Cloning and mapping of a candidate gene for germination and seedling vigour in yellow-seeded oilseed rape

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Abstract

Yellow coloured seeds are of particular interest for oilseed rape (*Brassica napus*) breeding because of their association with a thinner seed coat resulting in reduced dietary fibre content. This considerably improves the feed and protein quality of rapeseed meal after oil extraction. However, the yellow-seeded phenotype is also associated with negative agronomic characters including a reduced germination rate and inhibition of seedling development and vigour. In *Arabidopsis*, the gene *AtPirin1* has an analogous effect on seed germination and early seedling development, and a *B. napus* sequence belonging to the same *cupin* gene family has been found to be strongly over-expressed in yellow-seeded oilseed rape. Based on the hypothesis that this candidate gene is closely linked to the major partially dominant gene for yellow seed colour in *B. napus*, the aim of this project is to identify and map all homologs of the corresponding *cupin* gene in *B. napus* and study their expression in different yellow-seeded rapeseed lines. By comparing the map locations with QTL for germination rate, seedling development, vigour and relevant plant growth hormones (particularly abscisic acid and ethylene), the intention is to develop molecular markers for transcriptionally active copies of the gene that can be used for a marker-assisted selection (MAS) of yellow-seeded rapeseed lines with no linkage drag for germination rate and seedling vigour. Degenerated primers from the *cupin* -domain containing gene with similarity to *AtPirin1* were used to identify the corresponding sequences in oilseed rape by BAC library screening. Positive BAC clones are being analysed to determine gene structure and functions in *B. napus*.

Keywords: oilseed rape, *Brassica napus*, germination, seedling vigour, *cupin* gene family

Introduction

Present-day double-low (00) oilseed rape or canola *(Brassica napus)* represents a potentially valuable source of vegetable protein due to its favourable composition of essential amino acids. However, the use of rapeseed protein for human nutrition is presently not possible due to the presence of major anti-nutritive compounds, which also reduce the value of rapeseed meal as a source of animal feed. Especially relevant in this regard are dietary fibre and dark-coloured tannins. Yellow coloured seeds are of particular interest for oilseed rape breeding because of their association with a thinner seed coat and simultaneous reduction in dietary fibre and tannin content. This considerably improves the feed and protein quality of rapeseed meal after oil extraction.

However, the yellow-seeded phenotype is also associated with negative agronomic characters, the most serious of which is a significantly reduced germination rate and inhibition of seedling development (Table 1; Lühs et al. 2000, 2001, Neubert et al. 2003).

Table 1. Comparison of germination rate (%) and seedling vigour in doubled haploid (DH) and inbred rapeseed lines with different seed colour. For each genotype eight repetitions of 50 seeds each were tested. Seedling vigour was scored as reduced when the seedling fresh weight was significantly lower than the average fresh weight for the line at seven days after seedling emergence

Genotype	Seed colour	Germination rate (%)	Seedling vigour (%)	
			Normal	Reduced
DH 4-34-1	Black	98.0	95.5	2.5
DH 4-40-1	Dark brown	94.0	91.5	2.5
DH 4-245-3	Dark brown	96.0	95.5	0.5
DH 4-143-1	Light brown	96.5	93.0	3.5
Inbred Line Y	Dark yellow	93.0	85.0	8.0
Line 1012-9	Yellow	90.0	85.0	5.0

In *Arabidopsis,* the gene *AtPirin1* has been found to have an analogous effect on seed germination and early seedling development (Lapik and Kaufman 2003). Knockout mutants of *AtPirin1* were found to display reduced germination levels in the absence of stratification, and a delay in germination and early seedling development caused by abscisic acid (ABA). *AtPirin1* is a *cupin*-domain protein that interacts with the *Arabidopsis* G-protein alpha-subunit, named GPA1. According to Lapik and Kaufman (2003) the gene probably functions immediately downstream of GPA1 to regulate seed germination and early seedling development through production of ABA, which is recruited as an endogenous signal in seed dormancy (Karssen et al. 1983, Koornneef et al. 2002). In non-stressed plant development, ABA has been found to inhibit ethylene generation and hence impede the signalling mechanisms to shoot and shoot growth (Sharp 2002). For this reason, measurement of ABA levels in developing seedlings has been suggested as one of the most effective methods of quantifying

seedling vigour (Duryea et al. 1985). Quantification of ABA and ethylene in seedlings can be achieved by gas-chromatography and mass-spectrometry (Cancel and Larson 2002, Müller et al. 2002).

We hypothesise that the poor germination rate and inhibited seedling vigour in yellow-seeded *B. napus* may in fact be caused by linkage drag between a mutation of *a cupin*-family gene with a similar function to *AtPirin1* with the major dominant gene for yellow seed colour in *B. napus.* The aim of this project is to identify and map all orthologs of a *cupin* candidate gene that was found to be under-expressed in yellow-seeded *B. napus,* and to and study their expression in different yellow-seeded rapeseed lines. By comparing the map locations with QTL for germination rate, seedling development and levels of central signalling hormones including ABA and ethylene, the intention is to develop molecular markers for transcriptionally active orthologous copies of this *cupin* gene that can be used for marker-assisted selection (MAS) of yellow-seeded rapeseed lines with normal germination and seedling development.

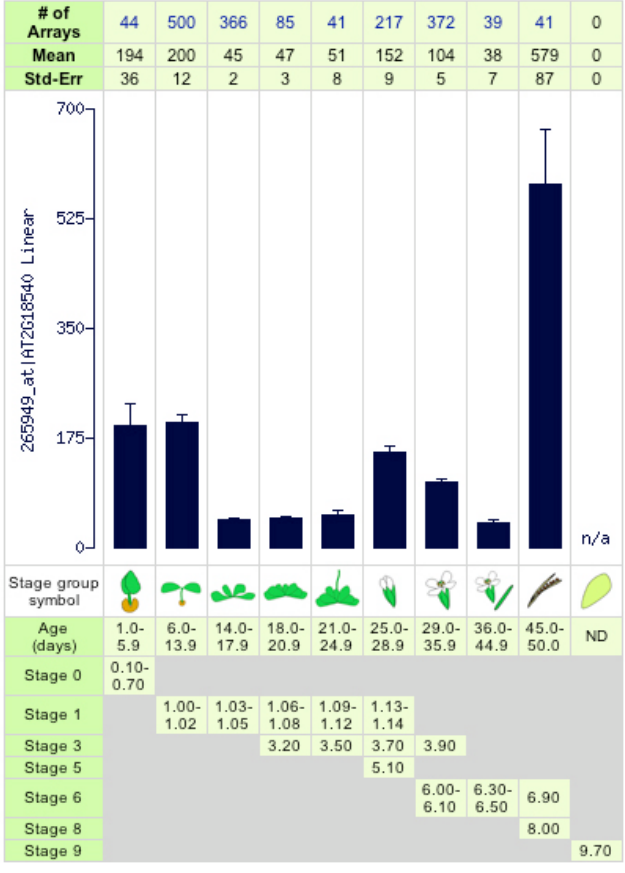
Materials and Methods

The yellow-seeded doubled haploid population (YE2-DH) of 166 DH lines derived from the cross between an inbred line of the black-seeded winter oilseed rape variety 'Express 617' (00-quality) and the yellow-seeded inbred line '1012-98' (00-quality) will be used for the QTL mapping of germination and seedling vigour as well as the isolation of orthologous *B. napus* copies of the *cupin* candidate gene.

The specific expression pattern of the cupin candidate gene was investigated in Arabidopsis using the Genevestig*at*or tools available at https://www.genevestigator.ethz.ch. Degenerate PCR primers from the genomic and flanking sequences harbouring the *cupin*-domain sequence were used to amplify the orthologous regions in *B. napus* with a PCR DIG probe synthesis kit (Roche). The DIG-labeled PCR products were used to probe an BAC library with 8x coverage of the genome of *B. napus* cultivar 'Express', the black-seeded parent of the mapping population.

Results and Discussion

Results from the Genevestig*at*or analysis of the cupin candidate gene confirm that this gene is an interesting candidate for germination and seedling development in oilseed rape. In particular, the gene shows strong expression in the late stages of seed development and during germination and early seedling development (Figure 1). Furthermore the "Response viewer" tool showed that the expression profile of this gene in Arabidopsis is particularly strongly influenced by plant growth hormones associated with germination, including ABA and IAA.



# of Arrays	44	500	366	85	41	217	372	39	41	0
Mean	194	200	45	47	51	152	104	38	579	0
Std-Err	36	12	2	3	8	9	5	7	87	0
Stage group symbol										
Age (days)	1.0-5.9	6.0-13.9	14.0-17.9	18.0-20.9	21.0-24.9	25.0-28.9	29.0-35.9	36.0-44.9	45.0-50.0	ND
Stage 0	0.10-0.70									
Stage 1		1.00-1.02	1.03-1.05	1.06-1.08	1.09-1.12	1.13-1.14				
Stage 3				3.20	3.50	3.70	3.90			
Stage 5						5.10				
Stage 6							6.00-6.10	6.30-6.50	6.90	
Stage 8									8.00	
Stage 9										9.70

Figure 1. Investigation of the expression profile of the *cupin*-family candidate gene for reduced germination rate and seedling vigour confirmed that this gene, which is under-expressed in yellow-seeded oilseed rape, has an expression pattern corresponding to a gene involved in these traits. Particularly high expression is seen during late seed development and early seedling growth (image from the Genevestigator "Respoinse Viewer" at https://www.genevestigator.ethz.ch).

A total of 56 positive BAC clones could be identified containing homeologous *B. napus* loci for the *cupin* candidate gene. BAC fingerprinting confirmed multiple copies of the gene in the polyploidy rapeseed genome, although this gene is single-copy gene in *Arabidopsis thaliana* (Lapik and Kaufman, 2003). Currently contig construction is being performed and one representative clone per contig will be used to isolate and sequence intron-extron boundaries and flanking sequences of the respective orthologs for the identification of locus-specific PCR primers. Using different primer combinations, we hope to identify sequence polymorphisms and differential expression between the yellow- and black-seeded mapping parents. This will open the possibility to study the role of the *cupin* candidate gene in germination and seedling vigour in *B. napus*.

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