Disruption of germination and seedling development in *Brassica napus* by mutations causing severe seed hormonal imbalance

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Introduction

We performed quantitative genetic analysis of germination and seedling development in a doubled haploid (DH) mapping population from a cross between a yellow-seeded oilseed rape (*Brassica napus*) mutant, 1012-98, with poor germination and low seedling vigour, and a normally germinating black-seeded winter oilseed rape variety, Express 617. Unexpectedly, the seed colour and germination traits showed no correlation in the DH offspring, indicating that they are controlled by independently segregating mutations. To further investigate the cause of the disturbed germination and seedling vigour, complete plant hormone profiles were analyzed by HPLC-MS/MS from ripe seeds and seedlings until 12 days after imbibation (DAI). Moreover, *Auxin Response Factor 10 (ARF10)*, an auxin (IAA) signalling regulator, was cloned to dissect its molecular functions and roles in the unusual accumulation of IAA and mutant phenotypes observed from the cross between Express 617 and 1012-98.

Materials and Methods

Plant materials

The *B. napus* homozygous inbred line Express 617 was derived by repeated self-pollination of the German winter oilseed rape variety Express (NPZ Lembke, Germany). Express 617 shows normal seed quality, germination and vigour. The B. napus line 1012-98 was derived by embryo rescue-assisted resynthesis from an interspecific cross between *Brassica rapa* (A genome, 2n=20) and *Brassica oleracea* (C genome, 2n=18), the two diploid parental species of the amphidiploid B. napus (AC, 2n=38). Due to the high relatedness of the *Brassica* A and C genomes, the chromosomes of resynthesised *B. napus* frequently contain non-reciprocal homologous translocations (Udall *et al.* 2005) that can lead to replacement or recombination of homoeologous gene copies in either the A or C genome. 1012-98 shows a yellow-seeded phenotype, atypical for *B. napus*, that is primarily attributable to a major quantitative trait locus (QTL) on chromosome N9 influencing testa thickness and flavonoid pigmentation (Snowdon *et al.* 2010) and thought to be caused by a non-homologous translocation leading to gene loss-of-function.

A population of 166 homozygous doubled haploid (DH) lines was generated by microspore culture (Weber *et al.* 2005) from an F1 plant derived from the cross between Express 617 and 1012-98. This segregating population was used to investigate the inheritance of the germination and vigour phenotypes of 1012-98. All seeds used for the investigations were harvested from self-pollinated plants grown under normal field conditions in a single environment. For determination of correlations between germination and seed quality traits, contents of fibre components and the seed colour were screened by near-infrared spectrophotometry using calibrations developed by Wittkop *et al.* (2009).

Germination and seedling development

Germination rate was assessed in vitro according to the recommendations of the International Seed Testing Association (ISTA). In each of three replications a total of 100 seeds each from Express 617, 1012-98 and the 166 DH lines were imbibed on moistened filter paper in Jacobsen germination vessels filled with 50 ml distilled water. Seeds were germinated in a growth chamber at a constant temperature of 25°C with 55% relative humidity and a photoperiod of 16 hours light/8 hours darkness (ISTA 2003).

Seedling development was assessed in 3 repetitions of 27 soil-sown seeds per genotype grown under controlled conditions in a climate-controlled greenhouse. Total seedling (root and shoot) biomass and hypocotyl length were measured at 3, 5, and 7 days after sowing (DAS). Mean trait values were calculated from all germinated seeds per genotype.

Sampling for hormone analysis

For comparison of hormone metabolite profiles in Express 617 and 1012-98, ten identical Jacobsen germination pots per genotype were prepared for sampling every 24 hours from one to eight days after imbibition (DAI) and every 48 hours from eight to twelve DAI. The experiment was conducted in three

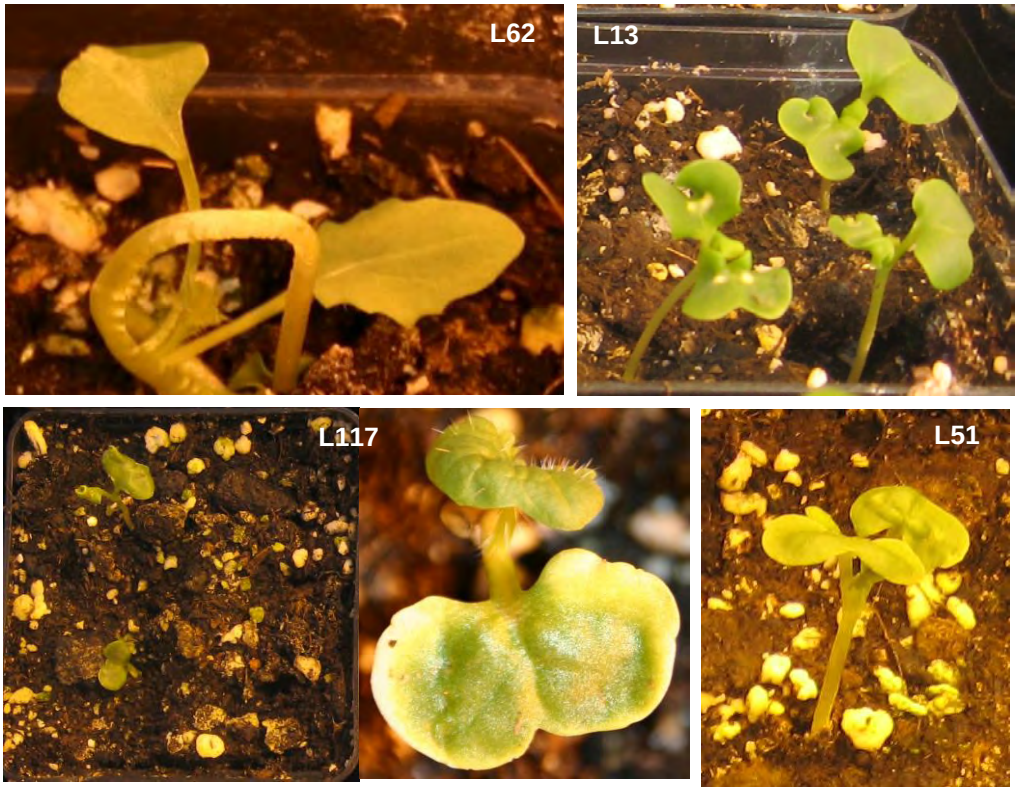
replications. A total of 15 seeds/seedlings per genotype per replication were pooled into 15 ml Falcon tubes, immediately immersed in liquid nitrogen and lyophilized for 24 hours.

Results and Discussion

The hormone profile of the mutant parent 1012-98 showed that both IAA and ABA contents were extremely high in the ripe seed and the first 3 DAI as comparing to the Express profile which is critical for transition from germination to post-germination development. Additionally, the catabolic pathways of these hormones occurred at relatively low rate until 3 DAI. In *Arabidopsis*, the *arf17* mutant, an *ARF10* overlapping function gene, is concomitant of high IAA accumulation due to the reduction in amino acid-, sugar-, peptide-linked conjugating pathways (Mallory *et al.*, 2005). The phenotypes observed in these IAA-regulator mutants are ABA hypersensitive which express out by arresting germination and retarding seedling development. In fact, DH lines with the disturbed hormone profiles also exhibited different levels of defective phenotypes e.g. twisted/curled cotyledons, “ostrich”-like seedlings, chlorosis/chlorotic leaves and stunted seedlings (Fig. 1) which are highly similar to mutant *Auxin Response Factors 10* (*ARF10*) seedlings in *A. thaliana* due to ABA hypersensitivity (Liu *et al.*, 2007). These evidences suggest the crosstalk between IAA and ABA in controlling seed germination and seedling establishment which is reviewed in Holdsworth *et al.* (2008).

In *Arabidopsis* the same phenotypes are observed in the miRNA-160 resistant form of *ARF10* involving in auxin signal transduction during many stages of plant growth development. Therefore, the full-length copies of *ARF10* in *Brassica* species (*BnARF10*) was cloned and 13 single nucleotide polymorphisms (SNPs) were detected between Express 617 and 1012-98 parental lines. Importantly, 1012-98 genotype harboured two importantly changed amino acids, R_{162} and L_{333} in functional domains (B3-DBD and ARF-Aux/IAA dimerization) which characterized for the *BnARF10* attributes as a transcriptional repressor (Guilfoyle and Hagen, 2007). Additionally, a recombinant allele between A and C genomes named 1012-ro was detected only in 1012-98. Detected SNPs can be served as potential information for marker development in mapping for seed germination and seedling-related traits in *B. napus* in the context of auxin regulation. Furthermore, ABA hypersensitive lines could provide an opportunity to generate hormone-coated seed that germinates normally but whose viability in the soil after harvest is considerably reduced. This might be of interest, for example, to hinder the potential weediness of herbicide-tolerant canola varieties, or to restrict the use of farm-saved seed from homozygous varieties.

Figures 1



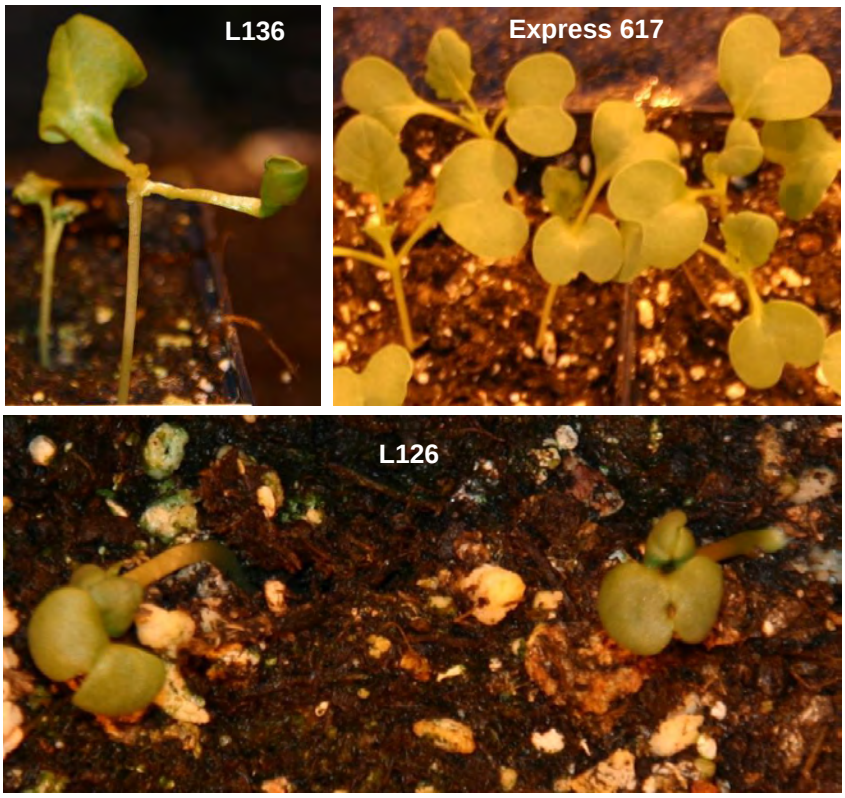


Figure 1. Typical phenotypic defects of six representative DH lines with high content IAA as comparing to Express 617 at 7 DAS. The mutant phenotypes vary from ostrich-like seedling (L62), severely chlorosis/chlorotics leaves (L13, 117, 136, 126), ectopic trichrome (L117) or twisted/curled cotyledon (L136)

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