canola-type inbred HOLL recombinants, with a content of about 78% of oleic acid and less than 3% of linolenic acid in seed oil. In order to significantly reduce the glucosinolates content in seed meal, HOLL lines were crossed with selected domestic lines of good agronomic value and a high oleic acid content and an extremely low glucosinolates content, and also with canola varieties with a high seed oil content. The obtained HOLL lines make a starting material for the development of a new cultivar. The new breeding forms are evaluated in field trials, in addition to the assessment of genetic similarities based on SSR marker analyses and association studies among a population of winter rapeseed genotypes collected at PBAI-NRI in Poznan, Poland.

Acknowledgments: this work was supported by the Polish Ministry of Agriculture and Rural Development funding for the research project: "Investigations for Biological Progress in Plant Production 2014-2020", Tasks No: 48 and 53.



PBAI-NRI, Research Division in Poznan

- ❖ Oilseeds, winter oilseed rape
- ❖ Starting materials for breeding stations
- ❖ Innovation, implementing new technologies into rapeseed breeding

R&D

Selection goals:

- ❖ Seed and oil yield
- ❖ Tolerance/ resistance to stresses
- ❖ New quality

# New seed oil quality

## ❖ HOLL

- Deep frying
- Biofuel



## ❖ Challenges

- Seed and oil yield of new genotypes
- Effective selection for the new HOLL genotypes
- Seed certification, effective quality control
- Protection of owner's rights
- Investment vs. profit

- High oleic (HO) and low linoleic (LL) mutant lines of winter oilseed rape developed by the chemical mutagenesis, EMS treatment, and following selection using gas-liquid chromatography of seed oil (Spasibionek, 2006)

'C18' fatty acid content [%]

| 'C18' Fatty acid  | canola | HO-mut | LL-mut |
|-------------------|--------|--------|--------|
| Oleic (C18:1)     | 62.0   | 76.6   | 61.0   |
| Linoleic (C18:2)  | 20.0   | 8.8    | 27.5   |
| Linolenic (C18:3) | 10.0   | 7.4    | 2.7    |

- Improving the agronomic value of HO-mut
  - Crosses of HO-mut with high yielding winter rapeseed cultivars and also with selected 00 lines characterized by high oleic acid content in seed oil and extremely low GLS content in seed meal

## Selection of HOLL recombinants (Spasibionek, unpublished)

|   |                       |             |       |       |   |   |                |       |       |            |
|---|-----------------------|-------------|-------|-------|---|---|----------------|-------|-------|------------|
| ♀ | Selected HO-type line |             |       |       | X | ♂ | LL mutant line |       |       |            |
|   | C 16-22:0             | C18:1       | C18:2 | C18:3 |   |   | C 16-22:0      | C18:1 | C18:2 | C18:3      |
|   | 5,0                   | <b>76,6</b> | 8,8   | 7,4   |   |   | 5,0            | 71,1  | 27,5  | <b>2,7</b> |

HOLL recombinant

00, canola-type cv.

|                                    |                |                  |                 |                |   |  |           |           |           |          |
|------------------------------------|----------------|------------------|-----------------|----------------|---|--|-----------|-----------|-----------|----------|
|                                    | C 16-22:0      | C 18:1           | C 18:2          | C 18:3         | X |  | C 16-22:0 | C18:1     | C18:2     | C18:3    |
| Generation                         | C 16-22:0      | C 18:1           | C 18:2          | C 18:3         |   |  | 7,0       | 60,3-64,1 | 18,4-21,8 | 8,9-10,1 |
| <b>F<sub>4</sub>-F<sub>3</sub></b> | <b>4,2-6,1</b> | <b>76,9-82,6</b> | <b>8,8-13,9</b> | <b>2,2-3,4</b> |   |  |           |           |           |          |

HOLL recombinant lines of improved agronomic value

|                                    |                |                  |                  |                |                  |                                      |
|------------------------------------|----------------|------------------|------------------|----------------|------------------|--------------------------------------|
|                                    | C 16-22:0      | C 18:1           | C 18:2           | C 18:3         | Fat [%]          | GLS<br>[μM g <sup>-1</sup> of seeds] |
| Generation                         | C 16-22:0      | C 18:1           | C 18:2           | C 18:3         | Fat [%]          | GLS<br>[μM g <sup>-1</sup> of seeds] |
| <b>F<sub>8</sub>-F<sub>6</sub></b> | <b>4,1-5,7</b> | <b>75,8-82,4</b> | <b>10,9-13,6</b> | <b>2,0-4,9</b> | <b>43,9-47,3</b> | <b>9,0-12,5</b>                      |



## Field trials



- Parental lines: 00, HO<sub>a</sub>, HO<sub>b</sub>, LL, and 12 recombinants were analyzed in 6 environments
- Quality traits and agronomic value were estimated
- Main effects of environment on linolenic and oleic acid contents were assessed

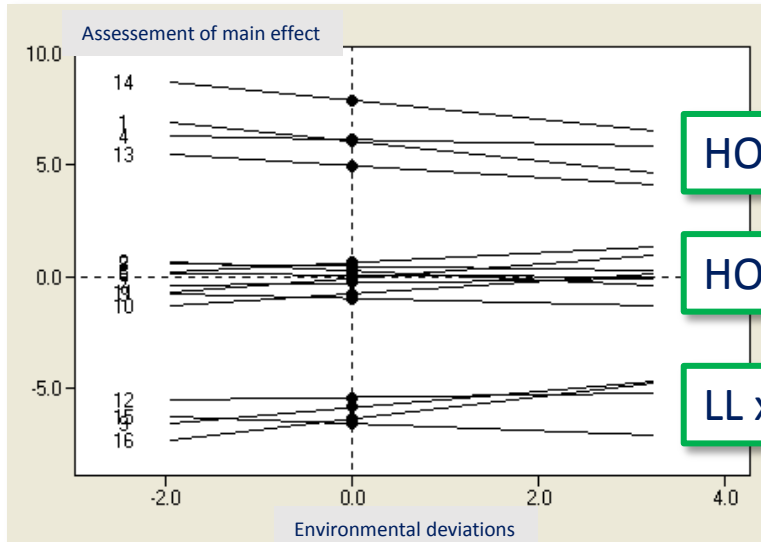


(Photos by Spasibionek)



## Oleic acid, C18:1

# Effect of environment on stability of oleic and linolenic acid content in parental and recombinant lines



$HO_a, HO_b, HO_a \times HO_b, HO_b \times HO_a$

$HO_b \times LL, OO \times HO_b, LL \times HO_b, HO_b \times OO, LL \times HO_a, OO \times HO_a, HO_a \times LL, HO_a \times OO$

$LL \times OO, OO \times LL, OO, LL$

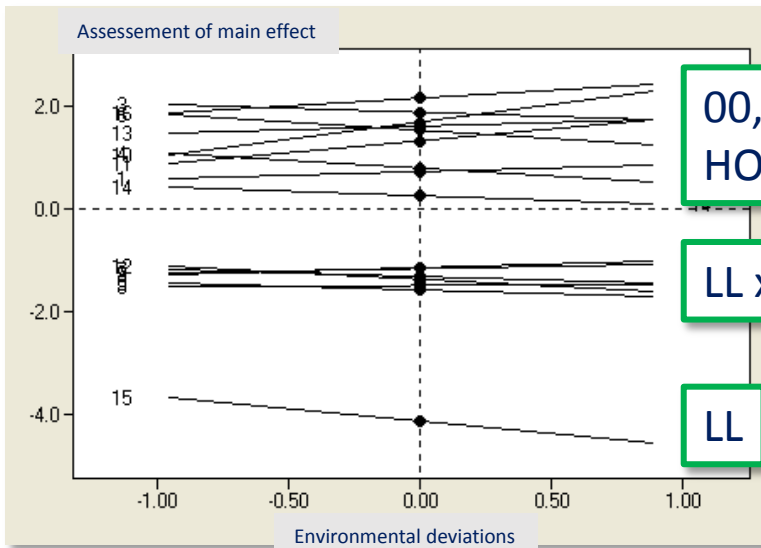
Parents:

OO, double low canola-type cv.

$HO_a HO_b$ , selected high oleic lines

LL, low linolenic mutant line

## Linolenic acid, C18:3



$OO, OO \times HO_a, HO_a, OO \times HO_b, HO_a \times OO, LL \times HO_b, HO_b \times OO, HO_a \times HO_b, HO_b \times HO_a, HO_b$

$LL \times OO, OO \times LL, LL \times HO_a, HO_a \times LL, HO_b \times LL$

LL

Linolenic acid content in seed oil was dependent on environment conditions – functional markers were necessary for effective selection

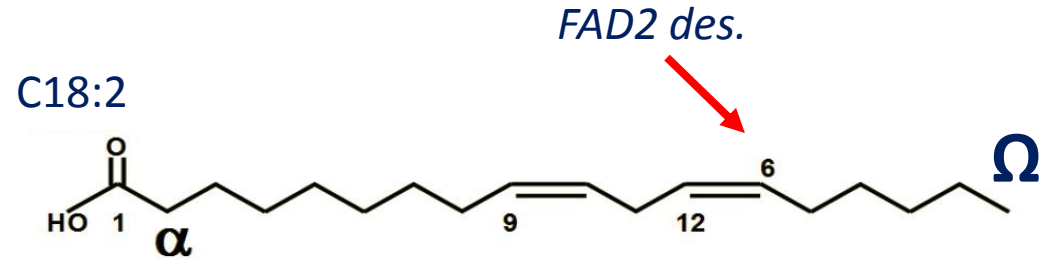


Development of allele-specific functional markers for monitoring LL-mut genotypes of oilseed rape

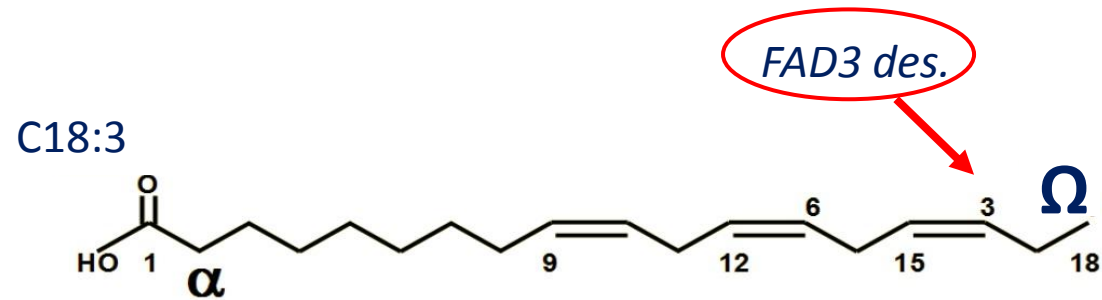
|        | C18:1 [%]   | C18:2 [%]   | C18:3 [%] |
|--------|-------------|-------------|-----------|
| Canola | 56 – 68     | 18 – 22     | 10 – 13   |
| LL-mut | 63.3 – 68.6 | 21.1 – 26.3 | 1.7 – 3.5 |



(Photo by S. Spasibionek)



Linoleic acid, Ω 6

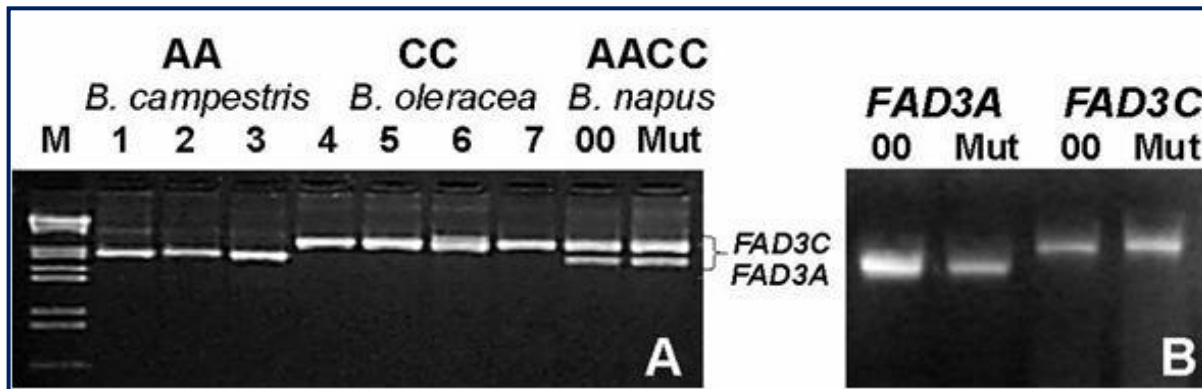
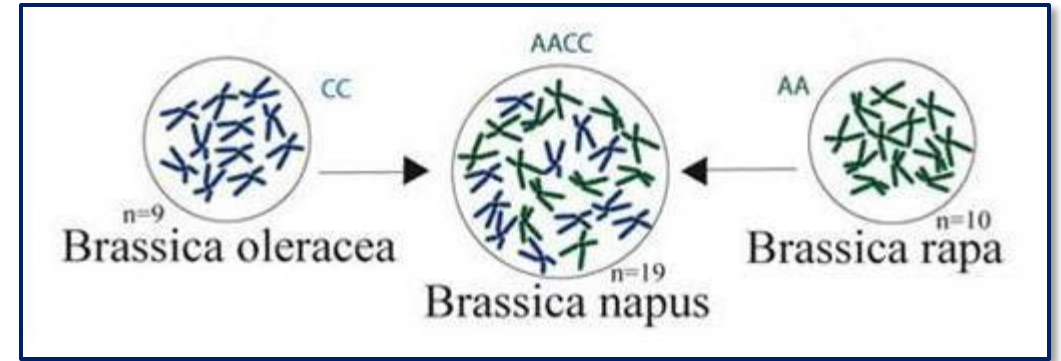


Linolenic acid, Ω 3

LLmut – low C18:3 and higher content of C18:2; *FAD3* desaturase gene was mutated (Jourden et al., 1998; Barret et al., 1999)

## 'candidate-gene' approach

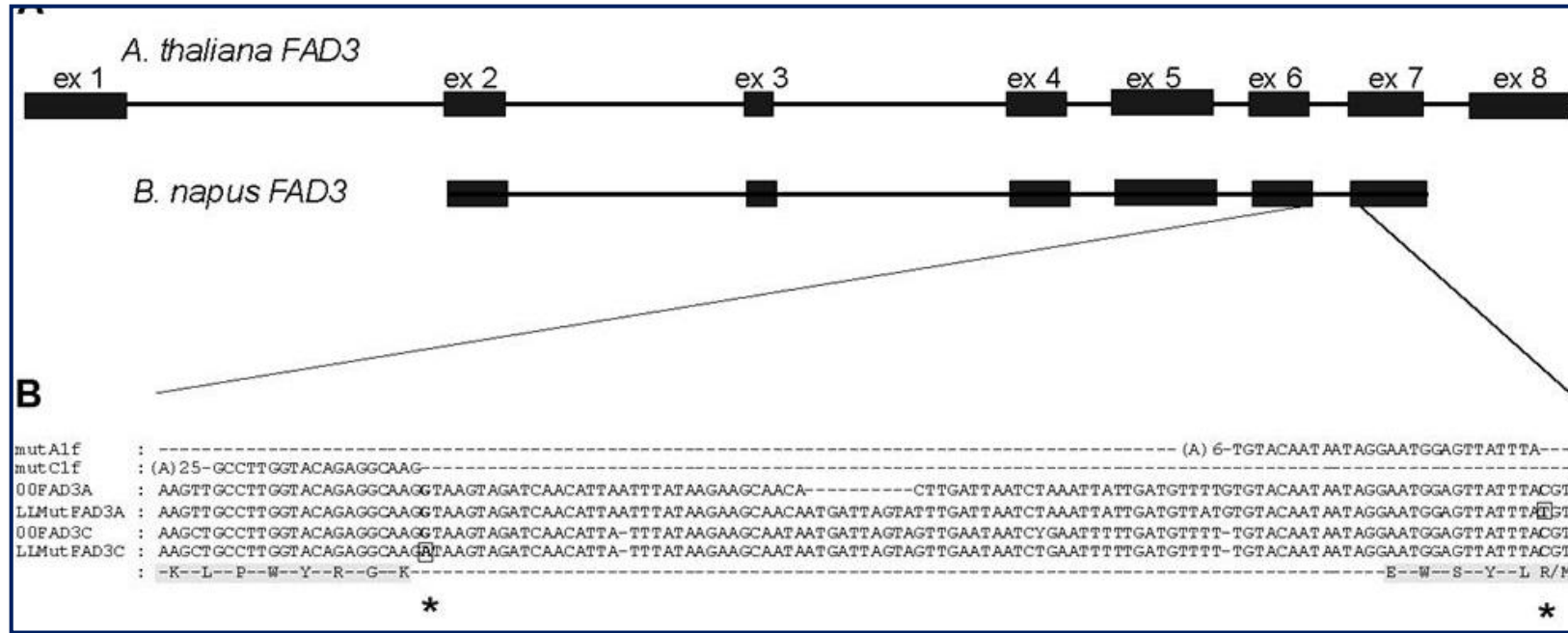
- Total DNA was isolated from wild-type and LL-mut winter oilseed rape genotypes
- *FAD3* desaturase alleles of the A and C genomes were cloned and sequenced
- Functional markers were developed



(Mikolajczyk et al., Plant Breeding 2010)

- PCR amplification of homeologous *B. napus* *FAD3* desaturase genes in the A and C genomes

- Two statistically important SNPs were identified in the selected LL-mut genotype of *B. napus*



(Mikolajczyk et al., 2010)

- Sequencing of RT-PCR amplification products confirmed the detected SNPs

C to T substitution caused Arg → Cys conversion in the 7th exon of the *BnaA.FAD3*  
 G to A, destruction of the 5' donor splice site of the 6th intron of the *BnaC.FAD3*

❑ The same SNPs in FAD3 genes were detected in:

➤ DowAgroScience spring oilseed rape HOLL, 'Apollo'-type mutant line

Hu X., Sullivan-Gilbert M., Gupta M., Thompson S. A. (2006) Mapping of the loci controlling oleic and linolenic acid contents and development of *fad2* and *fad3* allele-specific markers in canola (*Brassica napus* L.). *Theor Appl Genet* 113: 497-507

- QTL mapping using SSR and AFLP markers; N4 (A4) and N14 (C4) linkage groups
- candidate gene approach, cloning and sequencing of *fad2* and *fad3* genes
- Invader® assay for SNPs detection

➤ SW Hickory, a spring-type HOLL *B. napus* variety owned by Lantmännen SW Seed

Yang Q., Fan C., Guo Z., Qin J., Wu J., Li Q., Fu T., Zhou Y. (2012) Identification of FAD2 and FAD3 genes in *Brassica napus* genome and development of allele-specific markers for high oleic and low linolenic acid content. *Theor Appl. Genet* 125: 715-729

- QTL mapping using SSR markers; A4 and C4 linkage groups
- candidate gene approach, cloning and sequencing of *fad2* and *fad3* genes
- Development of SCAR markers for *fad2* and SNAP for *fad3* SNPs

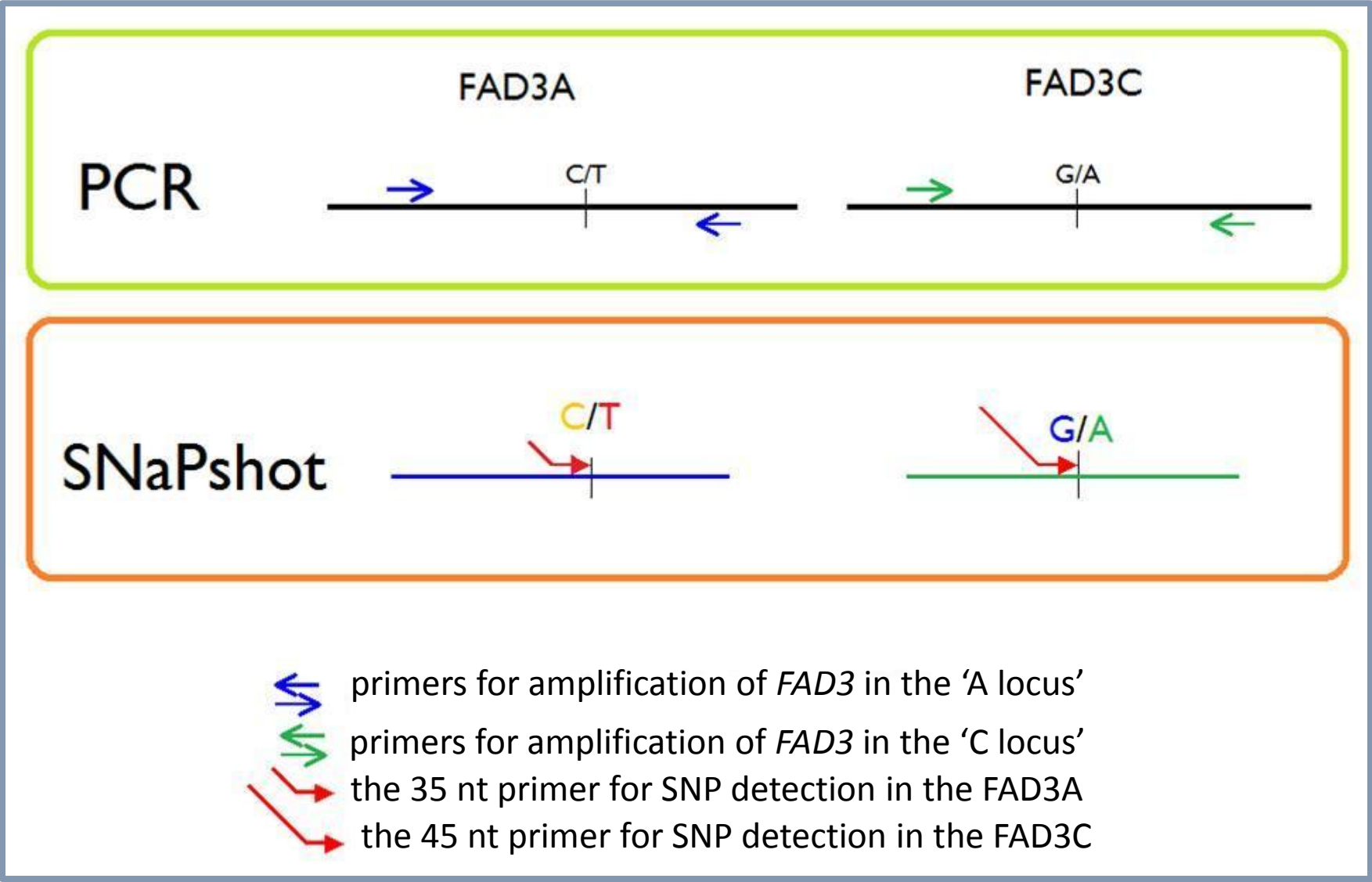


## ❑ Other oil crops, Soybean (*Glycine max* L.)

- HOLL; EMS mutants (Bilyeu et al., 2005):
  - G to A transition, identified in the 5' splice site of the 6th intron of the *Gmfad3a* gene conferred reduction of linolenic acid in seed oil
  - amino acid substitution, involving nonpolar glycine replacement with negatively charged glutamic acid to be responsible for the LL acid content in seeds of soybean with mutated *Gmfad3c* gene



# Detection of *B. napus* *FAD3* desaturase alleles



(M. Dabert, unpublished)

## Specificity of the developed SNaPshot assay

C18:3 content in seed oil depends on the allelic variant of *FAD3* genes in the A and C genomes:

| Locus | Regression coefficients | P values       | % of variation | Stand. error |
|-------|-------------------------|----------------|----------------|--------------|
| A     | 1.606                   | <0.001         | 35.6           | 1.580        |
| C     | 1.5738                  | <0.001         | 39.3           | 1.540        |
| A i C | 1.10, 1.15              | <0.001, <0.001 | 53.2           | 1.350        |

Additive (d) and dominance (h) effects of *FAD3* gene alleles in the A and C loci with respect to linolenic acid content:

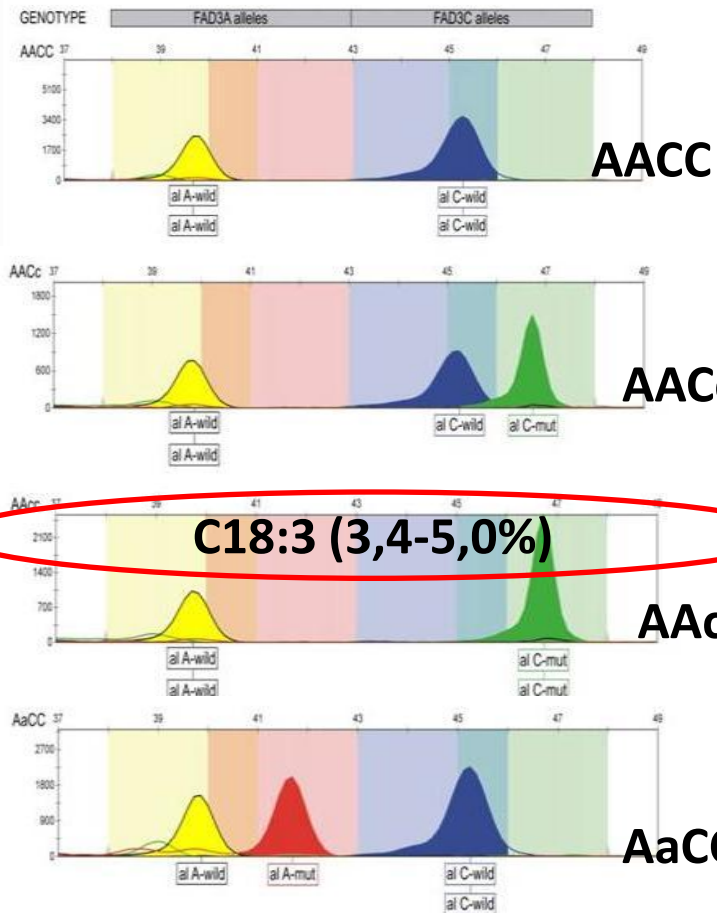
|       | FAD3A    |        | FAD3C    |        |
|-------|----------|--------|----------|--------|
|       | d        | h      | d        | h      |
| C18:3 | 1.595*** | -0.225 | 1.571*** | -0.180 |

\*\*\* - significance level 0.001

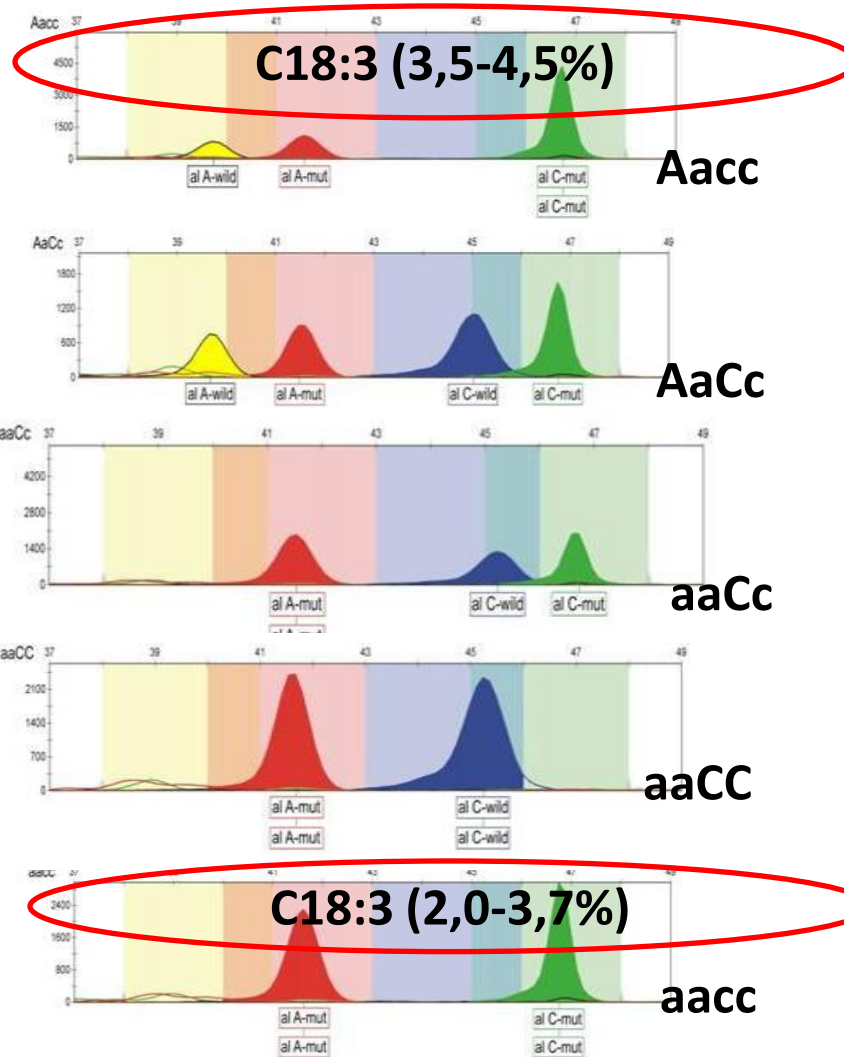
➤ The developed SNaPshot assay is highly specific

SNaPshot detection of allelic variants of FAD3 genes in the A and C genomes of *B. napus*

**AACC – wild type**



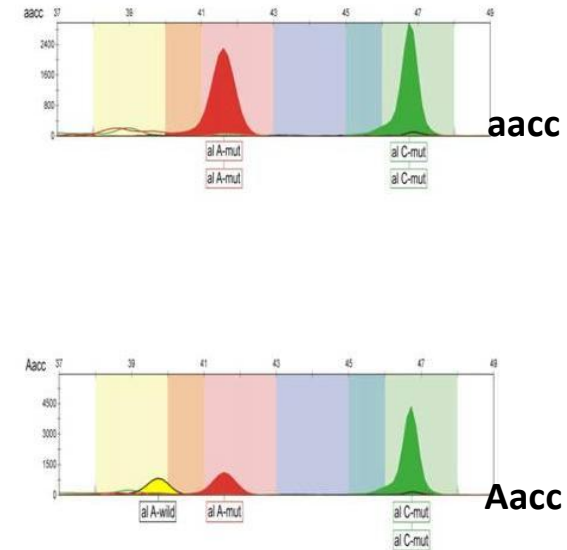
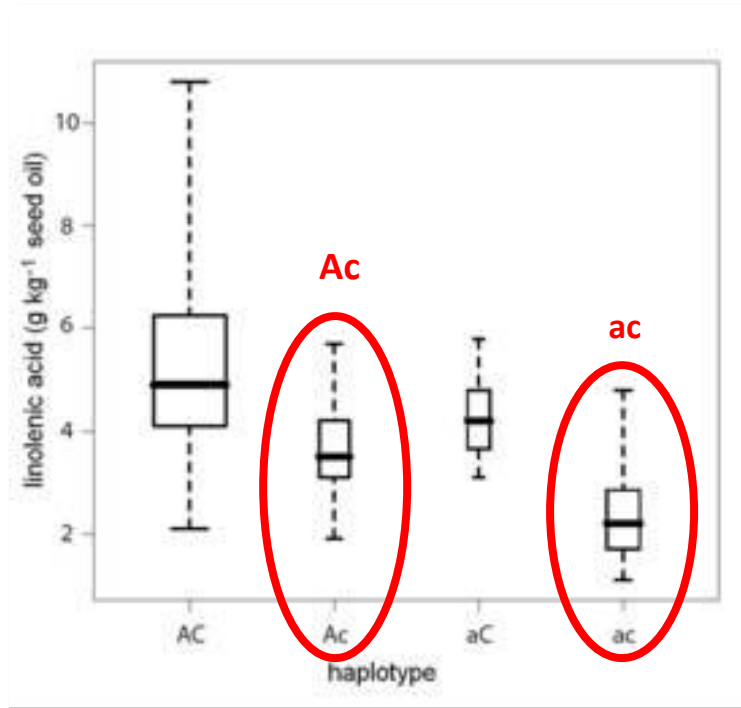
(Mikolajczyk et al. 2010, modified)



**aacc – LL mut**



The statistical significance of the observed negative effect of mutations in *BnaA.FAD3* and *BnaC.FAD3* on accumulation of linolenic acid in *B. napus* seeds



(Mikolajczyk et al. 2010, modified)

Progress in selection of HOLL recombinant lines – 2015/2016, first round of field trials including 25 objects  
(Spasibionek, unpublished)

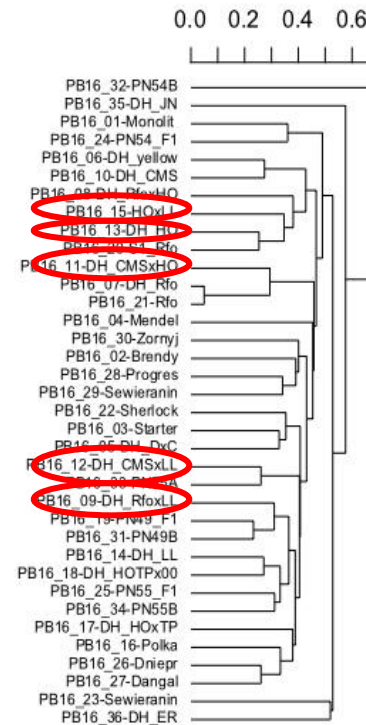
| Genotype                         | Seed yield dt/ha<br>(% of ref.) | % of<br>wintering | C18:1       | C18:2       | C18:3      | FAD3A     | FAD3C     |
|----------------------------------|---------------------------------|-------------------|-------------|-------------|------------|-----------|-----------|
| <b>Canola-type reference cv.</b> | <b>31,1 (100)</b>               | <b>68,0</b>       | <b>63,7</b> | <b>18,7</b> | <b>9,6</b> | <b>AA</b> | <b>CC</b> |
| LL                               | 13,8 (44,4)                     | 37,4              | 66,1        | 24,0        | 2,4        | aa        | cc        |
| HO                               | 9,2 (29,6)                      | 76,5              | 78,8        | 7,4         | 7,4        | AA        | CC        |
| <b>440/15</b>                    | <b>31,2 (100,3)</b>             | <b>62,3</b>       | <b>80,3</b> | <b>9,0</b>  | <b>3,8</b> | <b>AA</b> | <b>cc</b> |
| 837/15                           | 14,2 (45,6)                     | 67,7              | 77,6        | 12,7        | 2,4        | aa        | cc        |
| 878/15                           | 28,3 (91,0)                     | 50,1              | 76,6        | 13,4        | 2,8        | aa        | cc        |
| <b>880/15</b>                    | <b>32,0 (102,9)</b>             | <b>65,5</b>       | <b>80,1</b> | <b>9,7</b>  | <b>2,7</b> | <b>aa</b> | <b>cc</b> |
| 882/15                           | <b>29,5 (94,8)</b>              | <b>88,7</b>       | <b>76,7</b> | <b>8,3</b>  | <b>2,5</b> | <b>aa</b> | <b>cc</b> |
| 888/15                           | 24,2 (77,8)                     | 72,0              | 76,8        | 13,2        | 3,3        | AA        | cc        |
| 899/15                           | 27,0 (86,8)                     | 75,6              | 78,5        | 10,4        | 4,4        | AA        | cc        |
| 902/15                           | 22,8 (73,3)                     | 68,0              | 78,2        | 10,7        | 4,4        | AA        | cc        |
| F est.                           | 1,85 (-)                        | 2,87**            | 95,30**     | 125,06**    | 153,01**   | -         | -         |
| NIR 0.05                         | 4,49 (-)                        | 17,69             | 0,72        | 0,58        | 0,27       | -         | -         |

## Summary

- New HOLL winter rapeseed genotypes were obtained as a result of EMS mutagenesis followed by crosses with high yielding 00 cultivars
- The new lines are characterized by good agronomic value, incl. seed and oil yield, % of wintering and very low GLS content
- For effective selection of low linolenic recombinants, application of the developed allele-specific functional markers was necessary
- Field trials are continued to obtain starting materials for development of HOLL-type winter oilseed rape cultivar

## Prospects

- Protection of owner's rights by DNA fingerprinting; SSR, until now
- Implementing of NG techniques for effective high-throughput selection
- NG phenotyping for possible determination of functional biomarkers



Determination of genetic dissimilarities among collection of 36 rapeseed genotypes using 50 SSR loci, 185 polymorphic markers; HO, LL and their recombinants marked in red

## Acknowledgments

☐ Plant Breeding and Acclimatization Institute –  
NRI, Research Division in Poznan, Poland

Head: Professor Iwona Bartkowiak-Broda

Development of HO, LL and HOLL recombinants:

Dr. Stanisław Spasibionek

M. Sc. Teresa Pietka

DH lines:

Prof. Teresa Cegielska-Taras

Molecular markers and DNA analyses:

Dr. Katarzyna Mikolajczyk

M. Sc. Eng. Joanna Nowakowska

☐ Faculty of Biology, Adam Mickiewicz  
University, Poznan, Poland

DNA sequencing, development the  
SNaPshot assay:

Prof. Mirosława Dabert

bioinformatic analyses of *FAD3* genes  
nucleotide sequences, determining of  
statistically important SNPs:

Prof. Wojciech M. Karlowski

☐ Poznan University of Life Sciences

statistical analyses:

Prof. Jan Bocianowski

Research grants:

KBN: 3P06A01125 (2003 – 2006)

PB: 4201401 (2007 – 2012; 2014 - 2020)

PB: 4201705 (2007 – 2012; 2014 - 2020)