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Integrated Genetic and Metabolomic Analysis of Seed Germination and Seedling Vigour in Oilseed Rape (*Brassica napus* L.)

Inaugural Dissertation for a Doctorate Degree in Agricultural Sciences
in the Faculty of Agricultural Sciences, Nutritional Sciences and
Environmental Management

Examiners
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"If we knew what it was we were doing, it would not be called research, would it?"

- Albert Einstein -

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1 General Introduction

1.1 Origin and economic importance of oilseed rape

Oilseed rape (*Brassica napus* L.) is the most economically important species within the *Brassica* genus during the past 30 years. It is mainly cultivated as an oilseed crop (rapeseed and canola), but also includes swede and some fodder and vegetable crops. Recently it is also grown as a renewable feedstock for biodiesel. *B. napus* morphotypes show broad adaptation to diverse eco-geographical regions and is a model polyploid for study of crop evolution and adaptation. Rapeseed cultivation began in Europe during the Middle Ages and spread worldwide (Chalhoub et al., 2014).

The species *Brassica napus* L. (2n = 38, genome AACC) is a recent allopolyploid species derived from interspecific hybridizations between turnip rape *Brassica rapa* L. (2n = 20, genome AA) and cabbage *Brassica oleracea* L. (2n = 18, genome CC), resulting in an amphidiploid genome comprising the full chromosome complements of its two progenitors (Figure 1) (Allender and King, 2010). Because no wild *B. napus* forms are reported, it is assumed that the species arose relatively recently, in the coastal Mediterranean region, where both of its two parental species grow wild. The recent availability of reference genome sequences for *B. napus* (Chalhoub et al., 2014) has facilitated the confirmation of its two diploid progenitors *B. rapa* (Wang et al., 2011) and *B. oleracea* (Liu et al., 2014).

In 2017-2018 global production of rapeseed exceeded 74.71 (Figure 2a) making them the second most valuable source of vegetable oil and protein meal (40.12 Mt in Figure 2b) in the world after soybean. This rapid expansion in production is largely attributed to intensive breeding for reductions in erucic acid and glucosinolate content, greatly improving its worth as edible oil and a livestock feed respectively (Snowdon et al., 2007).

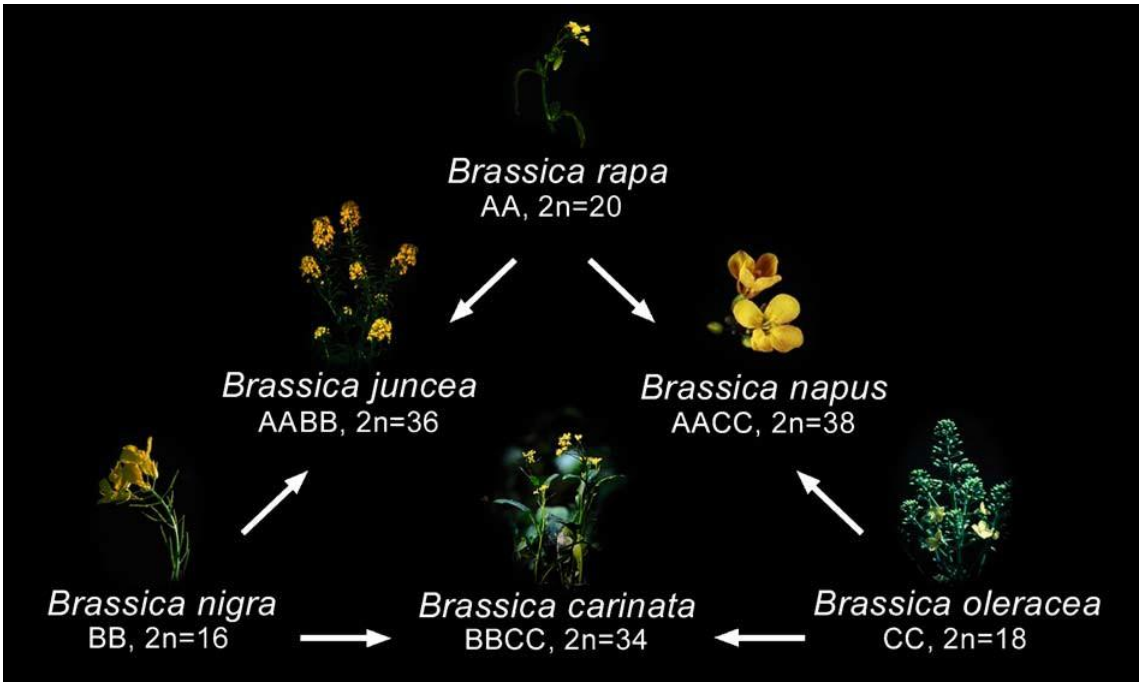

Figure 1: The *Brassica* triangle of species. *Brassica napus* (2n= 38) arose from spontaneous interspecific hybridisation between its two diploid progenitors. Reproduced from Snowdon (2007) *Chromosome Research* 15(1): 85-95. http://dx.doi.org/10.1007/s10577-006-1105-y. Copyright © 2007 Springer, Berlin, Heidelberg.

Today commodity rapeseed oil encompasses less than 2% erucic acid (Low Erucic Acid Rapeseed, LEAR) in its fatty acid profile and low glucosinolate (Kimber and McGregor, 1995; Mailer, 2009). With an optimal 2:1 proportion of linoleic to α-linolenic acid, rapeseed oil also contains the lowest proportion of saturated fatty acids of all the major vegetable oils, and the nutritionally significant monounsaturated fatty acid oleic acid comprises around 60% of the total fatty acids.

The combination of all these factors makes rapeseed oil arguably the nutritionally most valuable vegetable oil (Snowdon et al., 2007). Recently, high oleic/low linolenic acid (HOLL) varieties of *B. napus* have been developed for the production of vegetable oils suitable for frying at high temperatures and for producing margarine with reduced trans

fatty acids which play a role in the prevention and therapy of a number of chronic diseases, particularly in the reduction of the risk for coronary heart disease (Trautwein, 2001).

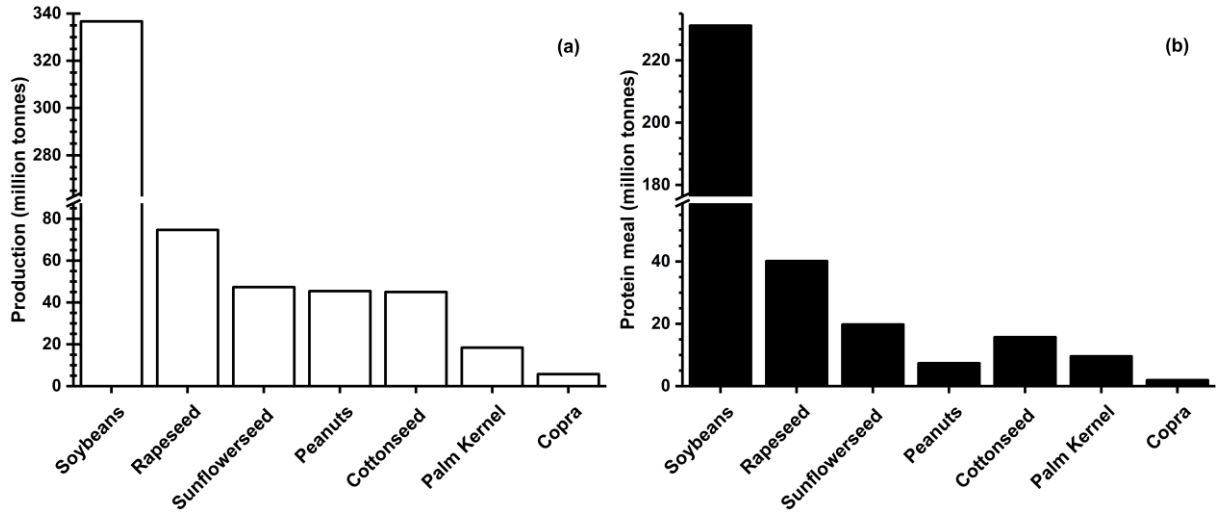

Figure 2: World production of major oilseed crops in 2017-2018 (FAOSTAT data, 2017-2018: http://faostat.fao.org/)

The next milestone in rapeseed breeding was the introduction of cultivars containing less than 30 micromoles of glucosinolate and its derivatives per gram of air-dry, oil free solid. The result was the release in 1974 of the first 00-quality spring rapeseed variety, Tower, with zero erucic acid and low glucosinolate content. With the introduction of double low (00) rapeseed cultivars, the meal by-product of oil extraction containing between 36 and 44% crude protein and a good balance of essential amino acids is a good source for animal feed (Snowdon et al., 2007). Besides the uses of human and animal nutrition, edible rapeseed is the most common oilseed used for biodiesel in Europe. Importantly, biodiesel made from rapeseed oil gels at a lower temperature than biodiesel produced from other feedstocks, making rapeseed biodiesel a more suitable fuel for colder regions (Peterson et al., 1997).

World production of rapeseed in 2017-2018 approached 68.86 million tons (MT), with the leading producers being Canada (18.42 MT), China (15.28 MT), India (6.80 MT), France (4.72 MT) and Germany (4.58 MT) in Figure 3. The top five rapeseed producers were

accounted for more than 72% of the world rapeseed production. Winter oilseed rape (WOSR) is cultivated predominantly in Europe and Asia, whereas in Canada, northern Europe and Australia only spring oilseed rape (SOR) are suitable. Accordance with the vernalization requirements for flowering initiation, selection of winter or spring varieties is largely attributed by climatic and phenological conditions (Snowdon et al., 2007). The world average yield of 2.04 (tonne/ha) in 2017-2018 covered a wide range, with up to 3.45 in Western Europe, 2.31 in Canada, 2.01 in China and 1.25 in Australia to 1.18 tonne/ha in India (FAOSTAT data 2017-2018 in Figure 3).

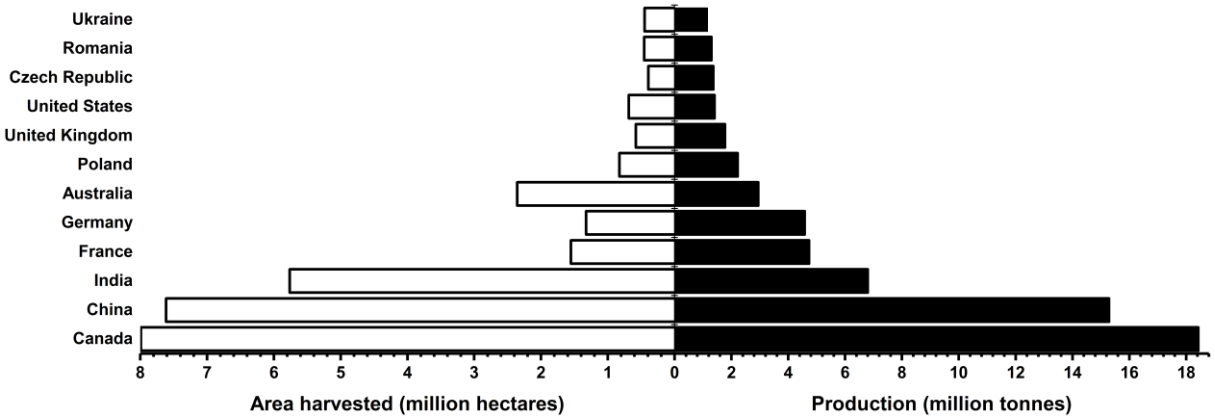

Figure 3: World leading producing countries of rapeseed in 2017-2018 (FAOSTAT data, 2017-2018: http://faostat.fao.org/)

1.2 Current breeding aims of oilseed rape

The general goals of oilseed rape breeding are summarized in Table 1. Four major goals with very high priority are tolerance to late planting and winter hardiness, plant height and lodging resistance, resistance to blackleg disease, *Verticillium* wilt and (if possible) *Sclerotinia*, very low contents of erucic acid and glucosinolates, high oil content and marketable seed yield (Friedt and Snowdon, 2010). Until recently good germination and seedling vigour are recently major breeding targets in *B. oleracea* (Bettey et al., 2000; Morris et al., 2016), *B. rapa* (Basnet et al., 2015) and *B. napus* (Hatzig et al., 2015;

Nguyen et al., 2016; Nguyen et al., 2018) because seedling vigour and prewinter crop establishment are closely associated with post-winter growth and seed yield.

Table 1: Four major fields and associated detail traits of oilseed rape breeding

Agronomic traits	**Disease and pest resistance**
○ Tolerance to late planting ○ Winter hardiness ○ Plant height and lodging resistance ○ Ripening time (early maturity) ○ Nutrient efficiency and drought tolerance ○ Shattering resistance ○ Herbicide tolerance ○ Good germination and seedling vigour	○ Phoma and *Vericillium* ○ Clubroot and *Cylindorsporium* ○ Sclerotinia (resistance to be identified) ○ Virus resistance (TuYV) ○ Various insects pests (pant resistances remain to be identified)
Yield potential	**Seed quality**
○ High oil content for edible and biofuel oils ○ Seed yield components ○ Harvest index ○ Total and marketable seed yield ○ Herbicide tolerance	○ Very low erucic acid content (C22:1 <0.2%) ○ Low glucosinolate content (<18 mmol/kg seed) ○ Reduced fibre (lignin) content and improved digestibility (monogastric animals)

Source: Modified from Friedt and Snowdon (2010) "Oilseed Rape." In *Handbook of Plant Breeding: Oil Crops*, edited by Johann Vollmann and Istvan Rajcan, 91-126. Copyright © 2010 Springer New York.

Current oilseed rape breeding programs focus on the development of new double low (00 in both erucic acid and glucosinolate content) *B. napus* cultivars with the balance of hormone metabolites especially auxin and ABA content and corresponding regulators

(*Auxin Response Factor 10, ARF10 and Auxin Response Factor 16, ARF16*), maximal germination capacity and seedling vigour.

1.3 The importance of germination and seed vigour and establishment

Seed germination and seedling vigour considered as two key components of the performance of crop seeds are very complex traits determined by the interaction of hormonal (Holdsworth et al., 2008), genetic and numerous environmental components including soil factors, water availability and temperature (Bentsink and Koornneef, 2009). The vigour of seeds is defined as their ability to germinate and establish seedlings rapidly, uniformly, and robustly across diverse environmental conditions (Finch-Savage and Bassel, 2016). International Seed Testing Association (ISTA) currently proposes the definition as 'Seed vigour is the sum of those properties that determine the activity and performance of seedlots of acceptable germination in a wide range of environments' (ISTA, 2018). In Europe, autumn-sown winter oilseed rape (WOSR) depends on a strong vigour and prewinter development to suppress weed competition and establish a good basis for winter survival. In all crops the number of plants established per unit area strongly correlates with total yield (Fig. 4a); therefore, if seedling emergence is inadequate, the amount of harvestable product is reduced (Bleasdale, 1967). Figure 4b shows that as plant density increases, the size of individual plants is reduced. In order to achieve the desired marketable size of plant produce, the plant density achieved must be precise. If seedling emergence is non-uniform in time, the size of seedlings has a greater spread (Fig. 4c) and fewer plants in the population achieve the desired size to produce a greater marketable yield.

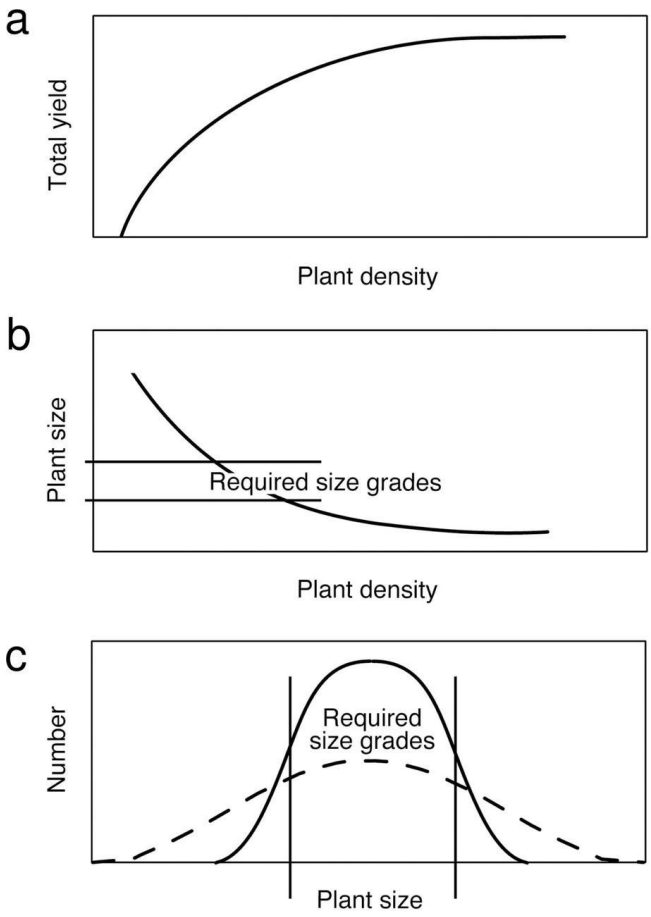

Figure 4: Crop establishment and crop yield. Schematics of the relationship of plant density on total yield (a) and plant size (b); and (c) the distribution of plant sizes resulting from uniform (solid line) and non-uniform (dotted line) seedling emergence. The vertical lines indicate the range of sizes acceptable for sale; thus uniform seedling emergence greatly increases the marketable yield (value) of the crop. Reproduced from Finch-Savage and Bassel (2016) *Journal of Experimental Botany* 67(3): 567-591. https://doi.org/10.1093/jxb/erv490. Copyright © 2016 Society for Experimental Biology

The importance of desired marketable size is more pronounced in high-value size grading crops (e.g., carrot and onion). Therefore, tightly controlled germination timing and seedling establishment is the highly-effective key to maximize both marketable yield potential and profit of crops. Successful seed germination and seedling establishment significantly impact final crop yields and resource use efficiency. As in other crops (Redoña and Mackill, 1995), good seed quality and seedling vigour are important traits in *Brassica* (Bettey et al., 2000), as seedling vigour is closely associated with growth and

yield (Ellis, 1992). Therefore, improving vigour to enhance crop growth and yield remains a primary objective of the agricultural industry and the seed and breeding companies. During the last few decades, extensive breeding efforts in *B. oleracea* (Bettey et al., 2000; Morris et al., 2016), *B. rapa* (Basnet et al., 2015) and *B. napus* (Hatzig et al., 2015; Nguyen et al., 2016; Nguyen et al., 2018) have focused on improving of germination and seedling establishment.

1.4 The genetic and hormonal control of seed germination and seedling vigour

Germination is the process by which the embryo wakes up from the state of dormancy and takes to active life. Germination commences with the uptake of water by imbibition of the dry seed, followed by embryo expansion. The visible sign of the completion of the germination process is the penetration of the structures surrounding the embryo by the radicle (Bewley, 1997; Kucera et al., 2005). Thereafter, seedling growth is underway (Figure 5). Seedling vigour is associated with many aspects of seed performance including rate and uniformity of seed germination and seedling growth, emergence ability of seeds under unfavourable environmental conditions, seed longevity and root length, shoot length, wet weight and dry weight in the early seedling growth process (Redoña and Mackill, 1995). Seed germination and seedling vigour are very complex traits (Bettey et al., 2000) determined by the interaction of hormonal (Holdsworth et al., 2008), genetic and numerous environmental components including soil factors, water availability and temperature (Bentsink and Koornneef, 2009). Several studies reported quantitative trait loci (QTL) for seed germination and seedling vigour traits in *Arabidopsis* (Clerkx et al., 2004), rice (Xie et al., 2014), *B. rapa* (Basnet et al., 2015), *B. oleracea* (Bettey et al., 2000; Morris et al., 2016) and *B. napus* (Hatzig et al., 2015; Nguyen et al., 2018).

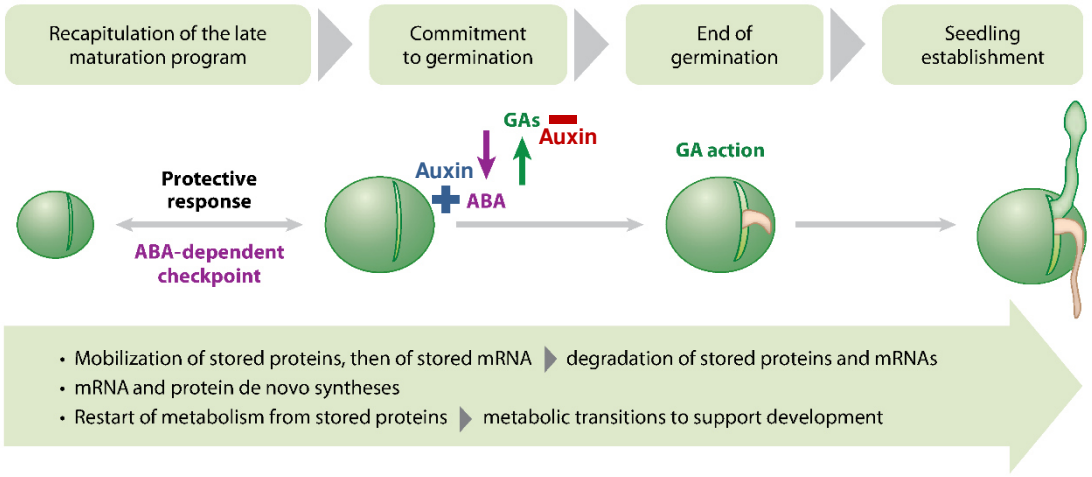

Figure 5: Phenomenology of seed germination. This panel includes the three phases of water intake, germination sensu stricto, and radicle emergence, and highlights the need for stored and de novo synthesized components for germination to occur. Auxin represses seed germination by enhancing (+) ABA biosynthesis, while impairing (-) GA biogenesis. Modified from Rajjou et al. (2012) *Annual Review of Plant Biology* 63(1): 507-33. http://dx.doi.org/doi:10.1146/annurev-arplant-042811-105550. Copyright © 2012 Annual Reviews, CA, USA.

Morris et al. (2016) used natural variation and fine mapping in the crop *B. oleracea* to show that allelic variation at three loci influence the key vigour trait of rapid germination. Two candidate genes named *BoLCVIG1* and *BoLCVIG2* were identified at the principal Speed of Germination QTL (*SOG1*) locus in *B. oleracea*. *BoLCVIG2* is a homologue of the alternative-splicing regulator while allelic variants of *BoLCVIG1* controls abscisic acid (ABA) sensitivity. The further QTL, Reduced ABscisic Acid 1 (*RABA1*) influenced ABA content is a homologue of the ABA catabolic gene *AtCYP707A2* in *Arabidopsis*.

The key contributor of seed germination success is the quality of the messenger RNAs and protein stored during embryo maturation on the mother plant. The stock of stored proteins is used to restart cellular activity, which is followed by mobilization of the stored mRNA pool following imbibition (Rajjou et al., 2012). The first study demonstrated that protein synthesis is directed by stored mRNAs during early cotton seed germination (Dure and

Waters, 1965). Genome-wide profiling of stored mRNA in *Arabidopsis thaliana* seed germination revealed that more than 12,000 stored mRNA species are present in mature dry seeds (Nakabayashi et al., 2005). Comprehensive combined transcriptome and metabolite profiling study that provides a foundation for the regulation at transcriptional, post-transcriptional, translational, and post-translational levels during rice (Howell et al., 2009) and rapeseed (Nguyen et al., 2016) seed germination.

Hormone imbalance affecting hormone biosynthesis or responses have been obtained in various plant systems by predicting phenotypes based on physiological information, by assaying hormone levels in plants with unexplained developmental defects or by selecting for resistance to hormones or their biosynthesis inhibitors. Upon imbibition massive metabolome changes occur, which are regulated by ambient temperature, light conditions, and plant hormones. Several plant hormones, including abscisic acid (ABA), gibberellins(GA), ethylene, brassinosteroids (BR),auxin and cytokinins(CK), interact to regulate germination (Kucera et al., 2005). The two prominent hormones in dormancy and germination control are the germinative inhibitor ABA and the germination promoter GA, respectively. Crosstalk at molecular levels of hormonal signaling influences the ABA:GA balance and thereby promotes or inhibits germination. Besides the ABA:GA balance, the individual sensitivity of the seed to these two hormones also plays a role in regulating dormancy and germination status (Finkelstein et al., 2008). Disruption in hormone signaling is responsible for diverse unexpected phenotypes including abolishing shoot regeneration abnormal seedlings and embryonic lethality in *Arabidopsis* (Chen et al., 2001; Cheng et al., 2013; Zhao et al., 2001) and stunted seedlings and germination disturbance in *Brassica* (Liu et al., 2007; Liu et al., 2013).

Germination is preceded by a decrease in ABA levels resulting from the activation of ABA hydroxylation predominantly at C-8′ position to 8-hydroxy ABA, PA, and DPA and of conjugation to ABA glucosylester (Nambara and Marion-Poll, 2005). Auxin is an

important phytohormone which mediates diverse development processes in plants. Recent studies have demonstrated that auxin induces seed dormancy. For example, exogenous application of auxin enhanced the inhibition of seed germination by ABA in *Arabidopsis* via *ABSCISIC ACID INSENSITIVE 3* (*ABI3*) transcription factor (Liu et al., 2007; Liu et al., 2013) and also delayed seed germination of wheat (Ramaih et al., 2003) and soybean through decreasing the GA/ABA ratio (Shuai et al., 2017). When auxin signaling is inactivated by low auxin level or signaling disruption, *Auxin Response Factor 10* (*ARF10*) and *Auxin Response Factor 16* (*ARF10*) are inactivated by the Aux/IAA repressors AXR2 and AXR3. *ABI3* expression cannot be maintained, and seed dormancy is released. With high auxin content or signaling activation, auxin binds to the auxin receptor TIR1/AFB F-box proteins and promotes the degradation of IAA7/AXR2 and IAA17/AXR3. The degradation releases the activity of *ARF10* and *ARF16* and maintains the expression of *ABI3*, which protects seed dormancy and inhibits seed germination (Liu et al., 2013). Under the effect of ABA on auxin-responsive genes, not only *ARF* genes but also early auxin-inducible *GH3.5* and *GH3.6* (for GRETCHENHAGEN-3) genes repress the *Arabidopsis* embryonic axis after germination by enhancing auxin signaling (Belin et al., 2009).

1.5 Methods for profiling and analysis of metabolome

Plant hormones are naturally occurring signaling molecules, which play key roles in the regulation of plant physiology, development, and adaptation to environmental stimuli at very low concentration. In fact, endogenous plant hormones are present at trace amounts in plants, usually at the level of 0.1–50 ng/g fresh weight (FW). In other words, plant hormones are chemical messengers that coordinate cellular activities of plants (Fleet and Williams). During the last decades, liquid chromatography-mass spectrometry (LC-MS), especially liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has

become the most widely used approach to plant hormone analysis (Floková et al., 2014; Li et al., 2016). LC-MS/MS can overcome some limitations of GC-MS for analyzing trace level plant hormones in complex plant samples, due to the good separation performance of LC and its versatility, without the need for derivatization. It is essential to reduce the influence of abundant interfering compounds present in plant matrices by rigorous purification of extracts before the instrumental analysis.

The whole analysis procedure of plant hormones based on LC-MS/MS recommended for typical plant material can be divided into five stages: (1) Sampling, (2) Grinding, (3) Extraction, (4) Purification, and (5) LC-MS/MS detection (Figure 6).

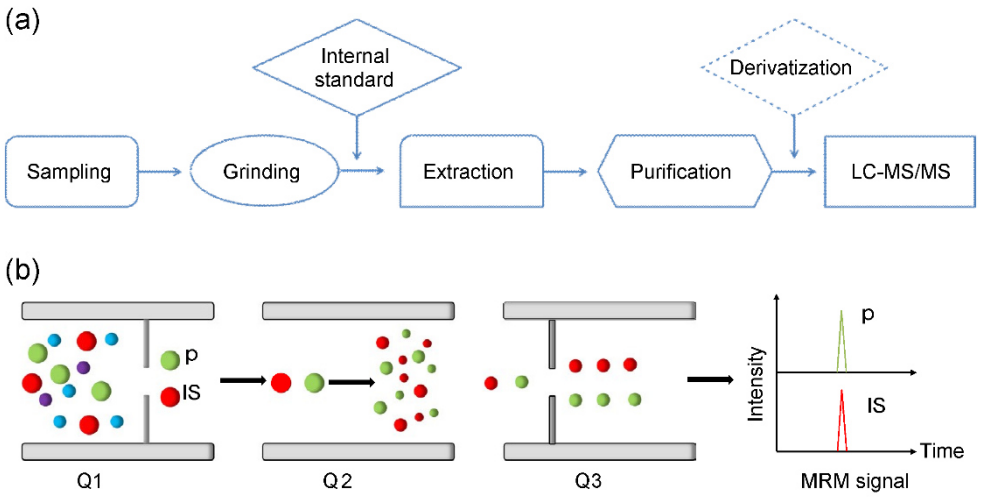

Figure 6: (a) Flow scheme for plant hormone quantification based on LC-MS/MS. (b) The principle of plant hormone quantification using a triple quadrupole MS in multiple reaction monitoring (MRM) mode. IS, internal standard; P, plant hormone. Reproduced from Chu et al. (2017) " Quantitative analysis of plant hormones based on LC-MS/MS" In *Hormone Metabolism and Signaling in Plants*, edited by J. Li, C. Li & S.M. Smith, 471-537. Copyright © 2010 Academic Press.

Obviously, a good method which can simultaneously quantify a wide range of plant signaling molecules of all known classes would greatly facilitate the investigation of hormone functions and networks. Using UHPLC-MS/MS, the most extensive hormone profiling covering 54 primary and secondary metabolites including auxins, ABA, salicylic

acid, jasmonates, GAs and cytokinins were simultaneously analyzed from a single rice (Cai et al., 2016) or wild tobacco (Schäfer et al., 2016) sample extract.

1.6 Aims of the thesis

The *B. napus* accession 1012-98 is of particular interest for oilseed rape breeding because it has a thin seed coat resulting in reduced dietary fiber content. This considerably improves the feed and protein quality of rapeseed meal after oil extraction. However, 1012-98 also exhibits negative agronomic characters including a reduced germination rate and inhibition of seedling development and vigour.

In the first part of the study, high-throughput UPLC-MS/MS profiling of hormone metabolites in seeds and seedlings before and after germination from the normal line Express 617 and the disturbed synthetic line 1012-98 parental lines and their segregating doubled haploid population (YE2-DH) were performed to identify the key hormones and their crosstalk causing the disruption of germination and seedling development in 1012-98 line. In addition, molecular cloning of their respective transcription factors *Auxin Response Factor 10* (*Bna.ARF10*) and early auxin-responsive genes *Bna.GH3.5,* and *Bna.GH3.6* were carried out to systematically analyze the copy number alteration and differentially transcriptional expression of key regulators in the regulation of germination and seedling development in *B. napus*.

In the second study, quantitative trait locus (QTL) analysis was performed to identified quantitative trait loci (QTL) related to germination, seedling vigour and seedling-regulated hormones in the YE2-DH mapping population from a cross between winter oilseed rape parents with high vigour (Express 617) and low vigour (1012-98). This will contribute to better understanding of the genetics and metabolomics of germination and seeding vigour in *B. napus*, and represent potential targets to breed high-vigour cultivars.

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

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Disruption of Germination and Seedling Development in *Brassica napus* by Mutations Causing Severe Seed Hormonal Imbalance

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The *Brassica napus* (oilseed rape) accession 1012-98 shows a disturbed germination phenotype that was thought to be associated with its lack of testa pigmentation and thin seed coat. Here, we demonstrate that the disturbed germination and seedling development are actually due to independent mutations that disrupt the balance of hormone metabolites and their regulators in the seeds. High-throughput UPLC-MS/MS hormone profiling of seeds and seedlings before and after germination revealed that 1012-98 has a severely disturbed hormone balance with extremely atypical, excessive quantities of auxin and ABA metabolites. The resulting hypersensitivity to abscisic acid (ABA) and a corresponding increase in dormancy often results in death of the embryo after imbibition or high frequencies of disturbed, often lethal developmental phenotypes, resembling *Arabidopsis* mutants for the auxin regulatory factor gene *ARF10* or the auxin-overproducing transgenic line iaaM-OX. Molecular cloning of *Brassica ARF10* orthologs revealed four loci in normal *B. napus*, two derived from the *Brassica* A genome and two from the C genome. On the other hand, the phenotypic mutant 1012-98 exhibited amplification of C-genome *BnaC.ARF10* copy number along with a chimeric allele originating from recombination between homeologous A and C genome loci which lead to minor increase of *Bna.ARF10* transcription on the critical timepoint for seed germination, the indirect regulator of *ABI3*, the germinative inhibitor. *Bna.GH3.5* expression was upregulated to conjugate free auxin to IAA-asp between 2 and 6 DAS. Functional amino acid changes were also found in important DNA binding domains of one *BnaC.ARF10* locus, suggesting that regulatory changes in *Bna.ARF10* are collectively responsible for the observed phenotpyes in 1012-98. To our knowledge, this study is the first to report disruption of germination and seedling development in *Brassica napus* caused by the crosstalk of auxin-ABA and the corresponding regulators *Bna.ARF10* and *Bna.GH3.5*.

Keywords: ***Brassica napus*, oilseed rape, seeds, hormones, germination, vigor**

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INTRODUCTION

Seed germination and seedling establishment are crucial processes in life cycles of seed plants. In fact, strong seedling vigor or rapid seedling growth is a major breeding target in *Brassica oleracea* (Bettey et al., 2000), rice, and other crops (Redoña and Mackill, 1995) as seedling vigor is closely associated with crop growth and yield (Ellis, 1992). Unfortunately, these traits are polygenic (Bettey et al., 2000) and determined by the interaction of hormonal (Holdsworth et al., 2008), genetic, and environmental components (Bentsink and Koornneef, 2009). Several plant hormones, including abscisic acid (ABA), gibberellins (GA), ethylene, brassinosteroids (BR), auxin and cytokinins (CK), interact to regulate germination (Kucera et al., 2005). The two prominent hormones in dormancy and germination control are the germinative inhibitor ABA and the germination promoter GA, respectively. Generally, ABA biosynthesis and sensitivity increases during seed development and maturation to prevent premature germination, whereas, GA accumulation and sensitivity dominate after seed imbibition, promoting the transition to germination (Hilhorst and Karssen, 1992; Finkelstein et al., 2008). Crosstalk at molecular levels of hormonal signaling influences the ABA:GA balance and thereby promotes or inhibits germination (Kucera et al., 2005; Finch-Savage and Leubner-Metzger, 2006; Finkelstein et al., 2008). Besides the ABA:GA balance, the individual sensitivity of the seed to these two hormones also plays a role in regulating dormancy and germination status (Finkelstein et al., 2008). Germination is preceded by a decrease in ABA levels resulting from the activation of ABA hydroxylation predominantly at C-8′ position to 8-hydroxy ABA, PA, and DPA and of conjugation to ABA glucosylester (Nambara and Marion-Poll, 2005). In fact, endogenous ABA contents decreased significantly within 6–24 h after the onset of imbibition in *Arabidopsis* (Ali-Rachedi et al., 2004) and rice seeds (Ye et al., 2011). In non-endospermic *Brassica* seeds, ABA does not inhibit testa rupture, but inhibits subsequent radicle growth of germinating process (Schopfer and Plachy, 1984; Kucera et al., 2005). Germinating seeds of lettuce (*Latuca sativa* L.) transiently accumulate high levels of ABA-GE and an increase of GA_1 to support germination and post-germinative growth (Chiwocha et al., 2003). Recent studies have suggested the potential involvement of auxin in regulation of seed dormancy and germination. For example, exogenous application of auxin enhanced the inhibition of seed germination by ABA in *Arabidopsis* via *ABSCISIC ACID INSENSITIVE 3* (*ABI3*) transcription factor (Brady et al., 2003; Liu et al., 2007, 2013) and also delayed seed germination of wheat (Ramaih et al., 2003). When auxin signaling is inactivated by low auxin level or signaling disruption, *Auxin Response Factor 10* (*ARF10*) and *Auxin Response Factor 16* (*ARF16*) are inactivated by the Aux/IAA repressors AXR2 and AXR3. *ABI3* expression cannot be maintained, and seed dormancy is released. With high auxin content or signaling activation, auxin binds to the auxin receptor TIR1/AFB F-box proteins and promotes the degradation of IAA7/AXR2 and IAA17/AXR3. The degradation releases the activity of *ARF10* and *ARF16* and maintains the expression of *ABI3*, which protects seed dormancy and inhibits seed germination (Liu et al., 2013). Under the effect of ABA on auxin-responsive genes, not only *ARF* genes but also early auxin-inducible *GH3.5* and *GH3.6* (for *GRETCHENHAGEN-3*) genes repress the *Arabidopsis* embryonic axis after germination by enhancing auxin signaling (Belin et al., 2009). Stronger dormancy and germination failure were observed in both auxin-overproducing transgenic *Arabidopsis* line *iaaM-OX* (Cheng et al., 2006) and exogenous auxin-applied wild-type *Arabidopsis* seeds (Liu et al., 2007, 2013). In addition, the auxin overproduction mutants *iaaM* and *YUC1-OX Arabidopsis* express long hypocotyls and epinastic cotyledons (Zhao, 2010).

Oilseed rape (*Brassica napus* L., $2n = 38$, genome AC), a recent amphidiploid species derived from interspecific hybridizations between *Brassica rapa* L. ($2n = 20$, genome A) and *B. oleracea* L. ($2n = 18$, genome C) is the second most important oilseed crop worldwide. Germination and seedling vigor are highly important aspects of seed quality, with a major impact on stand establishment and subsequent yield. Considering the economic importance of germination and vigor, these traits are still poorly understood in oilseed rape and many other important crops. Transfer of knowledge from related model systems into important crops like oilseed rape will greatly increase our ability to improve and manipulate germination and vigor through breeding.

The *B. napus* line 1012-98 is of particular interest for oilseed rape breeding because it has a thin seed coat resulting in reduced dietary fiber content (Badani et al., 2006). This considerably improves the feed and protein quality of rapeseed meal after oil extraction.However, 1012-98 also exhibits negative agronomic characters including a reduced germination rate and inhibition of seedling development and vigor. In contrast to 1012-98, the homozygous inbred line Express 617 has a normal seed coat, germinates normally and shows normal seedling development. To investigate the relationship between seed quality traits, germination and seedling vigor, we generated homozygous, microspore-derived doubled-haploid (DH) progenies from the cross between 1012-98 and Express 617 and screened these for seedling vigor traits and metabolic profiling. Regulation of germination and seed-to-seedling transition by phytohormones was investigated in the parental lines and their DH progenies by high-throughput UPLC-MS/MS profiling of hormone metabolites before and after germination. Molecular cloning and expression analysis of *B. napus* orthologs of the auxin-responsive genes *ARF10*, *GH3.5*, and *GH3.6* were performed to identify functional sequence polymorphisms with a potential impact on seed hormone regulation.

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 vigor. The *B. napus* line 1012-98 is a progeny of an interspecific hybrid derived by embryo rescue-assisted resynthesize from an interspecific cross between

B. rapa and *B. oleracea*. Due to the high relatedness of the *Brassica* A and C genomes, the chromosomes of resynthesized *B. napus* frequently contain non-reciprocal homeologous translocations (Udall et al., 2005) that can lead to replacement or recombination of homeologous gene copies in either the A or C genome. 1012-98 shows a yellow-seeded phenotype, atypical for *B. napus*. This phenotype is primarily attributable to a major quantitative trait locus (QTL) on chromosome A09 that influences testa thickness and flavonoid pigmentation (Snowdon et al., 2010) and is 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 a single 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 vigor phenotypes of 1012-98. All seeds used for the investigations were harvested from self-pollinated plants grown under normal field conditions in a common environment. For determination of correlations between germination and seed quality traits, contents of fiber components and the seed color 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, 2010). 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 h light/8 h darkness.

Seedling development was assessed in three 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 7 and 14 days after sowing (DAS). Mean trait values were calculated from all successfully germinated seeds per genotype.

Sampling for Hormone Analysis and Quantitative RT-PCR

For comparison of hormone metabolite profiles and differential expressions of *B. napus Bna.ARF10*, *Bna.GH3.5*, and *Bna.GH3.6* in Express 617 and 1012-98, 10 identical Jacobsen germination pots per genotype were prepared for sampling every 24 h from 1 to 8 days after sowing (DAS) and every 48 h from 8 to 12 DAS. The experiment was conducted in three replications, total of 15 (ca. 50 mg), and 50 (ca. 100 mg) seeds/seedlings per genotype and replication were pooled into 15 ml Falcon tubes, immediately immersed in liquid nitrogen and lyophilized for 24 h for hormone analysis and quantitative RT-PCR, respectively.

Internal Standards for Hormone Quantification

Calibration curves and quality controls for dihydrophaseic acid (DPA), abscisic acid glucose ester (ABA-GE), phaseic acid (PA), 7′-hydroxy-ABA (7′-OH-ABA), neo-phaseic acid (neoPA), and indole-3-acetic acid glutamate (IAA-glu) were created by the Plant Biotechnology Institute of the National Research Council of Canada (PBI-NRC, Saskatoon, SK, Canada). Details on all internal standards used for quantification of hormone metabolites are provided in **Supplementary Table S1**.

Extraction and Purification of Hormone Metabolites

Lyophilized plant tissue was homogenized in a bead mill for 2–6 min. A 100 µl aliquot containing all the internal standards, each at a concentration of 0.2 pg μ^{-1}, was added to around 50 mg of homogenized tissue. After addition of 3 ml of isopropanol:water:glacial acetic acid (80:19:1, v/v) the samples were agitated for 24 h at 4°C. Samples were then centrifuged and the supernatant was isolated and dried on a Buechi Syncore Polyvap (Buechi, Switzerland). Samples were reconstituted in 100 µl acidified methanol, adjusted to 1 ml with acidified water, and then partitioned against 2 ml hexane. After 30 min, the hexane layer was removed and the hexane partitioning was repeated. The aqueous layer was then isolated and dried. Dry samples were reconstituted in 800 µl acidified methanol and adjusted to 1 ml with acidified water. The reconstituted samples were passed through equilibrated Sep-Pak C18 cartridges (Waters, Mississauga, ON, Canada), the eluate being dried on a centrifugal evaporator. An internal standard blank was prepared with 100 µl mixture of the deuterated internal standards. A QC standard was prepared by adding 20 ng of each analyte to 100 µl of the internal standard. Finally, all samples, blanks and QCs were reconstituted in a solution of 40% methanol (v/v), containing 0.5% acetic acid, and 100 pg µl of each of the recovery standards.

Hormone Quantification by HPLC-ESI-MS/MS

The procedure for quantification of multiple hormones and metabolites, including auxins (IAA, IAA-asp, and IAA-glu), abscisic acid, and metabolites (ABA, PA, DPA, 7′-OH-ABA, neoPA, and ABA-GE), and CKs (2iP, iPA, Z, ZR, dhZ, dhZR, and Z-O-Glu) has been described in detail by Chiwocha et al. (2003; 2005). Samples were injected onto a Genesis C18 HPLC column (100 · 2.1 mm, 4 lm, Chromatographic Specialties, Brockville, ON, Canada) and separated by a gradient elution of water against an increasing percentage of acetonitrile that contained 0.04% acetic acid. Calibration curves were generated from the MRM signals obtained from standard solutions based on the ratio of the chromatographic peak area for each analyte to that of the corresponding internal standard, as described by Ross et al. (2004). QC samples, internal standard blanks, and solvent blanks were prepared and analyzed along with each batch of tissue samples. Mean minimum limits of quantification (LOQ) for each analyte were: 8 ng g^{-1} dry weight (DW) for Z, dhZ, Z-O-Glu, 2iP, iPA, and ABA; 60 ng g^{-1} DW for ZR, dhZR, IAA-asp, IAA-glu, IAA, and 7′-OH-ABA; 118 ng g^{-1} DWfor DPA; 78 ng g^{-1} DW for PA; 56 ng g^{-1} DW for ABA-GE; and 30 ng g^{-1} DW for neoPA.

Cloning of Full-Length *Brassica ARF10* Orthologs in *B. napus*, *B. oleracea* and *B. rapa*

Genomic clones harboring *Bna.ARF10* orthologs in Express 617 were isolated by hybridization to an 8x-coverage bacterial artificial chromosome (BAC) genomic library from Express 617. A deoxygenin-dUTP (DIG) labeled PCR amplicon from Express 617 was used a probe. The probe sequence, amplified by the PCR primer combination *ARF10* -ex1-1F/R (**Table 1**) corresponded to the conserved region between positions 416 to 600 in the *Arabidopsis thaliana ARF10* coding sequence (At2g28350, accession NM_128394). BAC filter hybridization and fluorescent detection was performed according to Garratt et al. (2001).

A total of 70 positive BAC clones were identified. To isolate all full-length *Brassica* A and C genome orthologs of *ARF10*, 100 bp from the 5′- and 3′-ends of the *AtARF10* coding sequence were first blasted against *Brassica* genomic sequences available from the *Brassica* Genome Gateway at http://brassica.bbsrc.ac.uk/. Highly matching accessions were selected corresponding to the 5′ (CC952958, ES905909, EV092049) and the 3′ ends (BH703182, EX084979, EV067938, ES907226, BH605387) of *AtARF10*. Based on these *Brassica* sequences, a pair of consensus PCR primers was designed (Bn*ARF10* F/R2, see **Table 1**) to amplify full-length *Bna.ARF10* orthologs in the *Brassica* A and C genomes. The Bn*ARF10* F/R2 primers were also used to screen all 70 positive BAC clones. Five BAC clones harboring putative full-length *Bna.ARF10* sequences were selected for sequencing of the gene region. Nomenclature for the *Brassica* orthologs follows (Ostergaard and King, 2008).

The BnARF10F/R2 primers were also used to amplify full-length *ARF10* sequences from genomic DNA of *B. napus* 1012-98, *B. oleracea,* and B. rapa, respectively. All PCR reactions were carried out using PCR Extender proofreading polymerase enzyme (5 PRIME GmbH, Hamburg, Germany) in accordance with the manufacturer's guidelines. Full-length fragments were cloned into TOPO TA vector (Invitrogen, Darmstadt, Germany) for DNA sequencing. Bidirectional sequencing reactions were conducted by Eurofins MWG (Ebersberg, Germany). Express 617 sequences were generated directly from the six *Bna.ARF10*-positive BAC clones. Six randomly chosen clones were sequenced from full-length *Bna.ARF10* amplicons from 1012-98, along with four positive clones each from *B. oleracea* and B. rapa, respectively. Sequence and cluster analyses were performed using Vector NTI Advance 9.0, BioEdit 7.0.5, and CLC Sequence Viewer 6.4.

Quantitative RT-PCR Analysis for *B. napus Bna.ARF10*, *Bna.GH3.5*, and *Bna.GH3.6*

Total RNA from 100 mg freshly harvested seeds and total seedlings were isolated using TRIzol (Invitrogen). The protocol details can be referred to the work of MacRae (2007). The first-strand cDNA strand was synthesized from 5 µg DNA-free total RNA using RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit (Fermentas GmbH, St. Leon-Rot, Germany) and an oligo (dT) primer following the manufacturer's instructions. Diluted cDNA (1 µl) was used in 10 µl PCR containing 200 nM of each primer, 0.2 µl of ROX low and 5 µl KAPA SYBR® FAST (PEQLAB Biotechnologie GmbH, Erlangen, Germany). Three independent biological replicates were used for each sample, and quantification was performed in technical triplicate. PCR was performed in the 7500 fast RT-PCR (Applied Biosystems, Darmstadt, Germany) with the following temperature program: 10 min at 95°C, then 40 cycles of 15 s at 95°C, and 1 min at 60°C. At the end of the PCR, the melting temperature of the product was determined to verify the specificity of the amplified fragment. PCR product was analyzed using 7500 Software version 2.0.6 (Applied Biosystems, Darmstadt, Germany). The RT products of *Bna.ARF10*, *Bna.GH3.5*, and *Bna.GH3.6* were subjected to semi-quantitative PCR using BnaA+C.ARF10 forward (5′-GGRCAAGCKTTCGAAGTTGTTT-3′)/ BnaA+C.ARF10 reverse (5′-TCACGTCGGAAGCCTTCAC-3′), BnaA+C.GH3.5 forward (5′- GGTGTGAACCTAAGGCCAC TTT-3′)/BnaA+C.GH3.5 reverse (5′-CGAAATAAGCCAT GGTCGGTAT-3′), and BnaA+C.GH3.6 forward (5′-TYTCACGCAATGCTGACGTT-3′)/BnaA+C.GH3.6 reverse (5′-WCTCRCGGTCGGTTCGT-3′) primers, respectively. Three classic housekeeping genes for vegetative stage in *Brassica* sp. namely β-actin (ACT2), tubulin (TUA), and ubiquitin (UBQ) were selected and analyzed using geNORM software package to identify the most stably expressed genes within a set of reference genes across three representative timepoints 4, 6, 8 DAS on the parental genotypes, Express 617 and 1012-98. For each reference gene, a stability value M was calculated; the lower the M-value the more stably the gene is expressed. ACT2 gene was selected as the best internal reference based on its stability in expression pattern in our test (data not show). Our test outcome is in agreement with Chandna et al. (2012) selection for the most suitable reference gene during vegetative stage in *Brassica juncea*. The relative expression levels of A- and C-copies of *B. napus ARF10* named *BnaA.ARF10.a.E617*, *BnaA.ARF10.b.E617*, *BnaA.ARF10.b.1012-98*, *BnaAC.ARF10.a.1012-98*, *BnaC.ARF10.a.E617*, *BnaC.ARF10.a.1012-98*, *BnaC.ARF10.b.E617*, *BnaC.ARF10.b.1012-98*, *BnaC.ARF10.c.1012-98*, and *BnaC.ARF10.d.1012-98 mRNAs* were calculated using the $2^{-\Delta\Delta Ct}$ method normalized to the internal reference ACT2.

RESULTS

Line 1012-98 has Disturbed Germination and Seedling Development

Figures 1, **2** compare the germination and seedling development in Express 617 and 1012-98. Whereas, Express 617 showed a normal germination rate of around 90%, the germination rate was severely depressed in 1012-98 with successful testa rupture and radicle emergence being observed in only around one quarter of the seeds (**Figure 1C** with $^{***}p < 0.001$). Express 617 also exhibited a considerably better seedling establishment than 1012-98 under greenhouse conditions. Seedlings of 1012-98 (**Figure 2C**) showed significantly increased etiolation and consequently longer hypocotyls than those of Express 617 ($^{*}p < 0.05$) which was also found in transgene-mediated auxin overproduction in *Arabidopsis* (Romano et al., 1995). However,

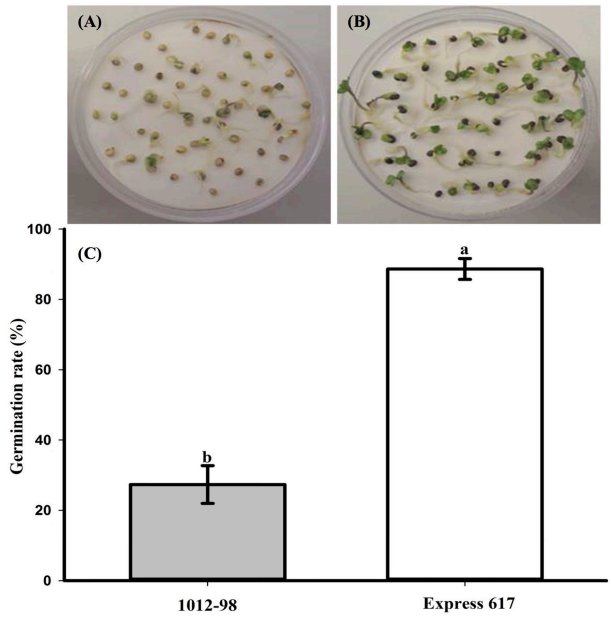

FIGURE 1 | Comparison of germination and germination rate (%) at 7 days after sowing in the phenotypically disturbed synthetic *B. napus* line 1012-98 (A, filled vertical bar C) and the normal line Express 617 (B, blank vertical bar C). Germination assessment was performed in Jacobsen chambers with 50 seeds per homozygous line under controlled conditions in a growth chamber. Values are presented as averages (± SEMs) with Student's *t*-test. Different letter superscripts between vertical bars indicate significant difference ($p < 0.001$).

TABLE 1 | Primer sequences and optimum annealing temperatures used to amplify a conserved *Brassica ARF10* exonic region (ARF10-ex1-1) for BAC library screening, and full-length *Brassica ARF10* orthologs (BnARF10), respectively.

Primer name	Sequence	PCR annealing temperature (°C)
ARF10-ex1-1F	5′-CGAGGCTTGATTACACGG-3′	54.4
ARF10-ex1-1R	5′-GCGGAGGAAGACGATTGA-3′	
BnARF10F	5′-AAAATGGAGCAAGAGAGAAG-3′	58.6
BnARF10R2	5′-ACAACCCAAACAAATAAAATT-3′	

no significant difference was observed between the two genotypes for mean shoot fresh weight of successfully germinated plants at 7 DAS.

Progenies from 1012-98 Express Abnormal Developmental Phenotypes

Figure 3 and **Supplementary Table S2** give details of all documented seed and seedling phenotypes in 1012-98, Express 617, and their offspring, respectively. The DH progenies derived from the cross between 1012-98 and Express 617 showed a quantitative segregation for the germination defect and exhibited diverse developmental abnormalities during seedling development. No significant correlation was found between germination rate and seed color in the DH population, suggesting that these two traits are controlled by mutations at independent loci in the defective parent 1012-98. Abnormal developmental phenotypes included ectopic trichromes on cotyledons, curled cotyledons, tricotyledons, chlorotic spots on cotyledons, or chlorotic first leaves. Examples are shown in **Figure 3**. Seedlings with a weak hypocotyl and consequent "ostrich" phenotype, where the cotyledons failed to emerge from the soil, were common in the defective-development DH lines, as were seedlings with variable levels of stunted growth. Where the growth deformation was too severe the phenotype was often lethal. A high degree of variation was observed among individuals of the same homozygous genotype, with different degrees of developmental retardation being commonly observed within each hormone-defective line. This is typical for epigenetic variation and could indicate potential functional defects in one or more transcription factors involved in hormone-driven developmental regulation.

1012-98 Seeds and Seedlings Show Severe Hormonal Imbalance

Hormone profiles of seeds and seedlings from 1012-98 and Express 617 until 12 DAS are described in **Figure 4**, with detailed data available in **Supplementary Table S2**. The desiccated, ripe seeds of the normal-germinating line Express 617 showed characteristically low levels of all major hormones and hormone metabolites. The dominant hormone in Express 617 was IAA, with a mean value of 68 ng/g dry weight (DW). In contrast, 1012-98 seeds contained ≈9-fold higher concentration of IAA and remained close to this proportion until 3 DAS, with an abnormally high level of 593 ng/g DW. IAA content in Express 617 declined gradually as compared to the sharply diminishing curve in 1012-98 within the first 3 DAS. The first 24 h after imbibition played a vital role in success of *B. napus* germination (Schopfer and Plachy, 1985). Despite of the remarkable reduction, IAA content in 1012-98 still remained 3.8-fold higher than the quantified amount in Express 617. The biggest difference in auxin profile between Express 617 and 1012-98 was extremely high levels of IAA-asp, IAA-glu and free IAA as late as 3 DAS on which is critical time for seed germination (Thakur and Sharma, 2016). An abnormally extreme concentration of more than 25,000 ng/g DW IAA-asp, higher than we have ever recorded in seeds of *Brassica* spp., increased to over 30,000 ng/g DW in 7 DAS before sharply declining to the lowest concentration of 454 ng/g DW at the end of study (see **Supplementary Table S2**).

The unusual levels of IAA conjugates were accompanied by uncharacteristically high seed GA levels in 1012-98. The only GA metabolites detected were GA_1 and GA_4, which in Express 617 appeared together in a brief burst around 4 DAS. In the normally germinating seeds of Express 617, the GA peak at 4 DAS preceded a sharp rise in IAA, DPA, ABA, and CK-ZR concentrations, at 5 DAS to support seedling growth. In 1012-98, on the other hand, abnormally high levels of GA_1 were detected in the desiccated seeds and levels remained uncharacteristically high until the onset of post-germinative growth. Almost no GA_4 was detected, with the exception of a minor peak at 5 DAS which

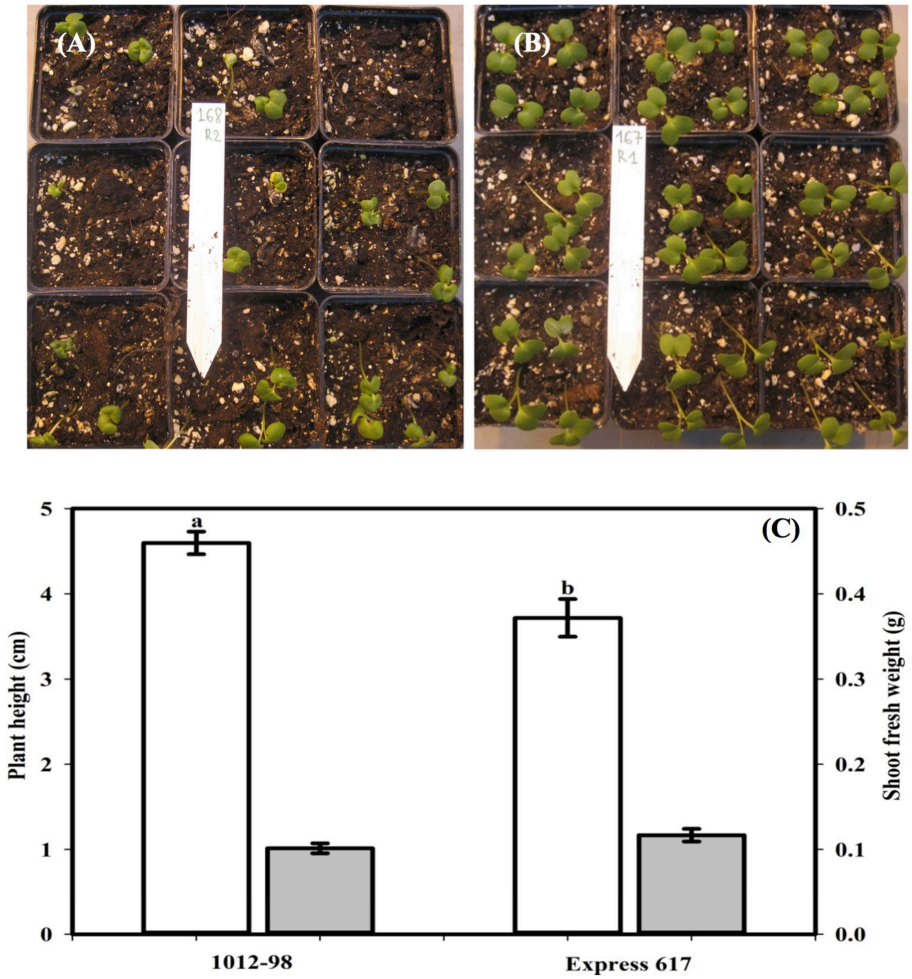

FIGURE 2 | Comparison of seedling vigor, plant height (cm, blank vertical bar), and shoot fresh weight (g, filled vertical bar) at 7 days after sowing in the phenotypically disturbed resynthesized *B. napus* line 1012-98 (A,C) and the normal line Express 617 (B,C). Seedling vigor was assessed in 36 soil-sown seeds per genotype grown under controlled conditions in a climate-controlled greenhouse. Values are presented as averages (±SEMs) with Student *t*-test. Different letter superscripts between vertical bars indicate significant difference ($p < 0.05$).

presumably corresponds to the GA peak at 4 DAS in Express 617. This appears to be the deciding time-point that determines whether endosperm weakening and testa rupture is successful in 1012-98.

The dried seeds of both genotypes contained similar quantities of ABA. *De novo* synthesized ABA in 1012-98 started immediately within 24 h after imbibition with the concentration of 69.7 ng/g DW then decreased gradually until 4 DAS. The normal seeds of Express 617 produced ABA transiently at 5 DAS, however the ABA appeared to be rapidly inactivated through oxidation at the 8′-carbon atom, followed by rapid reduction of PA to DPA. DPA catabolism is not tracked in our study but likely occurs through conjugation to DPA glycoside. There is no evidence of significant conjugation of ABA at 5 days in Express 617. In both Express 617 and 1012-98 the ABA, DPA, and ABA-GE levels were found to rise after 6 days, with metabolism at this stage through both conjugation to the glucose ester and oxidation through the 8′-oxidation, with DPA pools rising and then falling.

In the DH progeny from Express 617 × 1012-98 the lines showing the most severe phenotypic abnormalities showed abnormally high levels of IAA conjugates, like those seen in 1012-98 (see **Supplementary Table S2**). This indicates that the developmental phenotypes are heritable and associated with a genetically determined hormonal imbalance derived from 1012-98. The observed phenotypes are consistent with auxin-overdose symptoms, as described by Boerjan et al. (1995) and Romano et al. (1995).

Copy Number Amplification and Intergenomic Recombination in *Bna.ARF10* Orthologs

Each of the four sequenced *ARF10* clones from *B. rapa* and *B. oleracea*, respectively, gave identical sequences (hereinafter designated *BraA.ARF10* and *BolC.ARF10*, respectively). This indicates either a single locus of this gene in each of the two

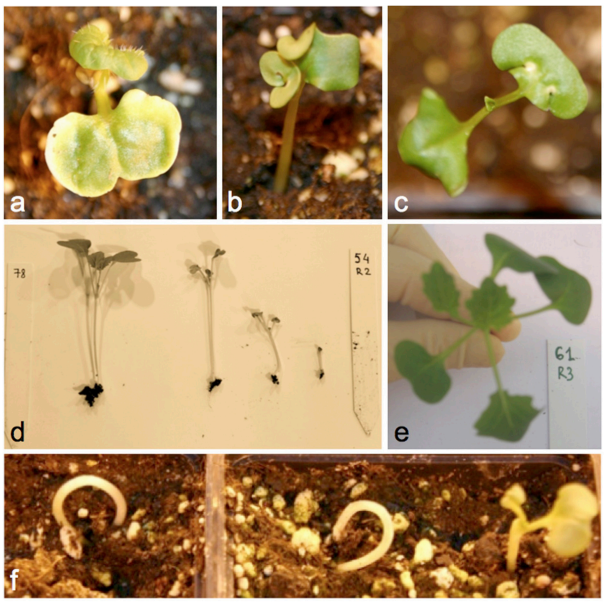

FIGURE 3 | Examples of commonly observed phenotypic abnormalities in seedlings of DH progenies from Express 617 × 1012-98 showing excessive IAA contents at 7 DAS. (A) Ectopic trichromes, **(B)** deformed cotyledons, **(C)** chlorotic spots, **(D)** different degrees of stunted growth in the same homozygous line, **(E)** tricotyledons, **(F)** "ostrich" phenotype.

diploid species, or complete identity of paralogs in the respective genomes, which are each considered to be ancestral tetraploids (Lysak et al., 2005).

The sequences we obtained from the amphidiploid *B. napus* were derived from homozygous inbred lines, hence each unique sequence represents an independent, homeologous *ARF10* locus. Two distinct loci with very high homology to *BraA.ARF10* were found (hereinafter referred to as *BnaA.ARF10*.a and *BnaA.ARF10*.b), suggesting a duplication event in the *B. napus* A genome following polyploidization. Interestingly, in place of *BnaA.ARF10.a* the resynthesized *B. napus* line 1012-98 contained a novel recombinant allele, here designated as *BnaAC.ARF10.a.1012-98*, that was derived from a concatenation of exon 1 from the *BraA.ARF10* locus with exons 2, 3, 4 from *BnaC.ARF10*. This recombinant allele likely resulted from a non-homeologous recombination event, a frequent phenomenon during the first meiosis of resynthesized *B. napus* genotypes (Udall et al., 2005; Szadkowski et al., 2010). In the UPGMA tree (**Figure 5**) the *BnaAC.ARF10.a.1012-98* sequence was found to cluster at an intermediate position between the A and C genome *ARF10* sequences, reflecting its origin as a chimeric locus.

Express 617 and 1012-98 shared two common C genome *ARF10* loci (hereinafter referred to as *BnaC.ARF10.a* and *BnaC.ARF10.b*), however in 1012-98 we unexpectedly detected two additional C-genome loci (designated here as *BnaC.ARF10.c* and *BnaC.ARF10.d*). As in the A genome, the C genome *ARF10* locus from *B. oleracea* also appears to have been duplicated during the evolution of natural *B. napus* (Express 617), while the resynthesized *B. napus* line 1012-98 shows additional copy number amplification from two to four C genome *ARF10* copies. Alignment of all detected *Bna.ARF10* copies against *Brassica* ESTs showed that all copies present in both Express 617 and 1012-98 have very high homology to expressed transcripts, and all have over 99% similarity to *BolC.ARF10*. This suggests that all C-genome copies could be potentially transcriptionally active. Therefore, the quadruplication event in 1012-98 might be expected to increase *ARF10* transcript abundance, thus causing an over-accumulation of m*ARF10* in auxin signal transduction throughout plant growth development. Liu et al. (2007) found that repression of *ARF10* is essential for normal seed germination and seedling establishment in *A. thaliana*, hence such an over-accumulation is expected to have a negative influence on these processes.

Strong Evolutionary Conservation of *Brassica* ARF10 Protein Domains

An overview of predicted structural parameters of all identified *Brassica ARF10* orthologs is given in **Table 2**, while **Supplementary Table S3** compares pairwise DNA and deduced protein sequence identities among all detected *Brassica ARF10* loci and *AtARF10*. A-genome alleles aligned with *BnaA07g13830D* gene (≥99% similarity) on chromosome A07 while C-genome alleles matched to BnaC04g15900D (≥98.7% similarity) gene chromosome C04 (Chalhoub et al., 2014). The complete amino acid sequences for all investigated loci are provided in **Figure 1**. The genomic and coding sequences of *BraA.ARF10* and *BolC.ARF10* were 97% identical, while the deduced protein alignments revealed even higher conservation at the protein level than at the nucleotide level. Protein sequence conservation of over 99% was observed within the respective A-genome and C-genome loci, with over 98% conservation observed between A and C genome loci in B. rapa, *B. oleracea* and *B. napus*, respectively. The *Brassica ARF10* genomic sequences showed 85% identity to the corresponding genomic sequence of *AtARF10*, with 88% identity among the coding sequences. Comparisons with the NCBI Conserved Domains Database (CDD) revealed that the predicted proteins from all *Brassica ARF10* orthologs share the same three highly-conserved domains with *AtARF10*: a conserved auxin responsive factor, a B3-DNA binding domain, and an AUX/IAA super-family domain.

1012-98 has Amino-Acid Substitutions in *BnaC.ARF10* DNA-Binding Domains

All *Brassica ARF10* sequences were submitted to NCBI with the accession numbers presented in **Table 2**. Frequent functional mutations compared to *A. thaliana*, *B. rapa* and *B. oleracea* were observed in *B. napus* A-genome *ARF10* sequences from both the mutant 1012-98 and also the normal line Express 617. This suggests that the C-genome homeologs of this gene family probably have more functional relevance in *B. napus* than the two A-genome homeologs. Besides the four native C-genome *ARF10* copies in 1012-98 (compared with only two in Express 617), the chimeric AC sequence that was found in 1012-98 in place of *BnaA.ARF10.a* also contains the DNA-binding

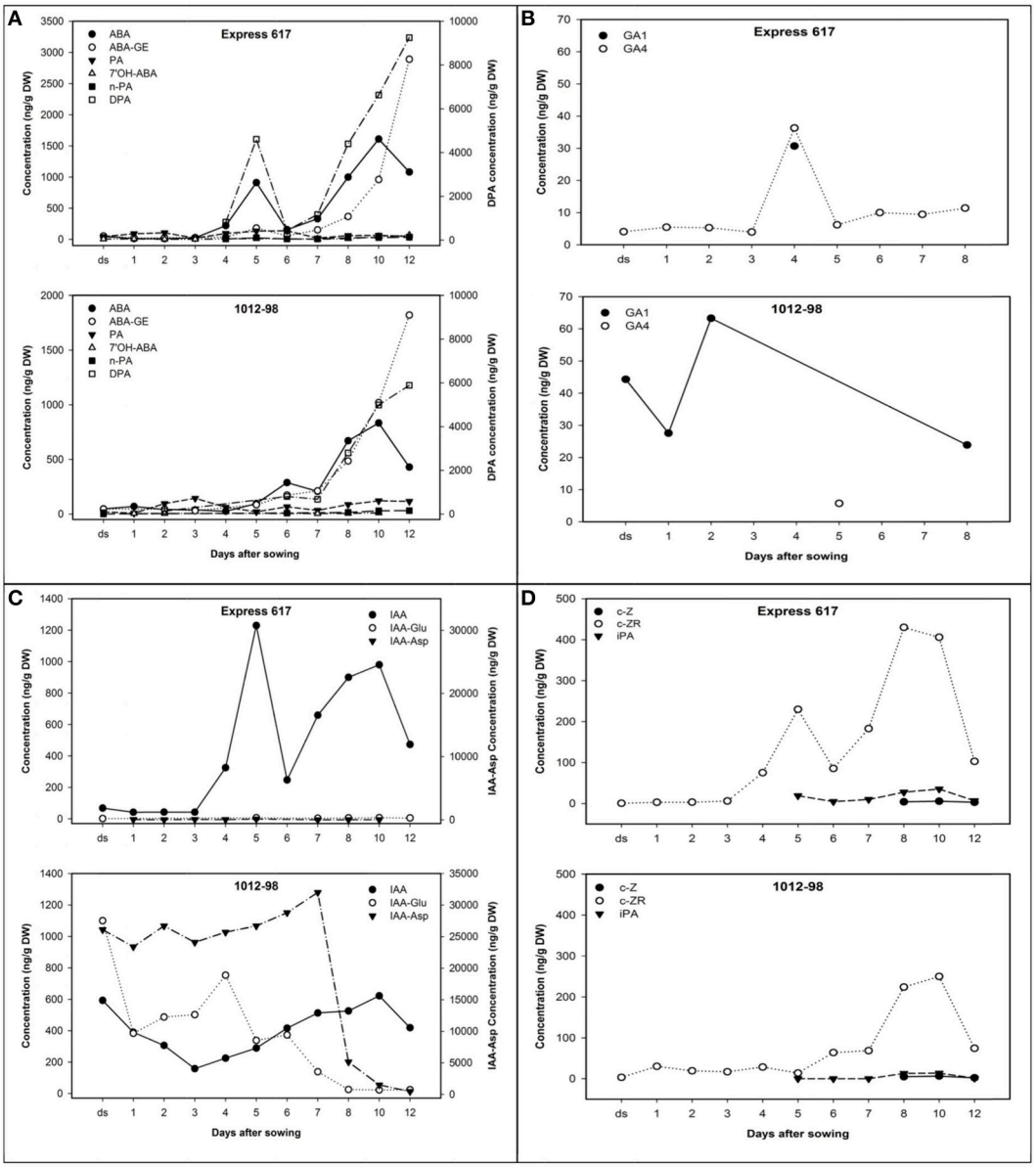

FIGURE 4 | Hormone profiles from dried seeds (ds) and germinating seedlings of Express 617 and 1012-98 after imbibition under 25°C, 55% RH and 16-h light. (A) ABA and ABA metabolites, **(B)** GA metabolites, **(C)** auxin metabolites, and **(D)** cytokinins. The right-hand axes in **(A)** show the DPA concentrations, while the right-hand axes in **(C)** show concentrations of IAA-Aspartate (Asp). The first radicle protrusion appeared ~24 h after imbibition.

domain (DBD) from the C-genome donor. We hypothesize that this substantial increase in copy number, from two to five *Bna.ARF10* copies with C-genome DBDs, strengthens repression of auxin response genes in 1012-98, leading to phenotypes that closely mimic the mi160 resistance described in *Arabidopsis* by (Liu et al., 2007).

Comparison of *Bna.ARF10* nucleotide sequences from Express 617 and 1012-98 to the *Brassica ARF10* consensus sequence (**Table 3**) revealed a total of eight SNPs in 1012-98 alleles from *BnaC.ARF10* loci, seven of which appear within the highly conserved DNA binding domains. Three SNPs in the AUX/IAA family domains of *BnaC.ARF10.b* and *BnaC.ARF10*.d do not result in amino acid substitutions, while a fourth is a putative non-functional A > G substitution in intron 4. In contrast, two SNPs in 1012-98 result in amino acid substitutions, one in the B3 DNA-binding domain (W162 to R162) and one in the ARF domain (S333 to L333), respectively. Express 617 exhibited no SNPs of putative functional relevance: An A > G substitution was observed in intron 4, a synonymous T > C substitution at C593 in the AUX/IAA family domain of *BnaC.ARF10.b* and three SNPs outside the functional domains of the A-genome allele *BnaA.ARF10.a*.

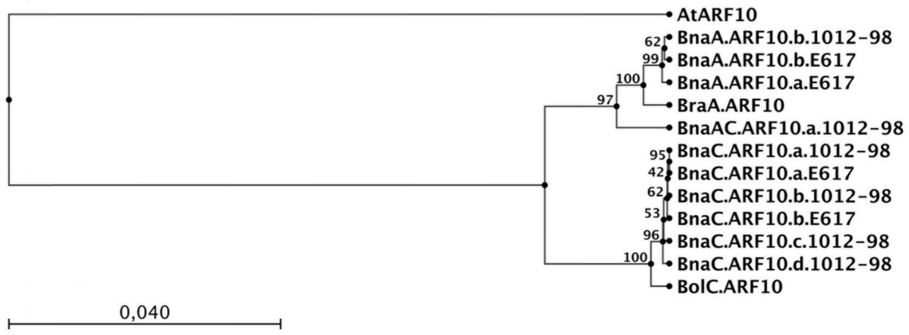

FIGURE 5 | UPGMA tree showing the relationship between genomic coding DNA sequences of *ARF10* orthologs from *A. thaliana* (At), *B. napus* (Bna) Express 617 (E617) and 1012-98, *B. rapa* (Bra) and *B. oleracea* (Bol). The capital letter following the 3-letter *Brassica* species code represents the A or C genome origin of the sequence. The tree was constructed using CLC Sequence Viewer 6.4. Arm lengths represent average genetic distances between clusters. Bootstrap values from 100 repetitions of the UPGMA cluster are shown on the nodes.

TABLE 2 | Predicted parameters of *ARF10* products from *A. thaliana* (*At*), *B. oleracea* (*Bol*), *B. rapa* (*Bra*), *B. napus* (*Bna*) Express 617 (E617), and *B. napus* 1012-98, respectively.

Locus	Origin of allele	Accession number	Chromosome/ Identity (%)	*B. napus* genes	Amino acids	MW (kDa)	pI	Conserved domains (NCBI CDD)		
								B3 DNA binding domain	Auxin response factor	AUX/IAA family
AtARF10	-	At2g28350	2		693	76.7	7.71	F_{115}-A_{216}	G_{284}-Q_{360}	S_{574}-I_{656}
BraA.ARF10	-	JX494287	A07/99	BnaA07g13830D	705	77.9	7.33	F_{112}-A_{213}	G_{288}-Q_{364}	G_{586}-I_{668}
BolC.ARF10	-	JX494286	C04/99.5	BnaC04g15900D	703	77.9	7.04	F_{112}-A_{213}	G_{287}-Q_{363}	G_{584}-I_{666}
BnaA.ARF10.a	E617	JX456096	A07/99	BnaA07g13830D	705	77.9	8.00	F_{112}-A_{213}	G_{288}-Q_{364}	G_{586}-I_{668}
BnaA.ARF10.b	E617	JX456097	A07/99.3	BnaA07g13830D	706	78.0	8.00	F_{112}-A_{213}	G_{288}-Q_{364}	G_{587}-I_{669}
	1012-98	JX456098	A07/99.1	BnaA07g13830D	706	78.0	8.00	F_{112}-A_{213}	G_{288}-Q_{364}	G_{587}-I_{669}
BnaAC.ARF10.a	1012-98	JX456089	C04/98.7	BnaC04g15900D	704	77.8	7.02	F_{112}-A_{213}	G_{288}-Q_{364}	G_{585}-I_{667}
BnaC.ARF10.a	E617	JX456090	C04/100	BnaC04g15900D	703	77.8	6.59	F_{112}-A_{213}	G_{287}-Q_{363}	G_{584}-I_{666}
	1012-98	JX456092	C04/100	BnaC04g15900D	703	77.8	6.59	F_{112}-A_{213}	G_{287}-Q_{363}	G_{584}-I_{666}
BnaC.ARF10.b	E617	JX456091	C04/100	BnaC04g15900D	703	77.8	6.59	F_{112}-A_{213}	G_{287}-Q_{363}	G_{584}-I_{666}
	1012-98	JX456093	C04/99.9	BnaC04g15900D	703	77.8	6.59	F_{112}-A_{213}	G_{287}-Q_{363}	G_{584}-I_{666}
BnaC.ARF10.c	1012-98	JX456094	C04/100	BnaC04g15900D	703	77.8	6.59	F_{112}-A_{213}	G_{287}-Q_{363}	G_{584}-I_{666}
BnaC.ARF10.d	1012-98	JX456095	C04/99.9	BnaC04g15900D	703	77.8	6.79	F_{112}-A_{213}	G_{287}-Q_{363}	G_{584}-I_{666}

The locus nomenclature is explained in the legend to ***Table 3****.*

High Accumulation of Active IAA and its Metabolites in 1012-98 Caused Minor and Major Up-Regulations of *Bna.ARF10* and *Bna.GH3.5*, Respectively

To learn about the differential expression of *Bna.ARF10*, *Bna.GH3.5,* and *Bna.GH3.6* between Express 617 and 1012-98 in response to auxin signaling, quantitative PCR was used with the specific primers BnaA+C.*ARF10* forward/reverse to amplify both A- and C-genome of *Bna.ARF10*, *Bna.GH3.5,* and *Bna.GH3.6* genes from 0 to 12 DAS. Both *Bna.ARF10* and *Bna.GH3.5* highly expressed in dried seed, declined rapidly within 24 h after imbibition and remained close to these levels in both genotypes to the end of this study (**Figures 6A,B**). Expression level of *Bna.ARF10* in 2 and 12 DAS was ∼2.7-fold higher in 1012-98 than in Express 617. Coupling of up-regulation *Bna.ARF10*, *Bna.GH3.5* transcripts in 1012-98 was 2.5-, 11-, and 3.5-fold higher on 2, 3, and 4 DAS as compared with those in Express 617, respectively. These upregulations of *Bna.GH3.5* corresponded to the reduction of active IAA and the increase of IAA-asp between 1 and 4 DAS. In contrast, *Bna.GH3.6* expression in ripe seeds and 12 DAS accumulated 3.3- and 8.1-fold higher in Express 617 than in 1012-98, respectively while non-significant difference in transcription levels was observed between two genotypes (**Figure 6C**).

DISCUSSION

Seed germination is controlled by genetics and the crosstalk of hormonal components mainly ABA and GA (Holdsworth et al., 2008). Recent studies have provided the evidence of auxin involvement in both germination completion and seedling establishment by increasing seed sensitivity to ABA (Ogawa et al.,

TABLE 3 | Detected SNPs (underlined) and corresponding amino acids (bold) in *Bna.ARF10* alleles from Express 617 and 1012-98 compared to the *Brassica ARF10* consensus sequence (see also Supplementary Figure S1).

Locus/allele[a]	Position in genomic/coding sequence	Codon/amino acid substitutions	Predicted functional relevance
BnaA.ARF10.a.E617	324/324	AAA/**K_{108}** → AAG/**K_{108}**	
BnaC.ARF10.d.1012-98	484/484	TGG/**W_{162}** → CGG/**R_{162}**	B3 DNA binding domain
BnaC.ARF10.b.1012-98	861/861	GGA/**G_{287}** → GGG/**G_{287}**	Auxin response factor
BnaC.ARF10.c.1012-98	930/930	GTG/**V_{310}** → GTA/**V_{310}**	Auxin response factor
BnaC.ARF10.d.1012-98	1001/1001	TCG/**S_{333}** → TTG/**L_{333}**	Auxin response factor
BnaA.ARF10.a.E617	1213/1136	GTT/**V_{379}** → GCT/**A_{379}**	
BnaA.ARF10.a.E617	1569/1492	TCC/**S_{498}** → CCC/**P_{498}**	
BnaC.ARF10.b.E617	1863/1779	TGT/**C_{593}** → TGC/**C_{593}**	AUX/IAA family
BnaC.ARF10.b.1012-98	1869/1785	GTT/**V_{595}** → GTC/**V_{596}**	AUX/IAA family
BnaC.ARF10.b.1012-98	1890/1806	GTT/**V_{602}** → GTA/**V_{602}**	AUX/IAA family
BnaC.ARF10.d.1012-98	2019/1935	GTT/**V_{645}** → GTC/**V_{645}**	AUX/IAA family
BnaC.ARF10.b.E617	2067 (intron 4)	A → G	
BnaC.ARF10.a.1012-98	2231 (intron 4)	A → G	

[a] *Locus/allele nomenclature follows the convention for Brassica spp. described by Ostergaard and King (2008): [Species—3 letter code; genome—A or C; gene—ARF10]. [locus—a, b, c or d]. [allele—in this case E617 or 1012-98].*

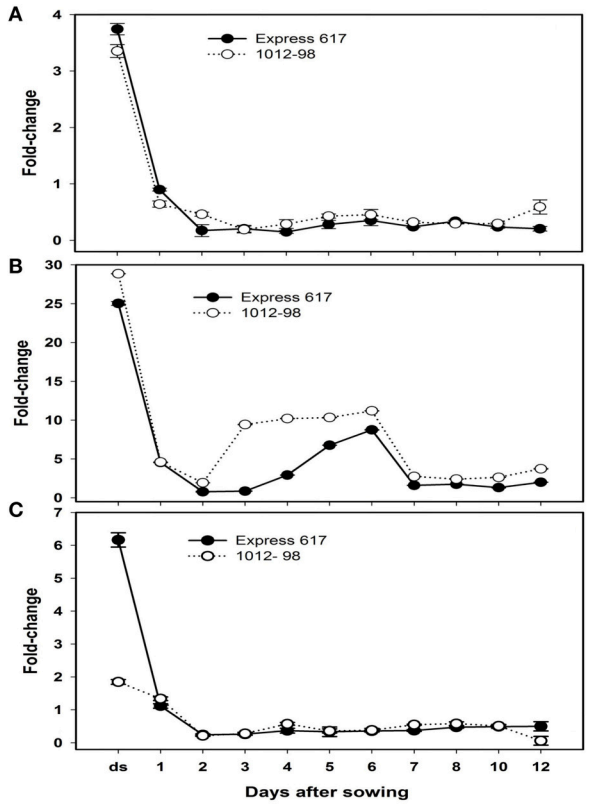

FIGURE 6 | Quantitative real-time PCR analysis of (A) *Bna.ARF10*, (B) *Bna.GH3.5*,and (C) *Bna.GH3.6* expression in Express 617 and 1012-98. Results were normalized against the expression of ACT2. Error bars denote SEMs.

2003; Liu et al., 2007). the present study, seeds of the mutant *B. napus* line 1012-98 produced higher content of ABA and IAA as late as 3 DAS, the critical timepoint for successful germination and emergence into post-germinative growth (Thakur and Sharma, 2016). Furthermore, 1012-98 and its hormone-defect offspring showed severe ABA-hypersensitivity phenotypes, and there was no evidence in 1012-98 during the first few days after imbibition for the necessary catabolism of ABA to less active PA and its inactive form, DPA. We found that the decline of ABA after imbibition in normal *B. napus* was accompanied by an increase in the level of less active phaseic acid (PA) conjugates, coinciding with the transition from germination to post-germinative growth. This suggests that 8′-OH ABA was formed first and then cyclized to form PA. This kind of ABA catabolism has been shown to be important in regulating germination potential (Kushiro et al., 2004; Okamoto et al., 2006). The high accumulation of free ABA in 1012-98 at 3 DAS was probably derived from *de novo* ABA biosynthesis, which is believed to play a key role in dormancy maintenance or delay of germination (Kushiro et al., 2004). In contrast, strong *de novo* synthesis of ABA in Express 617 occurred only post-germination.

ABA catabolism is primarily driven by 8′-hydroxylation encoded by the CYP707A gene family. The product of hydroxylation, 8′-hydroxy ABA, is converted to PA and subsequently inactivated as DPA (Nambara and Marion-Poll, 2005). The ABA decline at 5 DAS in *B. napus* Express 617 was not accompanied by an increase in concentration of its metabolites and/or conjugates, besides a very small accumulation of PA. On the other hand, the decrease in ABA content at 10 DAS coincided with 3-fold increase of ABA-GE. This suggests two independent ABA catabolic pathways, one fluxed through PA in the germinative phase and the other involved independently in post-germinative development.

Extreme Auxin Excess Inhibits Germination in *B. napus* 1012-98

Inhibition of germination by IAA was well-documented in lettuce over 50 years ago (Khan and Tolbert, 1966). In fact, auxin inhibits seed germination by enhancing ABA action driven by transcription factor ABSCISIC ACID INSENSITIVE 3 (*ABI3*), thereby adding secondary protective level of control in the

regulation of seed dormancy and germination. Auxin action in *Arabidopsis* seed germination requires the ABA signaling and the roles of auxin-ABA in seed dormancy are interdependent (Liu et al., 2013). The concentration of IAA and its metabolites in 1012-98 was remarkably high in dried seed and as late as 3 DAS on which success or failure of seed germination were determined. Additionally, IAA-asp remained at extreme levels until the transition into post-germination development. The high accumulation of endogenous auxin and ABA (over 4-fold higher) within 48 h after imbibition, these conditions probably created an ABA-hypersensitive phenotype in yellow-seeded 1012-98 line, thereby the seeds were failed to protrude radicles under favorable conditions. Additionally, successfully germinated seedlings of 1012-98 expressed significantly elongated hypocotyls under post-germinative auxin action (**Figure 2**). In *Arabidopsis*, both auxin-overproducing transgenic line *iaaM-OX* and exogenous auxin applied wild-type seeds did not germinate up to 30 DAS in germination assay due to inhibitory effect of ABA in a dose-dependent manner (Liu et al., 2007, 2013). Taken together, these results explains the extremely poor germination and seedling establishment observed in the *B. napus* mutant 1012-98, in which the characteristic IAA pattern was completely disturbed.

1012-98 Phenotypes are Consistent with Endogenous IAA Overdose

Auxin acts upstream of major regulator *ARF10*. Additionally, repression of *ARF10* by MIR160 is critical for seed germination and post-germination stages (Liu et al., 2007). The mutant phenotypes we observed in germinated 1012-98 seedlings and its hormone-defective offspring (**Figure 3**) correspond strikingly to those of *Arabidopsis* auxin overproduction or *arf10* mutants, which show similar developmental defects including elongated hypocotyl, small, and epinastic/deformed cotyledons, tricotyledon, reduced number of leaves, constricted apical end of hypocotyl, and curled stems (Boerjan et al., 1995; Romano et al., 1995; Liu et al., 2007). Disturbed apical hook development, causing the "ostrich" phenotypes we observed in 1012-98 and its offspring, has been most extensively connected with auxin. A common cause is auxin inhibition of cell expansion at the inner side GA promotion of cell division and expansion at the outer side of the hook (Vandenbussche et al., 2010; Mazzella et al., 2014). Stem curling and leaf malformation are also among the first tri-phasic responses of plants after application of the synthetic auxin herbicide 2, 4-D, followed by stunted growth, chlorosis, wilting, necrosis, and ultimately death. Excessive concentrations of endogenous IAA cause an imbalance in auxin homeostasis and in interactions with ABA and ethylene. Notably, the deformative and growth-inhibiting effects caused by application of synthetic auxin are also observed in IAA-overproducing transgenic plants (Grossmann, 2010). Irregular distribution of auxin leading to disturbed cell elongation is the most likely explanation for the hook-like seedlings, stem curvature, twisted petioles and other developmental phenotypes observed in Express 617 × 1012-98 DH lines despite successful germination.

The Hypersensitive-to-ABA Mutant 1012-98 Lacks Sufficient GA for Successful Germination

The balance of endogenous ABA and GA levels serves an important role in controlling seed germination (Finch-Savage and Leubner-Metzger, 2006). GA_1 levels in 1012-98 were unusually high in comparison with Express 617. In contrast the levels of GA_4, which in *Arabidopsis* seeds is around 10 times more active than GA_1 (Yang et al., 1995), were not detected until 5 DAS in the *B. napus* mutant. In the context of excess ABA content and extremely high auxin accumulation, the mutant 1012-98 genotype did not produce sufficient amount of active GAs to deactivate the ABA inhibition and complete germination. In fact, germination of 7B-1 ABA-hypersensitive mutant tomato line was able to restore with application of exogenous GA_3 (Fellner et al., 2001). The hormone profile of 1012-98 suggests that elevated levels of GA would be required to successfully promote germination in the presence of excessive ABA. In Express 617, the accumulation of free auxin and ZR at 5 and 10 DAS, respectively, appeared sufficient to successfully induce the transition from germination to post-germinative growth.

Molecular Links of Transcriptional Repressor *Bna.ARF10* to Auxin-Response Genes *Bna.GH3.5*, *Bna.GH3.6*, and High Auxin Accumulations

ARF10 is important for several aspects of plant growth and development, at least some of which appear to be dependent on GH3 gene family (Mallory et al., 2005; Guilfoyle and Hagen, 2007). Recently the crosstalk between auxin and ABA signaling in controlling *Arabidopsis* seed dormancy and germination have been proven and ABA function is largely dependent on the TIR1/AFB-AUX/IAA-ARF-mediated auxin signaling pathway. In fact, auxin acts upstream of the major regulator of seed dormancy, *ABI3*, by recruiting the *ARF10* and *ARF16* to control the *ABI3* expression during seed germination (Liu et al., 2007, 2013). In *Arabidopsis*, both *ARF10* and *ARF17* are classified as transcriptional repressors with an enrichment in serine (S), proline (P), leucine (L), and either glycine (G; ARF10), or theronine (T; ARF17) residues (Tiwari et al., 2003). The deduced protein sequences in *Brassica* in this study confirmed SPL-rich regions located just before and after the conserved domains. On the background of high accumulation of ABA and auxin as late as 3 DAS in yellow-seeded 1012-98 genotype, the copy number amplification of *Bna.ARF10* in 1012-98 leading to minor transcriptional increase presumably added another restriction on germinative process through the inhibitory action of *ABI3*. The upregulation of *Bna.GH3.5* between 2 and 6 DAS in ABA-hypersensitive 1012-98 corresponded to the second peak (4 DAS) of IAA-asp and the reduction of active IAA (3 DAS). In fact, the *GH3*-like gene family plays an essential role in maintaining optimal levels of endogenous IAA through the amino acid, sugar, and peptide-linked conjugating pathways in plant cells (Woodward and Bartel, 2005). Besides the previously described copy number variation for *BnaC.ARF10*, the major functionally relevant *Bna.ARF10* sequence divergence associated with the

developmental disruption in 1012-98 appears to be present in *BnaC.ARF10*.d. Mutations in the B3 D NA-binding domains might enhance binding activities of *BnaC.ARF10*.d to *AuxRE* and to *Aux/IAA* proteins in 1012-98. Taken together, this can be expected to lead to the germinative hyper-repressor in 1012-98, which in turn would cause the very poor germination and disrupted transition from germination to seedling development. It can be proposed that the crosstalk of auxin-ABA might have coevolved to synergically control seed dormancy to survive unfavorable environment. These mutations appear to have arisen independently during the *de novo* allopolyploidization of 1012-98 from an interspecific cross between *B. rapa* and *B. oleracea*.

AUTHOR CONTRIBUTIONS

TN designed, carried out research and wrote the manuscript. SA analyzed seedling metabolomics. CO designed the quantitative RT-PCR experiments. RS and WF participated in study design and revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.00322

Supplementary Figure S1 | Multi-alignment of *ARF10* proteins from *Arabidopsis thaliana* (At), *Brassica rapa* (Bra), *B. oleracea* (Bol), and the *B. napus* (Bna) lines Express 617 (E617) and 1012-98. The background shading represents the degree of similarity, with 100% conserved amino acids shown by black shading, strongly conserved amino acids in gray tones and variable positions in white. Locus/allele nomenclature follows the convention for *Brassica* spp. described by Østergaard and King (2008): [Species—3 letter code; genome—A or C]. [gene—*ARF10*; locus]. [allele—in this case E617 or 1012-98].

Supplementary Table S1 | Internal standards used for hormone quantification by HPLC-ESI-MS/MS.

Supplementary Table S2 | See and seedling phenotype data for 1012-98, Express 617 and 166 doubled haploid (DH) lines derived from their F_1 hybrid.

Supplementary Table S3 | Pairwise identities (%) in genomic/coding regions (above diagonal) and deduced protein sequences (below diagonal) from *ARF10* orthologs in *A. thaliana* (At), *B. rapa* (Bra), *B. oleracea* (Bol) natural *B. napus* (*Bna*) line Express 617 (E617) and resynthesized *B. napus* line 1012-98, respectively. Locus/allele nomenclature follows the convention for *Brassica* spp. described by Østergaard and King (2008): [Species—3 letter code; genome—A or C; gene—*ARF10*]. [locus]. [allele—in this case E617 or 1012–98].

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3 Quantitative trait locus analysis of seed germination, seedling vigour and seedling-regulated hormones in *Brassica napus*

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Quantitative trait locus analysis of seed germination, seedling vigour and seedling-regulated hormones in *Brassica napus*

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Abstract

Good germination and seedling vigour are major breeding targets in winter oilseed rape (*Brassica napus*), because seedling vigour and prewinter crop establishment are closely associated with postwinter growth and yield. Here, we identified quantitative trait loci (QTL) related to germination, seedling vigour and seedling-regulated hormones in a doubled haploid (DH) mapping population from a cross between winter oilseed rape parents with high vigour (Express 617) and low vigour (1012-98). By phenotyping in a climate-controlled glasshouse, we identified a total of 13 QTL on nine chromosomes for germination and seedling-related traits at 7 and 14 days after sowing (DAS), explaining up to 11.2% of the phenotypic variation for seedling vigour. Forty-seven metabolic QTL on 15 chromosomes were identified for auxin, abscisic acid (ABA) and dihydrophaseic acid (DPA) at 5 and 12 DAS, explaining up to 49.4% of phenotypic variation in seedling hormone composition. Multitrait QTL hot spots contribute to our understanding of the genetics and metabolomics of germination and seeding vigour in *B. napus*, and represent potential targets to breed high-vigour cultivars.

KEYWORDS

Brassica napus, germination, hormones, oilseed rape, quantitative trait loci mapping, seedling vigour

1 | INTRODUCTION

Oilseed rape (*Brassica napus* L., 2n = 38, genome AACC), a recent allopolyploid species derived from interspecific hybridizations between *Brassica rapa* L. (2n = 20, genome AA) and *B. oleracea* L. (2n = 18, genome CC), is a globally important oilseed. As in other crops (Redoña & Mackill, 1995), good seed quality and seedling vigour are important traits in *Brassica* (Bettey, Finch-Savage, King, & Lynn, 2000), as seedling vigour is closely associated with growth and yield (Ellis, 1992). In Europe, autumn-sown winter oilseed rape depends on a strong vigour and prewinter development to suppress weed competition and establish a good basis for winter survival.

The protrusion of the radicle from the seed is termed seed germination, while seedling vigour refers to the ability of a seed lot to establish seedlings after germination under a wide range of environmental conditions (Finch-Savage, Clay, Lynn, & Morris, 2010). Seed germination and seedling vigour are very complex traits (Bettey et al., 2000) determined by the interaction of hormonal (Holdsworth, Bentsink, & Soppe, 2008), genetic and numerous environmental components including soil factors, water availability and temperature (Bentsink & Koornneef, 2009). Germination is preceded by a decrease in abscisic acid (ABA) levels resulting from the activation of ABA hydroxylation predominantly at C-8′ position to 8-hydroxy ABA, phaseic acid (PA) and dihydrophaseic acid (DPA) (Nambara & Marion-Poll, 2005). Auxin is also implicated in regulation of seed dormancy and germination. For example, exogenous application of auxin enhanced the inhibition of seed germination by ABA in *Arabidopsis* via the transcription factor *ABSCISIC ACID INSENSITIVE 3* (*ABI3*) (Liu et al., 2013) and was also reported to delay seed germination in wheat (Ramaih, Guedira, & Paulsen, 2003). Several studies

reported quantitative trait loci (QTL) for seed germination and seedling vigour traits in *Arabidopsis* (Clerkx et al., 2004), rice (Xie et al., 2014), *B. rapa* (Basnet et al., 2015), *B. oleracea* (Bettey et al., 2000; Morris, Barker, Walley, Lynn, & Finch-Savage, 2016) and *B. napus* (Hatzig et al., 2015). However, none of these studies linked seed germination or seedling vigour to regulatory roles of phytohormones in controlling germination and seedling establishment. Identification of QTL controlling germination and seedling vigour via hormone regulation may help plant breeders select and combine positive early vigour traits, despite the strong environmental sensitivity and moderate-to-high heritability of germination and seedling vigour.

In this study, we identified QTL for germination and seedling-related traits at 7 and 14 days after sowing (DAS) in a doubled haploid (DH) mapping population from winter oilseed rape parents with high (Express 617) and low vigour (1012-98). By comparing these QTL to loci controlling auxin, ABA and DPA at 5 and 12 DAS provide mechanistic links that help explain the genetic control of early vigour. To our knowledge, this study is the first to report the colocalization of developmental and metabolic QTL relating to seed germination and seedling vigour in *B. napus*, improving our understanding of the genetics and metabolomics of these important traits and identifying potential targets for breeding of high-vigour cultivars.

2 | MATERIALS AND METHODS

2.1 | 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 canola seed quality (00), excellent germination and high seedling vigour. The *B. napus* line 1012-98 is a progeny from an interspecific hybrid derived by embryo rescue-assisted resynthesis from an interspecific cross between *B. rapa* and *B. oleracea* (Badani et al., 2006). Due to the high relatedness of the *Brassica* A and C genomes, the chromosomes of resynthesized *B. napus* frequently contain homeologous chromosome exchanges (Udall, Quijada, & Osborn, 2005) that cause widespread duplication and/or deletion of homeologous gene copies in the A or C subgenomes (Samans, Chalhoub, & Snowdon, 2017). 1012-98 shows a yellow-seeded phenotype, atypical for *B. napus*. This phenotype is primarily attributable to a major quantitative trait locus (QTL) on chromosome A09 that influences testa thickness and flavonoid pigmentation (Snowdon et al., 2010) and is thought to be caused by a non-reciprocal homeologous chromosome exchange in the parent 1012-98 leading to gene loss of function (Stein et al., 2017).

A population of 166 homozygous doubled haploid (YE2-DH) lines was generated by microspore culture (Weber, Ünker, & Friedt, 2005) from a single F_1 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 in the same field environment at Rauischholzhausen, Germany. 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, Snowdon, and Friedt (2009).

2.2 | Germination and seedling development

Germination rate was assessed in vitro according to the recommendations of the International Seed Testing Association (ISTA, 2010). An alpha-lattice design with three randomized replicates was applied in each case using 21 blocks comprising eight DH lines. 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-hr light/8-hr darkness.

Seedling development was assessed in three repetitions of 27 seeds per genotype sown in Fruhstorfer soil (Hawita Gruppe GmbH, Vechta, Germany) grown under controlled conditions in a climate-controlled glasshouse at a constant temperature of 25°C with 55% relative humidity and a photoperiod of 16-hr light/8-hr darkness. Total seedling (root and shoot) biomass and shoot length (SL) were measured at 7 and 14 DAS. Shoot length was measured from soil surface to shoot tip. Mean trait values were calculated from all successfully germinated seeds per genotype.

2.3 | Sampling for hormone analysis

Based on the hormone profiling of the parental lines Express 617 and 1012-98, 5 and 12 DAS were chosen because these time points showed the best differentiation in hormonal concentration especially for IAA, ABA and DPA (Nguyen, Obermeier, Friedt, Abrams, & Snowdon, 2016). For comparison of hormone metabolite profiles in DH lines with the best (n = 30) and the worst (n = 29) germination ability, ten identical Jacobsen germination pots per genotype were prepared for sampling at 5–12 DAS. The experiment was conducted in three replications. Subsequently, a total of 15 (ca. 50 mg) and 50 (ca. 100 mg) seeds/seedlings per genotype and replication were pooled into 15-ml Falcon tubes, immediately immersed in liquid nitrogen and lyophilized for 24 hr for hormone analysis.

2.4 | Internal standards for hormone quantification

Calibration curves and quality controls for DPA, abscisic acid glucose ester (ABA-GE), PA, 7′-hydroxy ABA (7′-OH-ABA), neo-phaseic acid (neoPA) and indole-3-acetic acid glutamate (IAA-glu) were created by the Plant Biotechnology Institute, National Research Council of Canada (PBI-NRC, Saskatoon, SK, Canada). Details on all internal standards used for quantification of hormone metabolites are provided in Table S1.

2.5 | Extraction and purification of hormone metabolites

Lyophilized plant tissue was homogenized in a bead mill for 2–6 min. A 100 μl aliquot containing all the internal standards, each at a concentration of 0.2 pg/μl, was added to around 50 mg of homogenized tissue. After adding 3 ml of isopropanol:water:glacial acetic acid (80:19:1, v/v), the samples were agitated for 24 hr at 4°C. Samples were then centrifuged and the supernatant was isolated and dried on a Buchi Syncore Polyvap (Büchi, Flawil, Switzerland). Samples were reconstituted in 100 μl acidified methanol, adjusted to 1 ml with acidified water and then partitioned against 2 ml hexane. After 30 min, the hexane layer was removed and the hexane partitioning was repeated. The aqueous layer was then isolated and dried. Dry samples were reconstituted in 800 μl acidified methanol and adjusted to 1 ml with acidified water. The reconstituted samples were passed through equilibrated Sep-Pak C18 cartridges (Waters, Mississauga, ON, Canada), the eluate being dried on a centrifugal evaporator. An internal standard blank was prepared with 100 μl mixture of the deuterated internal standards. A QC standard was prepared by adding 20 ng of each analyte to 100 μl of the internal standard. Finally, all samples, blanks and QCs were reconstituted in a solution of 40% methanol (v/v), containing 0.5% acetic acid and 100 pg μl of each of the recovery standards.

2.6 | Hormone quantification by HPLC-ESI-MS/MS

The procedure for quantification of multiple hormone metabolites, including auxins (IAA, IAA-asp and IAA-glu), abscisic acid and metabolites (ABA, PA, DPA, 7′ -OH-ABA, neoPA and ABA-GE), and CKs (2iP, iPA, Z, ZR, dhZ, dhZR and Z-O-Glu) has been described in detail by Chiwocha et al. (2003). Samples were injected onto a Genesis C18 HPLC column (100 × 2.1 mm, 4-μm, Chromatographic Specialties, Brockville, ON, Canada) and separated by a gradient elution of water against an increasing percentage of acetonitrile that contained 0.04% acetic acid. Calibration curves were generated from the MRM signals obtained from standard solutions based on the ratio of the chromatographic peak area for each analyte to that of the corresponding internal standard, as described by Ross et al. (2004). QC samples, internal standard blanks and solvent blanks were prepared and analysed along with each batch of tissue samples. Mean minimum limits of quantitation for each analyte were 8 ng/g dry weight (DW) for Z, dhZ, Z-O-Glu, 2iP, iPA and ABA; 60 ng/g DW for ZR, dhZR, IAA-asp, IAA-glu, IAA and 7′-OH-ABA; 118 ng/g DW for DPA; 78 ng/g DW for PA; 56 ng/g DW for ABA-GE; and 30 ng/g DW for neoPA.

2.7 | Statistical analysis

Analysis of variance (ANOVA) was performed using the GLM procedure of Statgraphics Centurion XVI (StatPoint, Inc., Warrenton, VA, USA). Broad-sense heritability (h^2) was calculated as:

$$h^2 = \sigma_g^2/(\sigma_g^2 + \sigma_e^2),$$

where σ_g^2, σ_e^2 and $\sigma_p^2 = \sigma_g^2 + \sigma_e^2$ are estimates of the variances of genotype, error and phenotype, respectively (Kelly & Bliss, 1975). Pearson's correlation coefficients between traits of interest were calculated using the Multiple-Variable Analysis Procedure of Statgraphics Centurion XVI (StatPoint, Inc., Warrenton, VA, USA). Histograms showing segregation patterns were performed for each trait using SigmaPlot version 12.0 for Windows (Systat Software, Inc., San Jose, CA, USA).

2.8 | QTL analysis

Genomic DNA was extracted from leaf samples of Express 617, 1012-98 and 166 YE2-DH lines using standard procedure (Doyle, 1991). The genetic maps for the YE2-DH populations contained 262 AFLP and 85 SSR markers over a total map length of 1,720.8 cM (Badani et al., 2006). Nineteen linkage groups were assigned according to the A-genome chromosomes A01-A10 (derived from *B. rapa*) and the C-genome chromosomes C01-C09 (derived from *B. oleracea*), according to the nomenclature suggested by the Multinational Brassica Genome Project (see www.brassica.info) and known genome positions of SSR markers. Linkage was calculated with a maximum recombination frequency of 0.4 and a logarithm of odds (LOD) score of >1.0. Significant QTL were localized by composite interval mapping using the software PLABQTL 1.2 (Utz & Melchinger, 1996), with LOD thresholds for QTL detection set to $p = .05$ and determined using permutation tests with 1,000 permutations (Churchill & Doerge, 1994). The QTL analysis was performed for each trait separately. QTL were defined as colocalized if peak markers were closer to each other than the optimal interval distance (Jacobs, Womack, Chen, Gharbi, & Elmer, 2017). Colocalized QTL were coded as "*Co-QTLk.m*" where *k* indicates the linkage group and *m* the QTL number.

3 | RESULTS

3.1 | Analysis of genetic variance

Variance components and the estimated broad-sense heritability for germination and seedling-related traits in YE2-DH population are given in Table 1. Moderate-to-high h^2 was observed for germination rate (GR), shoot length at 7 and 14 DAS (SL7 and SL14) and shoot fresh weight at 7 DAS (SFW7). In contrast, SFW at 14 DAS (SFW14) showed relatively low heritability.

3.2 | Correlation analysis

The phenotypic values, hormone content and correlation coefficients of YE2-DH lines are provided in Tables 2 and 3. Significant positive relationships between germination performance and SFW at 7 and 14 DAS were detected ($p < .001$). Similarly, strongly significant correlation between 7 and 14 DAS for SL and SFW was found

TABLE 1 Estimates of genetic variance (σ_g^2) and mean effective errors (σ_e^2) along with heritability (h^2) for traits measured in the population YE2-DH (n = 166) from a cross between Express 617 and 1012-98 grown in a growth chamber and climate-controlled glasshouse at a constant temperature of 25°C with 55% relative humidity and a photoperiod of 16-hr light/8-hr darkness, respectively

Trait	σ_g^2	σ_e^2	σ_p^2	h^2
GR	142.09	187.09	329.18	0.43
SL7	0.35	0.38	0.74	0.48
SFW7	0.00036	0.00032	0.00068	0.53
SL14	0.92	0.53	1.45	0.64
SFW14	0.006	0.021	0.027	0.24

GR indicates germination rate, SL7 and SFW7 indicate shoot length and shoot fresh weight at 7 days after sowing (DAS), and SL14 and SFW14 indicate shoot length and shoot fresh weight at 14 DAS.

(p < .001). In contrast, germination ability negatively correlated with SL at 14 DAS (p < .05). Significant negative correlations between SFW at 14 DAS and SL at both 7 and 14 DAS were also detected (p < .05).

The contents of IAA, ABA and DPA at 5 DAS correlated statistically with their corresponding content at 12 DAS (p < .05, p < .01 and p < .001, respectively). Significant correlations between IAA and ABA, IAA and DPA were detected at the two time points, 5 (p < .01) and 12 DAS (p < .05). While concentration of ABA and its metabolite DPA significantly strongly correlated at 5 and 12 DAS (p < .001), two time point relationships between ABA and DPA were also found at a lower level of significance (p < .05 and p < .01).

3.3 | Variation in GR, SL and SFW at 7 and 14 DAS

Five seed quality and seedling-related traits of the parental lines and the YE2-DH population are presented in Figure 1. The histograms showing segregation patterns were obtained for each trait using SigmaPlot version 12.0 for Windows. Results of statistical analyses, including mean value, range, standard deviation, skewness and kurtosis, are also presented in Table 1. Whereas Express 617 showed a normal GR of around 90%, the GR was severely depressed in 1012-98 to just 27.3% (***p < .001 in Table S2). Germination of YE2-DH lines with an average of 70.53 ± 1.18 (%) reflected the interparent variation and skewed towards 1012-98, indicating additive effects particularly from this parent. In contrast, the parental line 1012-98 had significantly higher SL at 7 DAS than Express 617 (*p < .05 in Table S2). Shoot length, SFW at 7 and 14 DAS in YE2-DH lines with mean values of 4.45 ± 0.05, 6.43 ± 0.08, 0.13 ± 0.00 and 0.52 ± 0.01, respectively, exhibited bidirectional transgressive variation, suggesting alleles with additive effects or complementation effect for these traits were distributed among the parents (Figure 1). From the skewness data in Table 2, it was determined that in all cases the extent of transgressive variation was towards higher values. Skewness and kurtosis values were <1.96 indicating the segregation pattern of most traits generally fitted a normal or near-normal distribution model (Kim, 2013) and controlling by multiple genes.

3.4 | Variation in ABA, IAA and DPA at 5 and 12 DAS

The ABA, IAA and DPA profiles and their corresponding histograms for 5 and 12 DAS of the 59 extreme DH lines are presented in Table 2 and Figure 2. Express 617 with normal seedling development had high to very high concentrations of ABA, IAA and DPA as compared to the seedling defective line 1012-98. In 59 extreme DH lines, the concentrations of ABA and DPA at 5 DAS in the extreme DH lines with an average 196.48 ± 13.94 and 635.75 ± 59.29 ng/g DW, respectively, showed interparent variations. Except for the content of IAA at 5 DAS, the content of IAA, ABA and DPA at 5 and

TABLE 2 Summary statistics of germination rate (GR), shoot length (SL) and shoot fresh weight at 7 (SFW7) and 14 (SFW14) DAS of Express 617, 1012-98 and their DH progeny

		Parents		DH lines			
Trait	Unit	Express 617	1012-98	Mean ± *SEM*	Range	Skewness	Kurtosis
GR	%	88.76 ± 2.96	27.33 ± 5.36	70.53 ± 1.18	15.00–96.67	0.98	1.15
SL7	cm	3.72 ± 0.22	4.60 ± 0.13	4.45 ± 0.05	2.86–6.63	0.63	0.61
SL14	cm	6.00 ± 0.07	6.96 ± 0.38	6.43 ± 0.08	4.21–9.06	0.28	0.50
SFW7	g	0.12 ± 0.01	0.10 ± 0.01	0.13 ± 0.00	0.07–0.19	0.15	0.13
SFW14	g	0.47 ± 0.05	0.39 ± 0.06	0.52 ± 0.01	0.26–0.84	0.27	0.08
IAA5	ng/g DW	1,230	289	208.97 ± 28.64	56–1,780	6.48	46.61
IAA12	ng/g DW	473	419	296.39 ± 14.55	123.00–584.00	0.77	0.02
ABA5	ng/g DW	910	95.7	196.48 ± 13.94	54.70–546.00	1.05	0.83
ABA12	ng/g DW	1,080	430	384.29 ± 22.41	148.00–1,130	1.58	4.79
DPA5	ng/g DW	4,610	506	635.75 ± 59.29	90.30–2,000	1.38	1.56
DPA12	ng/g DW	9,250	5,890	2,262 ± 148.53	556.00–6,660	1.45	3.59

Values are presented as averages ± *SEM*; n = 165 DH lines for germination and seedling-related traits and n = 59 DH lines (the extreme highest and lowest germination performance) for hormonal profiling.
DAS, days after sowing; DH, doubled haploid.

TABLE 3 Coefficients of pairwise correlations of germination seedling-related traits GR, SL7, SL14, SFW7 and SFW14 and seedling-regulated hormone content of IAA5, IAA12, ABA5, ABA12, DPA5 and DPA12 from YE2-DH population

	GR	SL7	SL14	SFW7	SFW14	IAA5	IAA12	ABA5	ABA12	DPA5
SL7	−0.062									
SL14	−0.269*	0.851***								
SFW7	0.608***	0.053	0.011							
SFW14	0.725***	−0.260*	−0.328*	0.747***						
IAA5	−0.033	0.102	0.185	−0.042	−0.069					
IAA12	−0.114	−0.111	0.105	−0.042	−0.006	0.304*				
ABA5	0.183	−0.113	−0.092	0.106	0.162	0.409**	0.162			
ABA12	0.068	−0.008	−0.024	0.012	0.092	0.165	0.261*	0.344**		
DPA5	0.184	−0.046	0.001	0.052	0.074	0.401**	0.085	0.719***	0.288*	
DPA12	0.002	0.024	−0.019	−0.245	−0.047	0.246	0.258*	0.389**	0.757***	0.339**

IAA5, IAA12, ABA5, ABA12, DPA5 and DPA12: content of indole acetic acid, abscisic acid and dihydrophaseic acid at 5 and 12 DAS (n = 59 the extreme highest and lowest germination performance YE2-DH lines).

GR, germination rate; SL7, SL14, SFW7 and SFW14, shoot length and seedling fresh weight at 7 and 14 DAS, respectively (n = 165 from YE2-DH lines); DAS, days after sowing; DH, doubled haploid.

*Significant at .05 probability level; **significant at .01 probability level; ***significant at .001 probability level.

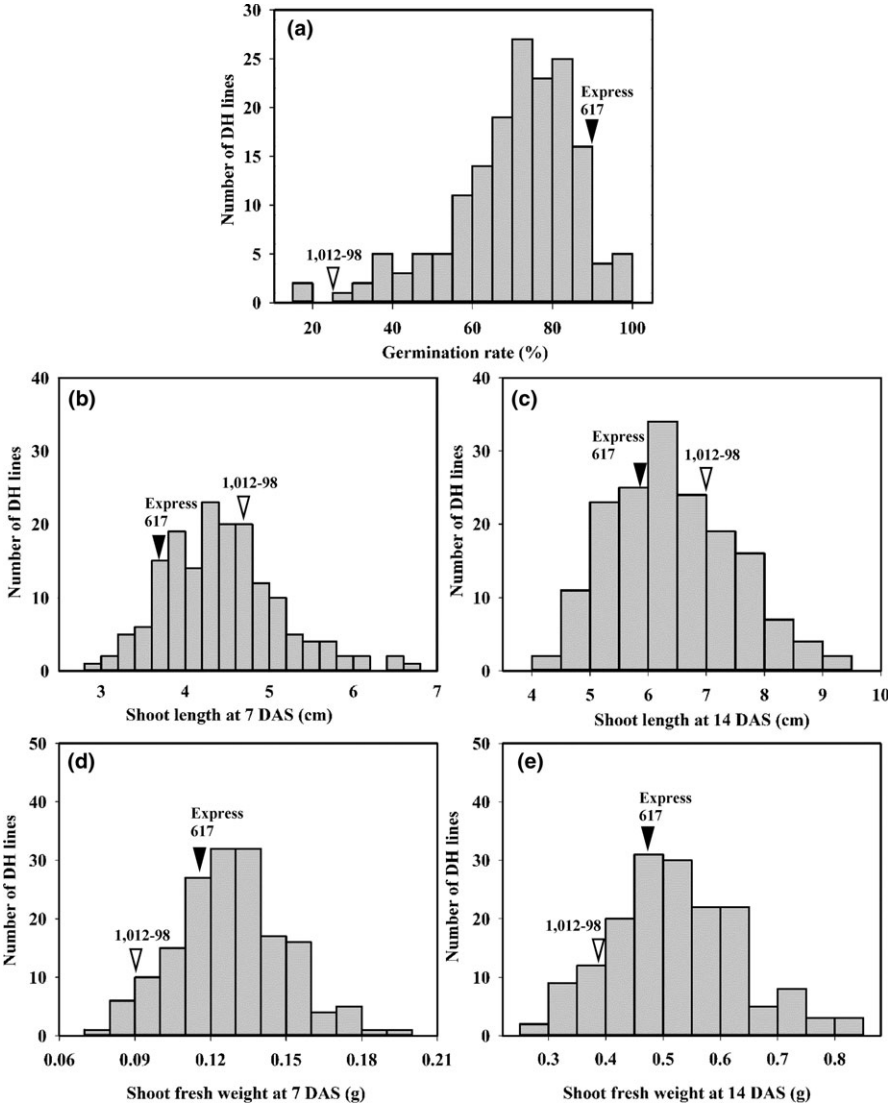

FIGURE 1 Frequency distribution of germination rate (a), shoot length at 7 days after sowing (DAS) (b) and at 14 DAS (c), and shoot fresh weight at 7 DAS (d) and 14 DAS (e) of n = 165 doubled haploid (DH) lines. Unfilled (▽) and filled (▼) triangles indicate the corresponding germination and seedling-related traits of parental lines 1012-98 and Express 617, respectively

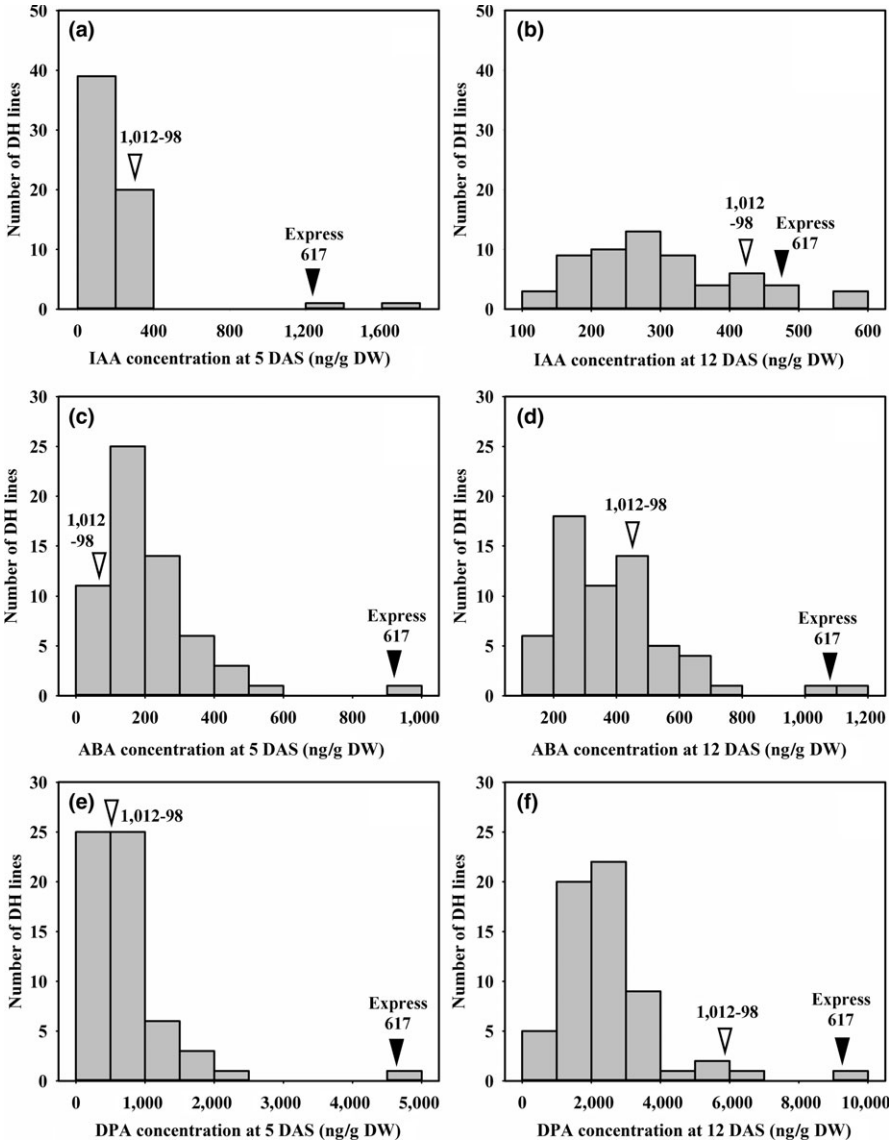

FIGURE 2 Frequency distribution of IAA concentration at 5 days after sowing (DAS) (a) and 12 DAS (b); ABA concentration at 5 DAS (c) and 12 DAS (d); DPA concentration at 5 DAS (e) and 12 DAS (f) of $n = 59$ extreme doubled haploid (DH) lines comprising the 30 best and the 29 lowest regarding germination. Unfilled (▽) and filled (▼) triangles indicate the corresponding hormonal concentration of parental lines 1012-98 and Express 617, respectively

12 DAS with mean values of 296.39 ± 14.55, 196.48 ± 13.94, 384.29 ± 22.4, 635.75 ± 59.29 and 2,262 ± 148.53 ng/g DW, respectively, expressed bidirectional transgressive variation and was strongly skewed towards the parental line 1012-98, indicating favourable alleles with additive effects from this parent. In addition, skewness and kurtosis values were <1.96 indicating that the segregation pattern of most traits fitted a normal or near-normal distribution model (Kim, 2013).

3.5 | QTL analysis for germination and seedling-related traits in the YE2-DH population

Quantitative trait loci mapping results are shown in Figure 3. In total, 13 QTL with a LOD threshold of >3.0 were detected for germination and seedling-related traits in the YE2-DH population ($n = 165$). Physical locations for ten of the 13 QTL (Table 4) could be confirmed in the *B. napus* reference genome sequence (Chalhoub et al., 2014) based on flanking sequences of SSR markers within or near the QTL. Two minor QTL were located on chromosomes A09 and C01 for GR, totally explaining 2.5%–6.3% of the phenotypic variation. One minor QTL was detected on chromosome A05 for SL at 7 DAS, explaining 7% of the phenotypic variation, whereas three minor QTL were located on chromosomes A03, C01 and C07 for SL at 14 DAS each explaining 4.5%–8.1% of the phenotypic variation. Two minor QTL were detected on chromosomes C09 and A02 for SFW at 7 DAS, totally explaining 4.5%–7.1% of the phenotypic variation, whereas five QTL were located on chromosomes A02, A10, C06 and C09 for SFW at 14 DAS with each explaining 6.0%–11.2% of the phenotypic variation (Table 4).

3.6 | Metabolic QTL analysis in 59 extreme DH lines

Metabolic QTL mapping results are shown in Figure 3. In total, 47 QTL with a LOD threshold of >3.0 were detected on chromosomes A01, A02, A04, A06, A07, A08, A09, C01, C03, C04, C05, C06, C07,

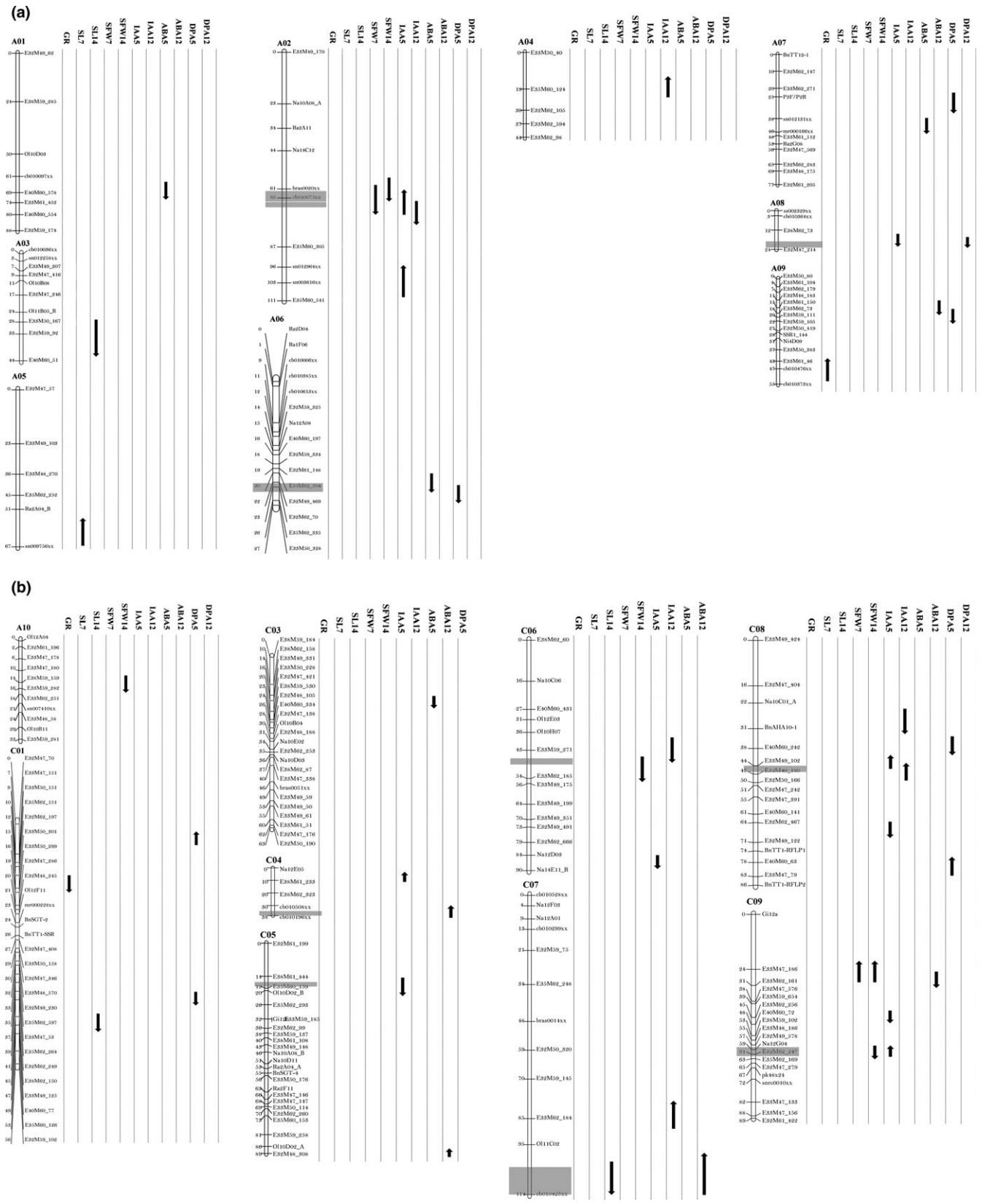

FIGURE 3 Quantitative trait loci (QTL) for germination rate (GR), shoot length at 7 (SL7) and 14 days after sowing (DAS) (SL14), seedling fresh weight at 7 (SFW7) and 14 DAS (SFW14), IAA concentration at 5 (IAA5) and 12 DAS (IAA12), ABA concentration at 5 (ABA5) and 12 DAS (ABA12), DPA concentration at 5 (DPA5) and 12 DAS (DPA12) in the YE2-DH population. Upward and downward arrows indicate additive effects of QTL in alleles contributed by the parents Express 617 and 1012-98, respectively. Grey blocks represent QTL clusters. (a) QTL on chromosomes A01, A02, A04 and A07. (b) QTL on chromosomes A10, C03, C06 and C08

TABLE 4 Identification of quantitative trait loci (QTL) associated with germination rate (GR), shoot length (SL), seedling fresh weight (SFW) at 7 and 14 DAS and their hormonal concentration of YE2-DH population

Trait	QTL	Chr[a]	Pos[b] (cM)	Left marker[c]	LOD	Part. R^2 [d](%)	Add[e]	Physical position (bp)	*Brassica napus* identifier	Gene description
GR	*GR.9.1*	A09	46	E33M61_46	3.63	2.5	423.77	9,725,515	GSBRNA2T00053170001	Leucine-rich repeat transmembrane protein kinase
	GR.11.2	C01	16	E33M50_301	4.26	6.3	−496.12	12,802,629–12,804,127	BnaA01g20900D	Unknown protein
SL7	*SL7.5.1*	A05	64	Ra2A04_B	4.06	7.0	−25.22	22,378,730–22,378,814	BnaA05g32740D	HSP20-like chaperones superfamily protein
SL14	*SL14.3.1*	A03	36	E32M59_92	3.11	7.2	26.42	21,343,615–21,343,949	BnaA03g42580D	CHD5-like protein
	SL14.11.2	C01	42	E32M62_249	3.16	4.5	30.90			
	SL14.17.3	C07	114	OI11C02	3.78	8.1	28.02	36,775,033–36,775,387	BnaC07g33680D	Tail-anchored protein insertion receptor WRB-like protein
SFW7	***SFW7.19.1***	C09	24	E33M47_186	3.75	4.5	0.73	2,719,553–2,719,695	BnaC09g04710D	Copper transport protein family
	SFW7.2.2	A02	66	cb010075	4.04	7.1	−0.63	2,357,741–2,358,308	BnaA04g03460D	Delta 1-pyrroline-5-carboxylate synthase 2
SFW14	*SFW14.10.1*	A10	14	E38M59_159	4.88	9.8	−3.60			
	SFW14.16.2	C06	52	E33M59_271	3.52	7.6	−2.99	5,082,992–5,083,159	BnaC06g04420D	Translation elongation factor EF1B
	SFW14.19.3	C09	24	E33M47_186	4.72	6.0	3.50	2,719,553–2,719,695	BnaC09g04710D	Copper transport protein family
	SFW14.19.4	C09	62	E32M62_247	5.88	11.2	−3.87	35,478,116–35,478,583	BnaC06g37450D	Unknown protein
	SFW14.2.5	A02	62	cb010075	3.18	8.3	−3.49	1,796,421–1,796,449	BnaC01g03460D	Basic-leucine zipper transcription factor
IAA5	***IAA5.8.1***	A08	22	E38M62_73	5.93	10.8	−1.19	1,372,194–1,372,637	BnaA08g30270D	Transmembrane protein
	IAA5.14.2	C04	10	E38M61_233	13.10	31.6	2.68			
	IAA5.15.3	C05	18	E38M61_444	7.45	14.4	−1.48	36,319,736–36,320,180	BnaC05g37220D	AMP-dependent synthetase and ligase
	IAA5.16.4	C06	90	Na12D03	4.18	10.7	−0.88	11,871,076–11,871,341	BnaC06g09960D	Tubby-like protein 7
	IAA5.18.5	C08	44	E33M49_102	12.01	40.2	3.76	3,044,258–3,044,360	BnaC08g03320D	Mannose-6-phosphate isomerase
	IAA5.18.6	C08	68	E32M62_467	10.62	40.3	−3.56			
	IAA5.19.7	C09	46	E33M62_256	17.52	49.0	−4.54			
	IAA5.19.8	C09	60	Na12G04	9.72	49.4	4.06	3,706,993–3,707,104	BnaC09g53760D	Dehydrodolichyl diphosphate synthase
	IAA5.2.9	A02	68	cb010075	8.20	22.9	1.86	2,357,741–2,358,308	BnaA04g03460D	Delta 1-pyrroline-5-carboxylate synthase 2
	IAA5.2.10	A02	106	sn003616	3.74	7.5	1.08			

(Continues)

TABLE 4 (Continued)

Trait	QTL	Chr[a]	Pos[b] (cM)	Left marker[c]	LOD	Part. R^2 [d](%)	Add[e]	Physical position (bp)	*Brassica napus* identifier	Gene description
IAA12	*IAA12.1.1*	A01	68	cb010097	3.33	3.6	0.80	1,705,602–1,705,867	BnaA01g03640D	Myosin-like proteins
	IAA12.4.2	A04	20	E35M60_124	5.08	11.4	1.16			
	IAA12.14.3	C04	10	E38M61_233	4.99	0.1	1.35			
	IAA12.16.4	C06	44	E33M59_271	6.50	14.6	−1.24	5,082,992–5,083,159	BnaC06g04420D	Translation elongation factor EF1B
	IAA12.16.5	C06	64	E33M49_199	3.11	4.9	0.78			
	IAA12.17.6	C07	86	E33M62_184	7.35	23.9	1.41			
	IAA12.18.7	C08	30	Na10C01_A	5.05	18.0	−1.23	13,076,937–13,077,184	BnaC08g08710D	Dirigent-like protein
	IAA12.18.8	C08	48	E32M49_180	7.99	21.1	1.66	3,044,258–3,044,438	BnaC08g03320D	Mannose-6-phosphate isomerase
	IAA12.2.9	A02	74	cb010075	5.26	5.8	−1.91	2,357,741–2,358,308	BnaA04g03460D	Delta 1-pyrroline-5-carboxylate synthase 2
	IAA12.2.10	A02	88	E35M60_305	3.01	0.2	−0.87			
ABA5	*ABA5.1.1*	A01	68	cb010097	4.92	16.1	−1.26	1,705,602–1,705,867	BnaA01g03640D	Myosin-like proteins
	ABA5.6.2	A06	22	E32M49_469	3.63	30.0	−1.68	12,884,494–12,886,372	BnaA06g20080D	SAUR-like auxin-responsive protein family
	ABA5.7.3	A07	46	sn012131	8.02	14.5	−1.79			
	ABA5.13.4	C03	16	E33M50_228	3.39	11.8	−0.97			
	ABA5.17.5	C07	4	Na12F03	3.39	3.6	−0.90	3,746,875–3,747,258	BnaA02g07900D	Temperature-induced lipocalin
								6,386,743–6,387,127	BnaC02g10990D	Temperature-induced lipocalin
ABA12	***ABA12.9.1***	A09	14	E32M59_105	3.98	8.2	−1.02	9,727,463–9,729,279	GSBRNA2T00053170001	Leucine-rich repeat transmembrane protein kinase
	ABA12.14.2	C04	38	cb010196	12.69	18.6	2.34	23,150,396–23,150,662	BnaA07g33870D	tRNA modification GTPase
	ABA12.15.3	C05	88	Ol10D02_A	6.03	14.0	1.95	56,252,903–56,253,363	BnaC03g66450D	Dynamin-related protein 3A
	ABA12.17.4	C07	86	E33M62_184	4.60	0.9	1.49			
	ABA12.17.5	C07	108	Ol11C02	3.28	20.8	1.35	36,775,033–36,775,387	BnaC07g33680D	GET1 membrane receptor homolog
	ABA12.19.6	C09	32	E33M62_161	4.42	11.1	−1.27	24,339,913–24,340,075	GSBRNA2T00110376001	Manganese superoxide dismutase 1
	ABA12.2.7	A02	46	Na14C12	4.45	1.5	1.33	19,803,130–19,803,390	BnaA02g26850D	O-fucosyltransferase family protein
DPA5	***DPA5.6.1***	A06	24	E32M62_70	15.98	32.7	−4.80	12,884,494–12,886,372	BnaA06g20080D	SAUR-like auxin-responsive protein family
	DPA5.7.2	A07	28	P3F/P2R	6.99	5.4	−2.37	14,973,302–14,974,311	BnaA07g18270D	Cupin domain-containing protein
	DPA5.9.3	A09	22	SSR1144	6.10	8.6	−1.99	19,968,384–19,968,806	BnaA09g26790D	SAUR-like auxin-responsive protein family
	DPA5.11.4	C01	14	E32M62_197	8.41	19.9	5.41			
	DPA5.11.5	C01	36	E35M62_597	4.85	17.1	−2.47			
	DPA5.13.6	C03	50	E33M49_59	8.82	1.2	3.93			
	DPA5.15.7	C05	30	E35M62_293	3.75	2.0	−1.89			
	DPA5.18.8	C08	38	E40M60_242	5.87	14.5	−2.41			
	DPA5.18.9	C08	82	E40M60_63	4.08	14.9	2.55			

(Continues)

TABLE 4 (Continued)

Trait	QTL	Chr[a]	Pos[b] (cM)	Left marker[c]	LOD	Part. R^2 [d](%)	Add[e]	Physical position (bp)	*Brassica napus* identifier	Gene description
DPA12	***DPA12.8.1***	A08	22	E38M62_73	7.45	5.2	−4.42	1,372,194–1,372,637	BnaA08g30270D	Transmembrane protein
	DPA12.13.2	C03	20	E32M47_421	10.48	4.2	9.92			
	DPA12.14.3	C04	32	cb010508	5.89	19.5	3.70	44,350,304–44,350,533	BnaC04g44220D	Ribosomal protein S5 family protein
	DPA12.15.4	C05	14	E38M61_444	6.12	14.1	3.97	36,319,736–36,320,180	BnaC05g37220D	AMP-dependent synthetase and ligase
	DPA12.17.5	C07	104	OI11C02	6.07	35.2	4.25	36,775,033–36,775,303	BnaC07g33680D	GET1 membrane receptor homolog
	DPA12.18.6	C08	36	BnAHA10_1	4.99	3.5	−3.80	20,887,790–20,892,333	BnaC08g17280D	H+-APTase gene family

Component QTL of consensus QTL were indicated in bold.
DAS, days after sowing; DH, doubled haploid.
[a]Chromosome number.
[b]Position of the peak marker.
[c]Left marker.
[d]Proportion of the phenotypic variance explained by each QTL.
[e]Additive effect: positive additivity indicates that the QTL allele originated from the parental line Express 617; negative additivity indicates that the QTL allele originated from the parental line 1012-98.

TABLE 5 List of consensus QTLs for the traits related to shoot length (SL), shoot fresh weight (SFW), content of IAA, ABA and DPA

	A02		A06	A08	A09	C04	C05	C06	C07	C08	C09	
Traits	Co-QTLA2.1	Co-QTLA2.2	Co-QTLA6.1	Co-QTLA8.1	Co-QTLA9.1	Co-QTLC4.1	Co-QTLC5.1	Co-QTLC6.1	Co-QTLC7.1	Co-QTLC8.1	Co-QTLC9.1	Co-QTLC9.2
SL14									*SL14.17.3*			
SFW7	*SFW7.2.2*										*SFW7.19.1*	
SFW14	*SFW14.2.5*							*SFW14.16.2*			*SFW14.19.3*	*SFW14.19.4*
IAA5	*IAA5.2.9*	*IAA5.2.9*		*IAA5.8.1*			*IAA5.15.3*			*IAA5.18.5*		*IAA5.19.8*
IAA12		*IAA12.2.9*						*IAA12.16.4*		*IAA12.18.8*		
ABA5			*ABA5.6.2*									
ABA12					*ABA12.9.1*	*ABA12.14.2*			*ABA12.17.5*		*ABA12.19.6*	
DPA5			*DPA5.6.1*		*DPA5.9.3*							
DPA12				*DPA12.8.1*		*DPA12.14.3*	*DPA12.15.4*		*DPA12.17.5*			

SL14.17.3, *SFW7.2.2*, *SFW7.19.1*, *SFW14.2.5*, *SFW14.16.2*, *SFW14.19.3* and *SFW14.19.4*: QTL for shoot length and seedling fresh weight at 7 and 14 DAS, respectively.
IAA5.2.9, *IAA5.8.1*, *IAA5.15.3*, *IAA5.18.5*, *IAA5.19.8*, *IAA12.2.9*, *IAA12.16.4* and *IAA12.18.8*: QTL for content of indole acetic acid at 5 and 12 DAS, respectively.
ABA5.6.2, *ABA12.9.1*, *ABA12.14.2*, *ABA12.17.5*, *ABA12.19.6*, *DPA5.6.1*, *DPA5.9.3*, *DPA12.8.1*, *DPA12.14.3*, *DPA12.15.4* and *DPA12.17.5*: QTL for content of abscisic acid and dihydrophaseic acid at 5 and 12 DAS, respectively.
QTL, quantitative trait loci; DAS, days after sowing.

C08 and C09 for hormone content in the YE2-DH population (n = 165), totally explaining 0.1%–49.4% of the phenotypic variation (Table 4). Among 47 metabolic QTL identified in this study, 44 could be assigned to corresponding chromosomal regions in the *B. napus* reference genome sequence (Chalhoub et al., 2014) using flanking sequences of SSR markers within or near the respective QTL. Of the QTL at 5 and 12 DAS, 20 QTL were located on chromosomes A01, A02, A04, A08, C04, C05, C06, C07, C08 and C09 for IAA content, explaining up to 49.4% of the phenotypic variation, whereas 12 QTL were detected for ABA content on A01, A06, A07, C07 and C09, totally explaining 0.9%–30.0% of the phenotypic variation. In addition, 15 QTL for DPA content were identified on A06, A07, A08, A09, C01, C03, C04, C05, C07 and C08, totally explaining 1.2%–35.2% of the phenotypic variation. Of the 47 detected QTL, seven major QTL for IAA, ABA and DPA content at 5 DAS were detected on C04, C08, C09 and A06, totally explaining 30%–49.4% of the phenotypic variation. In addition, one major QTL was located on chromosome C07 for DPA content at 12 DAS, explaining 35.2% of the phenotypic variation.

3.7 | Colocalization of QTL and candidate gene analysis

Twelve of QTL clusters were detected on chromosomes A02, A06, A08, A09, C04, C05, C06, C07, C08 and C09 (Figure 3 and Table 5). The QTL hot spots named *Co-QTLA2.1*, *Co-QTLC6.1* and *Co-QTLC9.2* on chromosomes A02, C06 and C09, respectively, include QTL for SFW and IAA (*SFW7.2.2*, *SFW14.2.5* and *IAA5.2.9*, *SFW14.16.2* and *IAA12.16.4*, and *SFW14.19.4* and *IAA5.19.8*, respectively). The QTL for SFW at 7 and 14 DAS showed negative additivity, indicating that these loci from parent 1012-98 contributed the favourable alleles. In contrast, the allele from Express 617 had both positive and negative effects for IAA content at *Co-QTLA2.1*, *Co-QTLC9.2* and *Co-QTLC6.1*, respectively. Colocalization of QTL for SFW and IAA content indicated the involvement of endogenous IAA in controlling SFW explaining up to 7.1%, 11.2%, 49.4% and 14.6% of phenotypic variance for SFW at 7 and 14 DAS and IAA content at 5 and 12 DAS, respectively. Similarly, the role of ABA in regulating SFW was confirmed by the colocalization of *Co-QTLC9.1* on C09, harbouring the QTL *SFW7.19.1*, *SFW14.19.3* and *ABA12.19.6* with explained phenotypic variation of 4.5%, 6.0% and 11.6%, respectively. In well-watered *Arabidopsis* plants, normal levels of endogenous ABA are required to maintain shoot growth by suppressing ethylene synthesis. Treatment with exogenous ABA resulted in the complete recovery of shoot growth in *aba2-1* mutant relative to the wild type (LeNoble, Spollen, & Sharp, 2004). Additionally, *Co-QTLC7.1* on C07 included QTL of *SL14.17.3*, *ABA12.17.5* and *DPA12.17.5*, totally explaining of 8.1%, 20.8% and 35.2% of the phenotypic variation, respectively. These results indicate that SL at 14 DAS was controlled by both ABA and DPA contents. Positive additivity at these loci indicated that the favourable allele was from Express 617.

Crosstalk between endogenous IAA and DPA was established by the QTL clusters *Co-QTLA8.1* and *Co-QTLC5.1*. These QTL hot spots harboured QTL *IAA5.8.1* and *DPA12.8.1* along with *IAA5.15.3* and *DPA12.15.4*, with each explained phenotypic variation of 10.8/5.2% and 14.4/14.1%, respectively. *Co-QTLA2.2* and *Co-QTLC8.1* on A02 and C08 included QTL *IAA5.2.9* and *IAA12.2.9*, totally explaining 22.9% and 5.8% of the phenotypic variation, respectively, along with *IAA5.18.5* and *IAA12.18.8* with each explained phenotypic variation of 40.2% and 21.1%, respectively (Table 5). This underlines the connection of IAA concentration between 5 and 12 DAS. At these loci, the Express 617 allele was favourable allele for IAA content at 5 DAS. In contrast, Express 617 contributed both favourable and unfavourable alleles for IAA content at 12 DAS. Hot spots *Co-QTLA6.1*, *Co-QTLA9.1* and *Co-QTLC4.1* on A06, A09 and C04 contained QTL (*ABA5.6.2* and *DPA5.6.1*), totally explaining 30.0% and 32.7% of the phenotypic variation, respectively, *ABA12.9.1* and *DPA5.9.3* with each explained phenotypic variation of 8.2% and 8.7%, respectively, and *ABA12.14.2* and *DPA12.14.3*, totally explaining 18.6% and 19.6% of the phenotypic variation, respectively. The Express 617 allele was unfavourable for both ABA and DPA contents on 5 and 12 DAS at the *Co-QTLA6.1*, *Co-QTLA9.1* loci, but favourable for ABA and DPA concentration on 12 DAS at the *Co-QTLC4.1* locus.

The candidate genes for 12 QTL clusters were analysed based on the annotations for the *Arabidopsis* reference genome (Table 4). The annotation included transcription factor, membrane transporter/receptor, auxin-responsive protein, protein biosynthesis, ATP binding, tRNA modification, ribosomal protein, etc. Some of the genes might be good candidates associated with seedling and hormonal traits according to the alignment results.

4 | DISCUSSION

Good seed germination and high seedling vigour are important traits in winter oilseed rape due to their positive influence on prewinter development as a basis for winter survival and postwinter establishment of the crop. Previous studies also emphasized the importance of germination and seedling vigour in the vegetable and oilseed brassicas *B. oleracea* (Bettey et al., 2000), *B. rapa* (Basnet et al., 2015) and *B. napus* (Hatzig et al., 2015). It is known that germination and seedling vigour are quantitative traits and are affected not only by environmental factors but also by intrinsic seed factors like hormonal and genetic components (Bentsink & Koornneef, 2009; Holdsworth et al., 2008). In this study, seed germination and seedling vigour were evaluated in a segregating DH population with strong variation for both traits. To obtain information on the interactions between genetic control of these traits with seed and seedling hormones, the content of auxin, ABA and their metabolites was analysed in 59 DH lines with extreme seed and seedling performance. This enabled identification of QTL regions and hormone metabolites associated with seed germination characteristics and seedling vigour. Thirteen QTL regions for traits related to seed germination and seedling vigour were detected. In addition, 47 mQTL for hormone content related to seed germination and seedling vigour were located. Based

on sequence information corresponding to SSR markers, some QTL could be related to potential positional and functional candidate genes involved in regulation of hormone balance and seedling development. Although these candidates can only be considered as preliminary without further fine-mapping and sequence analysis, they nevertheless represent an interesting first insight into the putative genetic mechanisms involved in hormone regulation, germination and seedling vigour in winter rapeseed. Importantly, germination, seedling-related traits and hormonal content generally fit to normal distributions and were consequently quantitative traits, presumably controlled by multiple genes. Therefore, a QTL mapping approach is required to characterize the genetics of these complex traits in *B. napus*.

Due to the very high costs of the detailed metabolite analysis, the QTL analysis for hormone traits was performed using preselected DH lines which showed extreme phenotypes for germination. Although the magnitude of QTL effects is frequently overestimated in small populations, the sample size (n = 59) was selected based to represent the tails of the phenotypic trait distribution for germination, using the procedure proposed by Paterson et al. (1988). Tails of phenotypic distributions are expected to be representative for significant QTL involved in related traits, and in accordance with this hypothesis, we were able to successfully colocalize hormone QTL in these lines with QTL for germination traits from the whole population (n = 166). Interestingly, we identified 12 major chromosome hot spots where QTL for germination, seedling vigour and seedling-regulated hormones were colocated, verifying this approach of selective metabolite phenotyping suggesting the strong involvement of hormone regulatory factors in these traits in rapeseed seeds and seedlings.

4.1 | Phenotypic variation, correlation and heritability of the traits

The GR of the YE2-DH population showed interparent variation with the favourable allele from 1012-98 line. In contrast, SL and SFW at 7 and 14 DAS exhibited bidirectional transgressive variations, suggesting that alleles with additive effects or complementation effect for seedling-related traits were more or less equally distributed among the parents (Figure 1). Table 3 shows a significant positive correlation between germination and SFW at 7 and 14 DAS (p < .001), while germination was negatively correlated to SL at 14 DAS (p < .05). Whereas Express 617 showed a normal GR of around 90%, the GR was severely depressed in 1012-98 with successful testa rupture and radicle emergence being observed in only around one quarter of the seeds (Nguyen et al., 2016). In contrast, seedlings of 1012-98 showed significantly increased etiolation and consequently longer hypocotyls than those of Express 617. In addition, SL was also negatively correlated to germination time in *B. oleracea* (Bettey et al., 2000). Akinyosoye, Adetumbi, Amusa, Olowolafe, and Olasoji (2014) found that the inheritance of germination in maize was not significantly related to seedling fresh weight or SL. In the present study, the time point of 7 DAS was positively correlated to 14 DAS for traits of SL and SFW (p < .001). This difference to maize may be related to the relatively small seed nutrient storage of *B. napus* seeds compared to the large energy reserve of maize kernels. A lower seed reserve at germination necessitates a rapid emergence to ensure a quick switch to self-sufficient growth via root uptake and photosynthesis. Rapid germination and fast growth of the coleoptile were also an important characteristic for rice varieties to allow stable and excellent seedling establishment, particularly at low temperature (Ogiwara & Terashima, 2001). In the present study, SFW at 14 DAS negatively was also correlated to SL at 7 and 14 DAS (p < .05). Heritability (h^2) was moderate-to-high for GR, SL at 7 and 14 DAS (SL7 and SL14) and SFW at 7 DAS (SFW7) while SFW at 14 DAS (SFW14) showed low heritability. The heritabilities in this study ranging from 0.24 to 0.64 (Table 1) are higher than the corresponding values for seedling-related traits in *B. oleracea* reported by Bettey et al. (2000). As a consequence, genetic factors determine as much as 64% of phenotypic variation for germination and seedling-related traits in the *B. napus* YE2-DH population.

Except for the concentration of IAA at 5 DAS, the contents of IAA, ABA and DPA at 5 and 12 DAS expressed bidirectional transgressive variations and were strongly skewed towards parental line 1012-98, indicating favourable alleles with additive effect from this parent. In addition, skewness and kurtosis values were <1.96, indicating that the segregation pattern of most traits generally fits a normal or nearly normal distribution model (Kim, 2013). Similar to seedling traits, the time point of 5 DAS was positively related to 12 DAS for traits of IAA, ABA and DPA (p < .05 and p < .01). At both 5 and 12 DAS, a significant positive crosstalk correlation was found between IAA and ABA, IAA and DPA (p < .01 and p < .05 for 5 and 12 DAS, respectively). In fact, a coordinating network of auxin and ABA signalling in regulating seed dormancy and germination has been reported in *Arabidopsis* (Liu et al., 2013) and in *B. napus* (Nguyen et al., 2016). Additionally, all possible significant correlations between the concentration of ABA and its metabolite, DPA, at both 5 and 12 DAS were detected (p < .05, p < .01 and p < .001). ABA catabolism is primarily driven through hydroxylation to form 8′-hydroxy ABA and is converted to PA and subsequently inactivated as DPA (Nambara & Marion-Poll, 2005).

4.2 | Major QTL hot spots for seed germination and seedling vigour suggest pleiotropic trait interactions

The colocalization of QTL, reflecting the significant intertrait correlations, suggests that one or several important genes participate in more than one pathway related to germination and vigour in *B. napus*. In *Arabidopsis*, auxin acts upstream of the major regulator of seed dormancy, *ABSCISIC ACID INSENSITIVE 3*, by recruiting the auxin response factors *AUXIN RESPONSE FACTOR 10* and *AUXIN RESPONSE FACTOR 16* to control the expression of ABI3 during seed germination (Liu et al., 2013). In the present study, 12 *Co-QTL* hot spots in *B. napus* were attributed to three functional groups corresponding to a seedling and hormone-related QTL group (*Co-QTLA2.1*, *Co-QTLC6.1*, *Co-QTLC7.1*, *Co-QTLC9.1* and *Co-QTLC9.2*), a hormonal

crosstalk group (*Co-QTLA8.1* and *Co-QTLC5.1*) and single-hormone group across different time points (*Co-QTLA2.2* and *Co-QTLC8.1* for *IAA*, *Co-QTLA6.1* and *Co-QTLA9.1* for ABA and ABA metabolites) (Table 5). In a previous study, we observed that the disruption of germination and seedling development in 1012-98 was caused by the crosstalk of auxin-ABA and amplification of C-genome *Bna-C.ARF10* copy number, leading to a minor increase in transcription factor *Bna.ARF10* at the critical time point for seed germination (Nguyen et al., 2016). In *B. napus*, two homeologous of transcription factor *Bna.ARF10* are located at the coding regions of GSBRNA2T00118741001, on chromosome A07, at physical position 12,221,970–12,224,345, and GSBRNA2T00089963001 on chromosome C04 at physical position 13,853,422–13,855,805 (Chalhoub et al., 2014). This region may correspond to the QTL *ABA5.7.3*, *DPA5.7.2*, *ABA12.14.2* and *DPA12.14.3*. In addition, SAUR-like auxin-responsive gene was mapped in the regions of *Co-QTLA6.1* and *Co-QTLA9.1* (Table 4) which provided strong evidence for the crosstalk of auxin-ABA in regulating germination and seedling development in *B. napus*. From the 12 *Co-QTL* hot spots, the *Co-QTLA2.1*, *Co-QTLC7.1* and *Co-QTLC9.2* were major QTL clusters for SFW, SL and IAA, ABA and DPA content, respectively. At these loci, the Express 617 allele is associated with higher phenotypic values of SL, content of IAA, ABA and its metabolite DPA, while the 1012-98 allele is favourable for SFW. In the regions of *Co-QTLA2.1*, *Co-QTLC7.1* and *Co-QTLC9.2*, delta 1-pyrroline-5-carboxylate synthase 2, GET1 membrane receptor and manganese superoxide dismutase 1 genes were potential candidates involving in osmotic regulation and ABA signalling (Zhu & Scandalios, 1994). In addition, *Co-QTLC5.1* with favourable alleles from 1012-98 (at QTL *IAA5.15.3*) and Express 617 (at QTL *DPA12.15.4*) is a major hormonal crosstalk QTL. Moreover, the *Co-QTLC8.1*, *Co-QTLA6.1* and *Co-QTLC7.1* with explained phenotypic variation value ranging 20.8–40.2 are hot spot regions for cross-time point correlation of IAA, ABA and DPA at 5 and 12 DAS.

Collectively, these results contribute to a better insight into the metabolomic and genomic mechanisms controlling germination and seedling development in *B. napus*, and identify genome regions and allelic variants with a putative positive role in enhancement of germination and vigour. Translation of the results to new genetic maps with high-density, sequence-annotated markers (e.g., Stein et al., 2017) will help to narrow down QTL intervals for identification and validation of putative candidate genes, and to develop robust markers for marker-assisted breeding of rapeseed varieties with superior germination and seedling growth.

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SUPPORTING INFORMATION

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4 Discussion

Seed vigour is a quantitative trait and a key factor controlling crop performance in the field. Strong seed germination and vigourous seedling establishment significantly impact final crop yields and resource use efficiency. Therefore, improving seed vigour to enhance crop growth and yield remains a primary objective of the agricultural industry and the seed and breeding companies. Several published studies have focused on the genetic dissection of seed germination and seedling vigour traits on *Arabidopsis* (Clerkx et al., 2004), rice (Xie et al., 2014), *B. rapa* (Basnet et al., 2015), *B. oleracea* (Bettey et al., 2000; Morris et al., 2016) and *B. napus* (Hatzig et al., 2018; Hatzig et al., 2015). In addition, hormonal regulation of dormancy and germination on *Arabidopsis* (Liu et al., 2007; Liu et al., 2013), wheat (Ramaih et al., 2003), rice (Zhao et al., 2018) and soybean (Shuai et al., 2017) has also been previously reported. However, a molecular link combining metabolomic and genetic approach to provide the powerful insights of metabolomic and genetic regulation of germination and seedling development in *B. napus* remains limited. This study aimed to assess an integrative approach to explore genetics modulating seed germination and seedling vigour and the link between seed metabolomics and genomics and seed vigour traits.

4.1 Improvement of germination and seedling establishment in oilseed rape by hormonal balance

4.1.1 Overproducing auxin as an indirect germinative inhibitor causes severe seed hormonal imbalance and leads to auxin-overdose phenotypes in *B. napus*

The transition between seed dormancy and germination represents a critically physiological stage in the plant life cycle and it is an important ecological and commercial trait. Seed vigour is controlled by genetics and the balance and crosstalk of hormonal components mainly ABA and GA (Holdsworth et al., 2008). Until now ABA is the sole plant hormone known to maintain seed dormancy and thus to inhibit germination. Recent

studies have provided the evidence of auxin involvement in both germination completion and seedling establishment by increasing seed sensitivity to ABA in *Arabidopsis* (Liu et al., 2007; Liu et al., 2013) and/or decreasing GA/ABA ratios in wheat and soybeans by enhancing ABA biosynthesis and impairing GA biogenesis. Microscope observation shows that auxin treatment delayed rupture of the soybean seed coat and radicle protrusion (Ramaih et al., 2003; Shuai et al., 2017).

In this study, high-throughput UPLC-MS/MS hormone profiling of seeds and seedlings before and after germination revealed that 1012-98 has a severely disturbed hormone balance with extremely atypical, excessive quantities of auxin and ABA metabolites. In *Arabidopsis*, both auxin-overproducing transgenic line *iaaM-OX* and exogenous auxin applied wild-type seeds did not germinate up to 30 DAS in germination assay due to inhibitory effect of ABA in a dose-dependent manner (Liu et al., 2007; Liu et al., 2013). The high accumulation of endogenous auxin and ABA as late as 3 DAS, these conditions probably created an ABA-hypersensitive phenotype in yellow-seeded 1012-98 line, thereby the seeds were failed to protrude radicles under favorable conditions. In fact, auxin inhibits seed germination by enhancing ABA action driven by transcription factor *ABSCISIC ACID INSENSITIVE 3* (*ABI3*), thereby adding secondary protective level of control in the regulation of seed dormancy and germination. Auxin action in *Arabidopsis* seed germination requires the ABA signaling and the crosstalk of auxin-ABA in seed dormancy are interdependent (Liu et al., 2013). There was no evidence in mutant line 1012-98 during the first few days after imbibition for the necessary catabolism of ABA to less active phaseic acid (PA) and its inactive form dihydrophaseic acid (DPA). ABA catabolism is primarily driven by 8′-hydroxylation encoded by the CYP707A gene family. The product of hydroxylation, 8′-hydroxy ABA, then converted to PA and subsequently inactivated as DPA (Nambara and Marion-Poll, 2005) has been shown to be important in

regulating germination potential in *Arabidopsis* (Kushiro et al., 2004; Okamoto et al., 2006).

In addition to extreme auxin excess and high content of ABA, GA_1 levels in 1012-98 were unusually high in comparison with Express 617. In contrast the levels of GA_4, which in *Arabidopsis* seeds is around 10 times more active than GA_1 (Yang et al., 1995), were not detected until 5 DAS in the *B. napus* mutant. In the context of excess ABA content and extremely high auxin accumulation, the mutant 1012-98 genotype did not produce sufficient amount of active GAs to deactivate the two-step germination inhibition by ABA and auxin and complete germination.

Moreover, the mutant phenotypes being observed in germinated 1012-98 seedlings and its hormone-defective offspring (Figure 3 in Chapter 2) are consistent with those of *Arabidopsis* auxin overproduction or *arf10* mutants. These abnormal developmental phenotypes in *B. napus* including elongated hypocotyl, small, and epinastic/deformed cotyledons, tricotyledon, reduced number of leaves, constricted apical end of hypocotyl, and curled stems are extensively described in *Arabidopsis* by Boerjan et al. (1995), Romano et al. (1995) and Liu et al. (2007). Disturbed apical hook development, causing the “ostrich” phenotypes we observed in 1012-98 and its offspring, has been most extensively connected with auxin. A common cause is auxin inhibition of cell expansion at the inner side GA promotion of cell division and expansion at the outer side of the hook (Mazzella et al., 2014; Vandenbussche et al., 2010). Stem curling and leaf malformation are also among the first tri-phasic responses of plants after application of the synthetic auxin herbicide 2,4-D, followed by stunted growth, chlorosis, wilting, necrosis, and ultimately death (Grossmann, 2010). Irregular distribution of auxin leading to disturbed cell elongation is the most likely explanation for the hook-like seedlings, stem curvature, twisted petioles and other abnormal developmental phenotypes observed in Express 617 × 1012-98 DH lines despite successful germination. Taken together, these results explain the

extremely poor germination and seedling establishment and developmental defects observed in the *B. napus* mutant 1012-98, in which the characteristic auxin pattern was completely disturbed. It has been proposed that auxin might act as a indirect negative regulator of seed germination by recruiting the *Bna.ARF10* to control the germinative inhibitory action *ABI3* during seed germination and seedling development in *B. napus* (Figure 7).

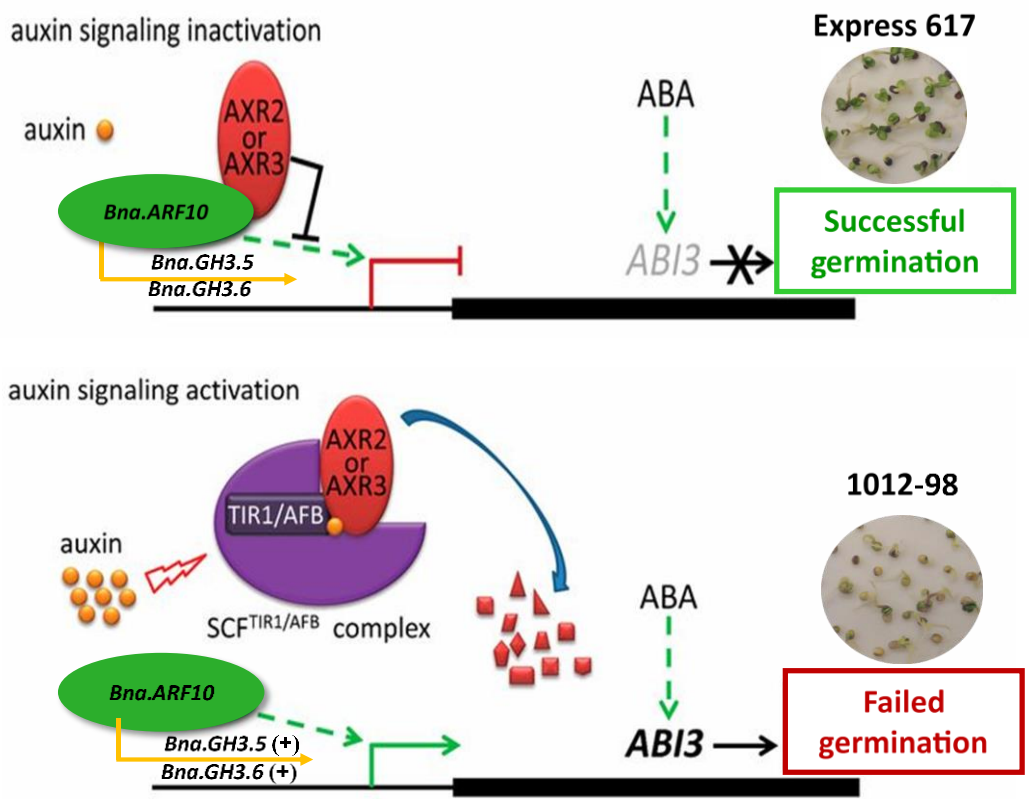

Figure 7: A proposed model for the effect of the ABA–auxin interaction on the control of seed dormancy/germination in *B. napus*. When auxin signaling is inactivated by low auxin level or signaling disruption, *Bna.ARF10* are inactivated by the Aux/IAA repressors AXR2 and AXR3. *ABI3* expression cannot be maintained, and seed dormancy is released. With auxin signaling activation, auxin binds to the auxin receptor TIR1/AFB F-box proteins and promotes the degradation of IAA7/AXR2 and IAA17/AXR3. The degradation releases the activity of *Bna.ARF10* and maintains the expression of *ABI3*, which protects seed dormancy and inhibits seed germination. The solid arrows and lines indicate direct regulation, and the dotted arrows indicate indirect regulation. The black box indicates the *ABI3* gene region. Modified from Liu et al. (2013) Proceedings of the National Academy of Sciences of the United States of America 110: 15485-15490. Copyright © 2013 National Academy of Sciences.

4.1.2 Molecular links of transcriptional repressor *Bna.ARF10* to auxin-response genes *Bna.GH3.5*, *Bna.GH3.6*, and high auxin accumulations

Early auxin-responsive genes *GH3* gene family and its transcription factors *ARF10* and *ARF16* play important roles in auxin signaling pathway and regulate many aspects of plant growth and development (Guilfoyle and Hagen, 2007; Mallory et al., 2005). Recently the crosstalk between auxin and ABA signaling in controlling *Arabidopsis* seed dormancy and germination have been proven and ABA function is largely dependent on the *TIR1/AFB-AUX/IAA-ARF*-mediated auxin signaling pathway. In fact, auxin acts upstream of the major regulator of seed dormancy, *ABI3*, by recruiting the *ARF10* and *ARF16* to control the *ABI3* expression during seed germination (Liu et al., 2007; Liu et al., 2013). In *Arabidopsis* and soybean, low free auxin and transcriptional repression of *ARF10* are two key components to support successful germination and normal developmental programs of root, leaf and flower organs (Liu et al., 2007; Liu et al., 2013; Mallory et al., 2005; Shuai et al., 2017). When auxin signaling is inactivated by low auxin level or signaling disruption, *ARF10* and *ARF16* are inactivated by the Aux/IAA repressors AXR2 and AXR3. *ABI3* expression cannot be maintained, and seed dormancy is released. With auxin signaling activation, auxin binds to the auxin receptor TIR1/AFB F-box proteins and promotes the degradation of IAA7/AXR2 and IAA17/AXR3. The degradation releases the activity of *ARF10* and *ARF16* and maintains the expression of *ABI3*, which protects seed dormancy and inhibits seed germination (Liu et al., 2013). Moreover, both *ARF10* and *ARF17* are classified as transcriptional repressors with an enrichment in serine (S), proline (P), leucine (L), and either glycine (G; ARF10), or theronine (T; ARF17) residues (Tiwari et al., 2003). The deduced protein sequences in *Brassica* in this study confirmed SPL-rich regions located just before and after the conserved domains. The transcription factors *ARF* and early auxin response *GH3* gene families are highly conserved in higher plants after speciation from *Arabidopsis thaliana* (Xie et al., 2015). On the background of high

accumulation of ABA and auxin as late as 3 DAS in mutant 1012-98 genotype, the copy number amplification of *Bna.ARF10* in 1012-98 leading to minor transcriptional increase presumably added another restriction on germinative process through the inhibitory action of *ABI3* (Figure 7).

In addition to minor upregulation of *Bna.ARF10*, the upregulation of *Bna.GH3.5* between 2 and 6 DAS in ABA-hypersensitive 1012-98 corresponded to the second peak (4 DAS) of IAA-asp and the reduction of active IAA (3 DAS). In fact, the *GH3*-like gene family plays an essential role in maintaining optimal levels of endogenous IAA through the amino acid, sugar, and peptide-linked conjugating pathways in plant cells (Woodward and Bartel, 2005).

Besides the previously described copy number variation for *BnaC.ARF10*, the major functionally relevant *Bna.ARF10* sequence divergence associated with the developmental disruption in 1012-98 appears to be present in *BnaC.ARF10.d*. Mutations in the B3 DNA-binding domains might enhance binding activities of *BnaC.ARF10.d* to AuxRE and to Aux/IAA proteins in 1012-98 line. Taken together, this can be expected to lead to the germinative hyper-repressor in 1012-98, which in turn would cause the very poor germination and disrupted transition from germination to seedling development. It can be proposed that the crosstalk of auxin-ABA might have coevolved to synergically control seed dormancy to survive unfavorable environment. These mutations appear to have arisen independently during the *de novo* allopolyploidization of 1012-98 from an interspecific cross between *B. rapa* and *B. oleracea*. Using the detailed phenotype data obtained in this study in combination with high-throughput genomic technologies, the wild-type *Bna.ARF10* genotypes combining non-disturbed hormone profiles will be successfully selected for breeding yellow-seeded oilseed rape lines with normal germination and seed vigour and high yield approaching those of elite, dark-seeded check varieties.

4.2 Major QTL hot spots for improving seed germination, seedling vigour and seedling-regulated hormones in *B. napus*

Seed germination and seedling vigour considered as two key components of the performance of crop seeds are very complex traits determined by the interaction of hormonal (Holdsworth et al., 2008), genetic and numerous environmental components including soil factors, water availability and temperature (Bentsink and Koornneef, 2009). Quantitative trait locus (QTL) mapping is currently the most commonly used approach to dissect the genetic factors underlying complex traits. The goal of QTL mapping is to identify genomic regions associated with a specific complex phenotype by statistical analysis of the associations between genetic markers and phenotypic variation (Doerge, 2002). Previous QTL studies also emphasized the importance of germination and seedling vigour in the vegetable and oilseed brassicas *B. oleracea* (Bettey et al., 2000; Morris et al., 2016), *B. rapa* (Basnet et al., 2015) and *B. napus* (Hatzig et al., 2018; Hatzig et al., 2015). However, none of these studies linked seed germination or seedling vigour to regulatory roles of phytohormones in controlling germination and seedling establishment. Therefore, identification of QTL controlling germination and seedling vigour via hormone regulation may help plant breeders select and combine positive early vigour traits, despite the strong environmental sensitivity and moderate-to-high heritability of germination and seedling vigour.

By phenotyping the doubled haploid (YE2-DH) mapping population from a cross between winter oilseed rape parents with high vigour (Express 617) and low vigour (1012-98) in a climate-controlled glasshouse, a total of 13 QTL on nine chromosomes for germination and seedling-related traits at 7 and 14 days after sowing (DAS), explaining up to 11.2% of the phenotypic variation for seedling vigour were identified. Forty-seven metabolic QTL on 15 chromosomes were identified for auxin, abscisic acid (ABA) and dihydrophaseic acid (DPA) at 5 and 12 DAS, explaining up to 49.4% of phenotypic variation in seedling

hormone composition. The colocalization of QTL, reflecting the significant intertrait correlations, suggests that one or several important genes participate in more than one pathway related to germination and vigour in *B. napus*. Interestingly, 12 major chromosome hot spots where QTL for germination, seedling vigour and seedling-regulated hormones were colocated, were identified. This verifies this approach of selective metabolite phenotyping and suggests the strong involvement of hormone regulatory factors in these traits in rapeseed seeds and seedlings. Importantly, 12 *Co-QTL* hot spots in *B. napus* were attributed to three functional groups corresponding to a seedling and hormone-related QTL group (*Co-QTLA2.1*, *Co-QTLC6.1*, *Co-QTLC7.1*, *Co-QTLC9.1* and *Co-QTLC9.2*), a hormonal crosstalk group (*Co-QTLA8.1* and *Co-QTLC5.1*) and single-hormone group across different time points (*Co-QTLA2.2* and *Co-QTLC8.1* for IAA, *Co-QTLA6.1* and *Co-QTLA9.1* for ABA and ABA metabolites) (Table 5 in Chapter 3). In a previous study, we observed that the disruption of germination and seedling development in 1012-98 was caused by the crosstalk of auxin-ABA and amplification of C-genome *BnaC.ARF10* copy number, leading to a minor increase in transcription factor *Bna.ARF10* at the critical time point for seed germination (Nguyen et al., 2016). In *B. napus*, two homeologous of transcription factor *Bna.ARF10* are located at the coding regions of GSBRNA2T00118741001, on chromosome A07, at physical position 12,221,970–12,224,345, and GSBRNA2T00089963001 on chromosome C04 at physical position 13,853,422–13,855,805 (Chalhoub et al., 2014). Several orthologous GH3 family genes (BnaA07g08630D and BnaC03g46000D) were also mapped on chromosome A7 or C03 (Shen et al., 2018). This important region may correspond to the QTL *ABA5.7.3*, *DPA5.7.2*, *ABA12.14.2* and *DPA12.14.3*. Interestingly, *Co-QTLC4.1* (consisting of *ABA12.14.2* and *DPA12.14.3*) on chromosome C04 in *B. napus* is confirmed by QTL *Reduced ABscisic Acid 1 (RABA1)* on chromosome C04 influencing ABA content in *B. oleracea* (Morris et al., 2016). Functional activity of *RABA1* locus is predicted from a

homologue of the key ABA catabolic gene *AtCYP707A2*. In addition, *Bna.YUCCA6* involved in auxin biosynthesis (Wang et al., 2016) and SAUR-like auxin responsive gene was mapped in the regions of *Co-QTLA6.1* and *Co-QTLA9.1* (Table 4 in Chapter 4). QTL *ABA5.13.4*, *DPA5.13.4* and *DPA2.13.3* were co-located with two new candidate *Bna.SAUR* genes (BnaC03g14890D and BnaC03g16420D) and other two auxin-related genes (BnaA03g10890D and BnaA03g11170D) (Shen et al., 2018). Collectively, these results provided strong evidence for the crosstalk of auxin-ABA in regulating germination and seedling development in *B. napus*.

From the 12 *Co-QTL* hot spots, the *Co-QTLA2.1*, *Co-QTLC7.1* and *Co-QTLC9.2* were major QTL clusters for SFW, SL and IAA, ABA and DPA content, respectively. At these loci, the Express 617 allele is associated with higher phenotypic values of SL, content of IAA, ABA and its metabolite DPA, while the 1012-98 allele is favourable for SFW. In the regions of *Co-QTLA2.1*, *Co-QTLC7.1* and *Co-QTLC9.2*, delta 1-pyrroline-5-carboxylate synthase 2, GET1 membrane receptor and manganese superoxide dismutase 1 genes were potential candidates involving in osmotic regulation and ABA signaling (Zhu and Scandalios, 1994). In addition, *Co-QTLC5.1* with favourable alleles from 1012-98 (at QTL *IAA5.15.3*) and Express 617 (at QTL *DPA12.15.4*) is a major hormonal crosstalk QTL. Moreover, the Co-*QTLC8.1*, *Co-QTLA6.1* and *Co-QTLC7.1* with explained phenotypic variation value ranging 20.8–40.2 are hot spot regions for cross-time point correlation of IAA, ABA and DPA at 5 and 12 DAS.

Collectively, these results contribute to a better insight into the metabolomic and genomic mechanisms controlling germination and seedling development in *B. napus*, and identify genome regions and allelic variants with a putative positive role in enhancement of germination and vigour. With the current development in metabolomic and high-throughput sequencing technologies, translation of the results to new genetic maps with

high-density, sequence-annotated markers will help to narrow down QTL intervals for identification and validation of putative candidate genes, and to develop robust markers for marker-assisted breeding of rapeseed varieties with superior germination and seedling growth.

5 Summary

With the rapidly growing human population and greater climate extremes, the importance of seed vigour is increasing with time because the vigour is closely associated with crop yield and resource use efficiency. Therefore, the issue of seed vigour is of central importance to agriculture and the seed industry but it is still poorly understood. Oilseed rape is the most cultivated and economically important species within the *Brassica* genus during the past 30 years. In 2017-2018 global production of rapeseed exceeded 74.71 making them the second most valuable source of vegetable oil and protein meal (40.12 Mt) in the world after soybean. Unfortunately, most of the double low (00) rapeseed cultivars are associated with agronomic problems, such as lower seed yield, poor germination, and reduced seedling growth rate. This study aimed to use an integrative approach to explore key genetic and metabolomic factors modulating seed germination and seedling vigour and the molecular link between these factors to breed new rapeseed varieties with superior germination and seedling growth.

Seed germination and seedling vigour are quatitative traits determined by the interaction of hormonal, genetic and numerous environmental components. During the last decade, liquid chromatography-mass spectrometry (LC-MS), especially liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has become the most widely used approach to plant hormone analysis. In addition, gene cloning has made it possible for oilseed breeders to develop genotypes/cultivars with high yield potential, improved oil content and oil quality and improved agronomical traits. In the first study, high-throughput UPLC-MS/MS hormone profiling of seeds and seedlings before and after germination in the high vigour (Express 617) and low vigour (1012-98) lines and their offspring was analyzed to identify key hormone components and their crosstalk causing poor germination and seedling development. This study is the first to report disruption of germination and seedling

development in *B. napus* caused by the crosstalk of auxin-ABA and the corresponding regulators *Bna.ARF10* and *Bna.GH3.5*.

Quantitative trait locus (QTL) mapping is currently the most commonly used approach to dissect the genetic factors underlying complex traits. The goal of QTL mapping is to identify genomic regions associated with a specific complex phenotype by statistical analysis of the associations between genetic markers and phenotypic variation. In the second study, QTL mapping was used to identify quantitative trait loci related to germination, seedling vigour and seedling-regulated hormones in the same mapping population in the first study. A total of 13 QTL on nine chromosomes for germination and seedling-related traits at 7 and 14 days after sowing were detected. Forty-seven metabolic QTL on 15 chromosomes were identified for auxin, abscisic acid (ABA) and dihydrophaseic acid (DPA) at 5 and 12 DAS. Multitrait QTL hot spots contribute to our understanding of the genetics and metabolomics of germination and seeding vigour in *B. napus*, and represent potential targets to breed high-vigour cultivars.

The innovative value of this work relies on the identification of key genetic and metabolomic components contributing to further development of robust markers for marker-assisted breeding of rapeseed varieties with superior seed vigour. With the astounding current developments in high-throughput genomics and metabolomic technologies, translation of the work results to new high-density genetic and metabolic maps to develop rapeseed varieties with hormonal balance and superior seed vigour.

6 Zusammenfassung

Aufgrund der schnell wachsenden Weltbevölkerung und zunehmenden Klimaextremen nimmt die Bedeutung der Triebkraft von Saatkörnern zu, da die Keimfähigkeit und Keimlingsentwicklung vor allem unter suboptimalen Bedingungen eng mit dem Ernteertrag und der Ressourcennutzungseffizienz zusammenhängt. Für die Landwirtschaft und die Saatgutindustrie besitzen Keimungseigenschaften daher eine zentrale Bedeutung, jedoch ist dieses Merkmalskomplex bei den meisten Kulturpflanzen noch immer nicht vollständig aufgeklärt. Raps gehörte in den letzten 30 Jahren zu den am intensivsten gezüchteten und wirtschaftlich bedeutendsten Arter innerhalb der Gattung *Brassica*. Im Zeitraum 2017-2018 übertraf die weltweite Rapsproduktion 74.71 M ha und ist damit nach Sojabohnen die zweitwichtigste Ölpflanze der Welt. Allerdings treten in der Sortenzüchtung immer wieder Probleme mit z.B. schlechter Keimfähigkeit oder verzögerte Keimlingswachstum auf. Diese Studie zielte deswegen darauf ab, einen integrativen Ansatz zu verwenden, um die wichtigsten genetischen und metabolischen Faktoren zu untersuchen, die die Keimung und Keimkraft von Rapssamen sowie die molekularen Verbindungen zwischen diesen Faktoren beeinflussen. Die Ergebnisse sollten neue Informationen liefern, um Rapssorten mit überlegener Keimfähigkeit und Keimlingswachstum zu züchten.

Samenkeimung und Keimlingsvitalität sind quantitative Merkmale, die durch das Zusammenspiel von hormonellen, genetischen und zahlreichen Umweltkomponenten bestimmt werden. Während des letzten Jahrzehnts hat sich die Flüssigchromatographie-Massenspektrometrie (LC-MS), insbesondere die mit Tandem-Massenspektrometrie (LC-MS/MS) gekoppelte Flüssigchromatographie (LC-MS/MS), zum am weitesten verbreiteten Ansatz der Pflanzenhormonanalyse entwickelt. Darüber hinaus hat das Klonen von Genen die Entwicklung von Sorten mit hohem Ertragspotenzial, verbessertem Ölgehalt und

Ölqualität sowie verbesserten agronomischen Eigenschaften ermöglicht. In dieser Arbeit wurden zunöchst UPLC-MS/MS-Hormonprofile aus Samen und Keimlingen vor und nach der Keimung in Winterrapsgenotypen mit hoher (Express 617) bzw. niedriger Vitalität (1012-98) sowie deren spaltenden Kreuzungsnachkommen analysiert, um die wichtigsten Hormonkomponenten zu identifizieren und deren genetischen Zusammenhang mit der Keimung und Keimlingsentwicklung zu erläutern. Diese Studie berichtet erstmals über eine Störung der Keimung und Keimlingsentwicklung in *B. napus*, die offensichtlich durch eine Überexpression von Auxin-ABA aufgrund von Mutationen in den regulatorischen Genen *Bna.ARF10* und *Bna.GH3.5* verursacht wurde.

Quantitative Trait Locus (QTL) Kartierung ist derzeit der am häufigsten verwendeten Ansatz zur Analyse von genetischen Faktoren, die komplexen Eigenschaften zugrunde liegen. Das Ziel der QTL-Kartierung besteht darin, mit einem spezifischen komplexen Phänotyp assoziierte genomische Regionen durch statistische Analyse der Assoziationen zwischen genetischen Markern und phänotypischen Variationen zu identifizieren. Hier wurde in der o.g. Kartierungspopulation aus der Kreuzung zwischen Express 617 und 1012-98 eine QTL-Kartierung durchgeführt, um Loci zu identifizieren, die mit der Keimung, der Keimlingsvitalität und den mit Keimlingen regulierten Hormonen zusammenhängen. Insgesamt wurden 13 QTL auf neun Chromosomen für Keimfähigkeit bzw. für Keimlingseigenschaften 7 und 14 Tage nach der Aussaat detektiert. Darüber hinaus konnten 47 metabolische QTL auf 15 Chromosomen identifiziert, welche mit der Konzentration von Auxin, Abscisinsäure (ABA) und Dihydrophasesäure (DPA) bei 5 und 12 DAS zusammenhingen. *Multitrait-QTL-Hotspots* tragen zu einem besseren Verständnis der Genetik und Metabolomik der Keimung und der Keimkraft von *B. napus* bei und stellen potenzielle Ziele für die Züchtung von Sorten mit hohem Wuchs dar.

Der innovative Wert dieser Arbeit beruht auf der Identifizierung der wichtigsten genetischen und metabolischen Komponenten, die zur weiteren Entwicklung robuster Marker für die markergestützte Züchtung von Rapssorten mit verbesserter Keimkraft beitragen. Angesichts der rasanten aktuellen Entwicklungen in den Hochdurchsatz-*Omics*-Technologien kann die Umsetzung dieser Ergebnisse in Form von neuen, hochdichten genetischen und metabolischen Karten zur Entwicklung von Rapssorten mit verbessertem Hormonhaushalt und Triebkraft führen.

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8 Appendices

Appendix I: Supplementary materials from the following publication

Nguyen, T.C.T, C. Obermeier, W. Friedt, S.R. Abrams, and R.J. Snowdon, 2016: Disruption of germination and seedling development in *Brassica napus* by mutations causing severe seed hormonal imbalance. Frontiers in Plant Science **7**, 1-13.

Supplementary Table S1.

Internal standards used for hormone quantification by HPLC-ESI-MS/MS.

Metabolite	Abbreviation	Source
Abscisic acid	ABA	Sigma-Aldrich, St. Louis, MO, USA
Indole-3-acetic acid	IAA	
Indole-3-acetic acid aspartate	IAA-asp	
Zeatin	Z	
Zeatin riboside	ZR	
Isopentenyl adenosine	iPA	
Isopentenyl adenine	2iP	
Dihydrozeatin	dhZ	Olchemim Ltd., Olomouc, Czech Republic
Dihydrozeatin riboside	dhZR	
Zeatin-O-glucoside	Z-O-Glu	
Duterated forms	d3-DPA	NRC-PBI, Saskatoon, SK, Canada
	d5-ABA-GE	
	d3-PA	
	d4-70-OHABA	
	d3-neoPA	
	d4-ABA	
	d3-IAA-Asp	
	d3-IAA-Glu	
	d5-IAA	Cambridge Isotope Laboratories, Andover, MA, USA
	d3-dhZ	Olchemim Ltd., Olomouc, Czech Republic
	d3-dhZR	
	d5-Z-O-Glu	
	d6-iPA	
	d6-2iP	
Recovery standards	d6-ABA	NRC-PBI, Saskatoon, SK, Canada
	d2-ABA-GE	

ABA & metabolites

ABA	Abscisic acid
ABAGE	Abscisic acid glucose ester
DPA	Dihydrophaseic acid
PA	Phaseic acid
7'OH-ABA	7'-Hydroxy-abscisic acid
neoPA	neoPhaseic acid

Auxins

IAA	Indole-3-acetic acid
IAA-Asp	N-(Indole-3-yl-acetyl)-aspartic acid
IAA-Glu	N-(Indole-3-yl-acetyl)-glutamic acid
IAA-Ala	N-(Indole-3-yl-acetyl)-alanine
IAA-Leu	N-(Indole-3-yl-acetyl)-leucine

Cytokinins

t-ZOG	(*trans*) Zeatin-O-glucoside
c-ZOG	(*cis*) Zeatin-O-glucoside
t-Z	(*trans*) Zeatin
c-Z	(*cis*) Zeatin
dhZ	Dihydrozeatin
t-ZR	(*trans*) Zeatin riboside
c-ZR	(*cis*) Zeatin riboside
dhZR	Dihydrozeatin riboside
2iP	Isopentenyladenine
iPA	Isopentenyladenosine

Gibberellins

GA1	Gibberellin 1
GA3	Gibberellin 3
GA4	Gibberellin 4
GA7	Gibberellin 7

Colour coded phenotypes

- DH lines with more than 75% of seedlings expressed stunted growth, chlorotic/unopened cotyledons
- DH lines with 50-75% of seedlings expressed stunted growth, chlorotic/unopened cotyledons
- DH lines with 25-50% of seedlings expressed stunted growth, chlorotic/unopened cotyledons
- DH lines with less than 25% of seedlings expressed stunted growth, chlorotic/unopened cotyledons

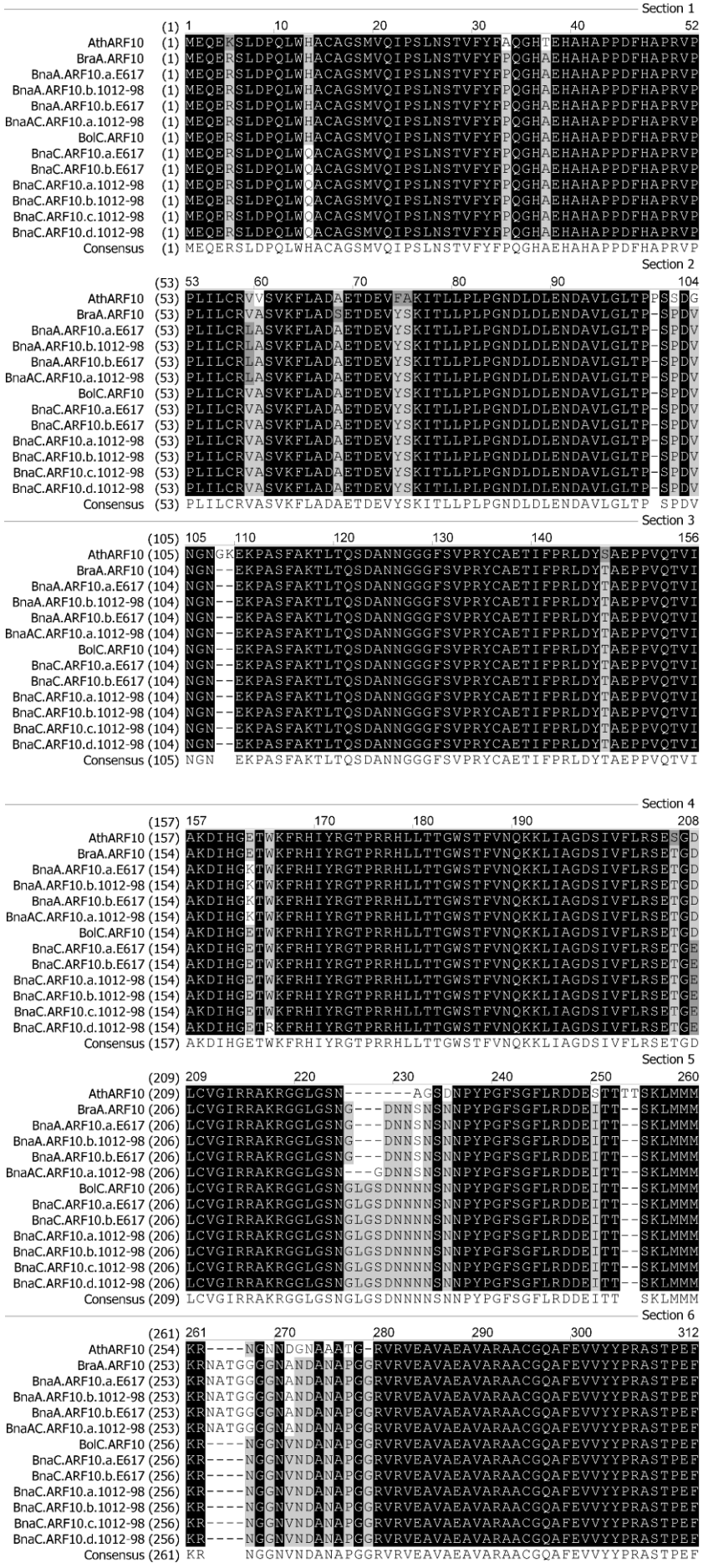
#	DH lines	PH7 (cm)	FW7 (g)	ABA metabolism: ABA (ng/g DW)	ABA metabolism: DPA (ng/g DW)	ABA metabolism: ABAGE (ng/g DW)	ABA metabolism: PA (ng/g DW)	ABA metabolism: 7'OH-ABA (ng/g DW)	ABA metabolism: n-PA (ng/g DW)	Auxins: IAA (ng/g DW)	Auxins: IAA-Ala (ng/g DW)	Auxins: IAA-Asp (ng/g DW)	Auxins: IAA-Glu (ng/g DW)	Auxins: IAA-Leu (ng/g DW)	Cytokinins: t-ZOG (ng/g DW)	Cytokinins: c-ZOG (ng/g DW)	Cytokinins: t-Z (ng/g DW)	Cytokinins: c-Z (ng/g DW)	Cytokinins: dhZ (ng/g DW)	Cytokinins: t-ZR (ng/g DW)	Cytokinins: c-ZR (ng/g DW)	Cytokinins: dhZR (ng/g DW)	Cytokinins: 2iP (ng/g DW)	Cytokinins: iPA (ng/g DW)	Gibberellins: GA1 (ng/g DW)	Gibberellins: GA3 (ng/g DW)	Gibberellins: GA4 (ng/g DW)	Gibberellins: GA7 (ng/g DW)
	Express61	3.72	0.12	910	4610	181	133	14.8	27.5	1230		126	6.73		< 26	< 26	< 1	< 2	< 3	< 4	230	1.78	1.06	19.2	< 128	< 23	6.21	< 42
	1012-98	4.6	0.10	95.7	< 506	84.7	21.1	< 4	< 4	289		> 26700	338		17.4	< 10	< 0.8	< 2	n.d.	n.d.	13.9	n.d.	< 0.9	< 4	< 48	< 20	5.71	< 38
1	YE2-DH005	3.69	0.12	167	503	< 150	36.8	< 7	6.66	151	< 3	19.7	< 10	< 3	< 10	< 11	< 3	< 4	< 5	< 5	< 12	n.d.	< 4	< 2	< 296	< 129	15.4	6.22
2	YE2-DH009	4.41	0.12	190	559	115	51.2	< 41	< 14	136	< 5	36.7	< 16	< 4	< 12	< 12	< 5	< 8	< 3	< 2	39.8	< 2	< 0.8	2.14	< 104	< 115	14.3	4.25
3	YE2-DH026	5.16	0.14	95.1	124	< 204	28.5	< 14	1.39	156	< 6	34.1	< 6	< 3	< 29	< 30	< 2	< 3	< 2	n.d.	11.1	< 0.8	< 3	1.51	< 207	< 98	9.51	2.39
4	YE2-DH031	3.74	0.12	83.3	< 452	< 151	58	< 9	< 4	124	< 5	36.6	< 9	< 3	< 22	< 23	< 2	< 4	< 4	< 4	12.8	< 2	< 0.4	< 2	17.7	< 162	8.77	2.34
5	YE2-DH036	4.26	0.17	181	510	< 181	80.6	< 17	4.79	186	< 2	26.1	< 11	< 4	< 18	< 19	< 2	< 4	< 3	< 2	14.6	< 2	n.d.	< 2	< 108	< 29	< 15	2.15
6	YE2-DH040	3.13	0.15	249	781	151	74.6	< 10	7.31	200	< 4	46.3	< 20	< 4	< 32	< 33	< 4	< 6	< 4	< 8	12	< 3	n.d.	1.22	< 340	< 50	12	1.91
7	YE2-DH041	4.58	0.13	180	459	< 162	61	< 20	4.82	177	< 9	60.1	< 7	< 3	< 10	< 10	< 6	< 9	< 3	2.84	13.8	n.d.	< 0.8	< 2	24.8	< 139	8.17	1.51
8	YE2-DH059	4.68	0.16	230	659	< 443	39.5	7.4	5.68	175	< 8	< 28	< 10	< 3	< 12	< 12	< 3	< 5	< 3	< 3	17.6	< 0.8	< 0.4	1.58	< 266	< 83	< 11	< 2
9	YE2-DH063	4.57	0.15	151	872	135	89.2	< 38	8.07	139	< 5	< 39	< 8	< 4	< 17	< 17	< 2	< 4	< 2	< 1	13	n.d.	< 1	< 2	< 351	< 71	< 9	2.24
10	YE2-DH064	4.30	0.14	297	622	< 254	72.8	< 12	< 14	271	< 7	31.1	< 14	< 3	15.2	< 9	< 3	< 5	< 2	< 3	18.8	< 3	< 1	< 2	< 481	21.3	< 9	1.51
11	YE2-DH066	6.44	0.17	127	229	< 95	63.7	< 32	5.53	139	< 11	38.4	< 10	< 3	< 60	< 62	< 1	< 2	< 2	< 2	11.7	< 6	< 0.3	1.69	< 638	< 160	< 10	1.63
12	YE2-DH079	4.28	0.13	75.9	141	< 255	15.5	< 26	< 18	177	< 3	< 26	< 7	< 3	< 21	< 22	< 1	< 2	< 2	< 2	8.19	< 2	< 39	< 2	< 164	< 19	< 8	< 1
13	YE2-DH080	3.76	0.14	334	855	< 184	80.4	< 9	13.2	231	3.93	< 33	< 12	< 3	< 17	< 18	< 0.8	< 1	< 2	< 2	26.6	< 3	< 4	< 2	23.2	< 65	< 18	< 2
14	YE2-DH088	4.77	0.15	162	514	< 359	33.1	< 19	9.45	235	< 2	36.7	< 7	< 4	< 21	< 22	< 2	< 4	< 3	< 2	12	< 3	< 3	1.34	< 300	< 19	< 8	< 2
15	YE2-DH091	5.94	0.16	166	287	< 146	85.7	< 15	< 11	176	< 4	< 39	< 8	< 3	< 45	< 46	< 1	< 2	< 3	< 2	10.2	< 2	< 1	< 2	< 308	< 67	< 20	< 2
16	YE2-DH094	3.96	0.13	62.9	184	< 185	46.7	< 7	2.11	78.9	< 3	< 18	< 13	< 3	< 19	< 19	< 1	< 2	< 1	1.25	7.91	< 1	< 2	< 2	< 390	< 19	< 9	< 1
17	YE2-DH096	4.09	0.13	106	< 869	< 129	104	< 5	< 8	132	n.d.	< 39	< 10	< 3	< 80	< 83	< 3	< 4	< 3	< 4	12.6	< 3	< 0.2	< 2	< 432	< 8	14.8	5.24
18	YE2-DH097	3.61	0.15	55.7	166	< 76	34.5	< 6	< 3	93.1	< 2	< 29	< 11	< 3	< 16	< 17	< 2	< 3	1.33	< 4	< 12	< 2	n.d.	< 3	< 208	< 58	10.8	3.25
19	YE2-DH100	3.79	0.16	167	311	137	138	< 22	3.83	170	< 4	< 31	< 9	< 3	< 35	< 36	< 5	< 8	< 3	< 2	8.66	< 2	< 39	< 3	< 300	< 82	< 10	2.36
20	YE2-DH112	5.14	0.13	176	1290	362	66.9	< 48	11.6	222	< 8	< 26	< 13	< 3	< 15	< 16	< 0.8	< 1	< 2	< 7	19.5	n.d.	< 2	1.74	43.1	< 118	14.8	< 2
21	YE2-DH113	3.64	0.13	90.6	262	116	21.5	< 8	5.29	97.4	2.57	< 29	< 22	< 3	< 32	< 33	< 2	< 4	< 2	< 5	< 9	< 3	n.d.	1.3	< 651	< 61	< 9	< 2
22	YE2-DH115	3.57	0.11	264	613	< 254	75.6	< 25	10.4	318	< 8	47.7	< 11	< 4	< 6	< 6	< 0.7	< 1	< 2	< 3	17.2	< 1	< 0.4	< 2	< 193	< 214	< 12	1.47
23	YE2-DH121	3.43	0.19	351	586	< 431	194	< 19	8.08	212	< 3	44.5	< 16	< 3	< 65	< 67	< 5	< 9	< 2	< 7	16.6	< 3	< 0.6	< 2	< 439	< 19	< 9	< 2
24	YE2-DH130	4.11	0.12	342	1130	< 1386	61.9	< 22	12.2	324	2.86	66.1	< 14	< 4	< 6	< 6	< 2	3.42	1.19	2.49	< 21	< 1	< 1	< 2	< 901	< 109	< 12	< 2
25	YE2-DH132	5.57	0.13	148	757	< 305	59.8	< 20	< 8	247	< 14	445	9.81	< 4	< 7	< 7	< 3	< 4	< 1	< 2	16.5	< 2	< 4	2.01	< 240	< 316	11.6	4.92
26	YE2-DH137	4.62	0.14	241	1000	< 603	96.5	< 25	8.64	174	3.55	18.5	< 5	< 4	< 27	< 28	n.d.	< 7	< 5	< 7	20.6	< 8	n.d.	< 3	< 343	< 177	10.7	2.88
27	YE2-DH138	4.84	0.11	241	304	< 1038	138	< 16	7.87	242	< 2	37.1	< 7	< 3	< 17	< 18	< 2	< 4	< 2	1.72	8.04	< 1	< 37	< 1	< 179	< 83	7.44	2.25
28	YE2-DH139	3.99	0.15	546	1700	< 337	290	< 74	24.1	299	< 2	15.5	< 9	< 4	< 21	< 21	< 2	< 4	< 1	< 6	38.3	< 3	< 1	< 3	< 498	< 28	< 6	1.87
29	YE2-DH143	3.84	0.16	136	275	< 343	42.5	< 12	4.76	111	< 5	108	< 10	< 3	< 46	< 47	< 1	< 2	< 2	< 1	9.98	< 1	< 0.2	< 2	< 96	< 161	< 8	< 2
30	YE2-DH161	4.32	0.14	369	2000	< 600	93.2	< 29	19.3	189	2.43	< 16	< 10	< 4	< 18	< 19	< 1	2.72	< 1	< 5	33	< 2	< 39	1.93	< 604	< 237	< 7	1.35
31	YE2-DH002	3.58	0.09	151	516	< 190	213	< 16	< 15	197	< 9	309	39.3	< 3	< 33	< 34	< 2	< 3	< 3	2.68	18.1	n.d.	< 1	< 2	< 10	< 129	10.1	1.58
32	YE2-DH008	4.90	0.12	349	1300	< 455	104	< 16	< 76	200	< 4	53.3	< 10	< 3	< 9	10.1	< 2	< 4	< 2	< 2	30.3	0.86	< 2	2	< 284	< 136	10.5	< 1
33	YE2-DH012	5.08	0.11	365	990	< 459	< 120	< 22	< 17	133	< 5	< 26	< 9	< 3	< 14	< 14	< 3	< 5	< 3	< 4	24	< 3	< 4	< 4	< 110	< 22	11.8	1.62
34	YE2-DH013	3.91	0.13	410	1790	297	275	< 32	25.2	361	< 6	35.8	< 18	< 4	< 12	< 12	< 0.8	< 1	< 0.9	< 6	35.7	< 2	< 0.8	3.31	< 377	< 282	< 6	< 3
35	YE2-DH024	4.33	0.10	143	516	< 440	57.5	< 41	< 17	146	< 2	< 65	< 6	< 3	19	< 13	< 2	< 4	< 3	< 3	11.5	< 2	< 0.5	< 2	< 400	< 534	< 9	< 1
36	YE2-DH028	4.76	0.12	240	1330	< 261	82.2	< 21	12.8	193	< 3	< 39	< 14	< 3	15.2	< 6	< 7	< 12	< 3	< 3	27.7	n.d.	< 2	2.13	< 247	< 237	< 8	< 1
37	YE2-DH042	3.87	0.09	123	205	< 229	156	< 29	< 19	278	< 7	189	16.2	< 4	< 13	< 13	< 2	< 4	3.52	1.83	9.74	< 2	< 12	< 2	< 756	< 160	< 21	< 4
38	YE2-DH043	5.33	0.09	400	626	< 204	133	< 46	14.7	306	< 8	159	19.4	< 4	< 16	< 16	< 2	< 3	< 1	< 2	42.8	< 2	n.d.	1.63	< 600	< 234	8.75	< 3
39	YE2-DH046	4.48	0.13	200	820	< 212	81.5	< 34	8.54	174	< 3	< 21	< 5	< 3	< 38	< 39	< 5	< 8	< 5	< 34	< 73	n.d.	< 38	1.65	< 318	< 368	9.16	< 0.9
40	YE2-DH051	6.15	0.15	105	327	< 162	107	< 22	5.5	1780	3.83	24750	67.3	< 3	< 32	< 33	< 3	< 5	< 6	< 3	10.9	< 4	< 39	1.29	< 537	< 127	11.7	< 0.8
41	YE2-DH054	3.75	0.08	73.8	90.3	< 322	40.6	< 17	< 7	165	< 12	158	< 13	< 3	< 46	< 48	< 8	< 14	< 5	< 4	9.15	n.d.	< 2	< 3	< 447	< 99	9.49	1.32
42	YE2-DH058	3.15	0.14	209	492	< 219	54.7	< 19	6.25	218	< 2	129	8.17	< 2	< 27	< 28	< 2	< 3	< 5	< 6	16.2	n.d.	< 39	< 3	20.5	< 216	< 9	< 1
43	YE2-DH060	3.97	0.12	144	< 641	< 676	117	< 52	< 24	193	< 4	47.8	< 10	< 3	< 23	< 24	< 3	< 5	< 5	< 6	< 13	n.d.	n.d.	< 2	< 164	< 20	< 7	< 1
44	YE2-DH062	3.91	0.12	127	524	141	89.3	< 17	< 6	240	6.41	81.1	13.3	< 3	< 24	< 25	< 3	< 6	< 3	< 6	12.5	< 3	< 0.5	< 2	< 841	< 59	< 5	< 1
45	YE2-DH067	5.05	0.13	75.7	206	< 207	68.8	< 8	< 5	84.2	< 6	52.1	5.41	n.d.	< 18	< 19	< 2	< 4	< 3	< 1	9.94	< 2	< 4	1.48	< 685	< 158	< 8	< 2
46	YE2-DH069	3.88	0.10	102	237	< 127	56.3	< 11	< 7	133	< 3	190	17.1	< 3	< 33	< 34	< 2	< 3	< 2	< 1	13.8	< 1	< 1	< 1	< 1094	< 117	7.03	< 1
47	YE2-DH076	4.34	0.11	263	726	< 231	76.2	< 16	10.4	230	< 7	< 34	< 14	< 3	< 12	< 12	< 5	< 9	< 1	< 3	23.2	n.d.	< 2	< 2	< 235	< 63	< 8	< 1
48	YE2-DH104	4.41	0.10	66.9	228	< 193	111	< 7	4.74	76.6	< 7	37	7.36	< 3	< 15	< 15	< 0.8	< 1	< 1	< 5	16	< 1	n.d.	< 1	< 199	< 18	7.1	< 1
49	YE2-DH117	4.87	0.10	127	237	< 495	36.7	< 37	< 6	174	5.07	157	< 19	< 4	< 35	< 36	< 4	< 7	< 5	< 10	27.6	n.d.	< 1	< 2	< 237	< 153	< 8	< 3
50	YE2-DH119	4.51	0.11	188	265	< 364	78.1	< 29	5.12	152	< 4	156	< 18	< 4	< 23	< 24	< 2	< 4	< 4	1.07	14.8	< 5	< 9	< 2	< 160	< 37	< 8	< 2
51	YE2-DH126	5.13	0.09	108	< 1903	< 693	114	< 12	5.49	372	< 8	> 30200	2200	< 5	< 11	< 11	< 2	< 4	< 2	< 6	15.1	< 3	< 7	< 3	< 10	< 19	< 9	< 4
52	YE2-DH128	4.58	0.12	142	403	943	20.2	< 25	8.51	104	< 4	193	< 7	< 3	< 54	< 56	< 3	< 6	< 3	< 6	< 12	n.d.	< 2	< 2	< 304	< 28	< 7	< 1
53	YE2-DH133	3.64	0.15	416	1290	816	142	< 32	< 21	130	< 10	88.9	< 12	< 3	< 25	< 25	< 3	< 5	< 2	< 5	31	< 2	< 40	< 3	< 438	< 189	< 6	< 2
54	YE2-DH135	5.34	0.16	106	273	424	63.7	< 38	< 7	56	< 2	56.1	< 11	< 3	< 38	< 40	< 1	< 2	< 2	< 3	13	< 3	< 38	< 2	< 397	< 19	9.71	< 1
55	YE2-DH136	4.72	0.07	217	504	591	54	< 21	< 21	195	< 7	83.6	< 9	< 4	< 12	< 13	< 2	< 3	< 2	< 2	17.2	< 8	< 0.2	< 2	55.1	< 78	< 10	< 2
56	YE2-DH148	4.70	0.13	87.7	428	968	< 50	< 11	6.46	126	< 3	33.7	< 7	< 3	< 31	< 32	< 3	< 5	< 3	< 2	11.3	< 2	< 2	< 2	< 78	< 114	< 10	< 1
57	YE2-DH157	4.30	0.09	208	635	609	59	< 7	8.69	156	< 2	45	< 8	< 3	< 19	< 20	< 2	n.d.	< 2	2.28	14.2	< 4	< 2	< 2	< 205	< 34	9.45	1.22
58	YE2-DH158	4.46	0.10	208	395	682	74.6	< 6	6.28	75.5	< 4	1540	< 14	< 4	< 33	< 34	< 3	< 5	< 2	< 6	12.4	< 3	< 2	< 1	< 346	< 19	8.78	< 1
59	YE2-DH163	4.00	0.12	253	598	872	70	< 11	13.4	98.5	< 3	45.6	< 7	< 2	< 13	< 13	< 3	< 5	< 2	1.94	24.7	< 3	0.34	1.72	< 336	< 139	< 10	< 1

#	DH lines	PH14 (cm)	FW14 (g)	ABA metabolism					Auxins			Cytokinins								Gibberellins			
				ABA (ng/g DW)	DPA (ng/g DW)	ABAGE (ng/g DW)	PA (ng/g DW)	7'OH-ABA (ng/g DW)	n-PA (ng/g DW)	IAA (ng/g DW)	IAA-Ala (ng/g DW)	IAA-Asp (ng/g DW)	IAA-Glu (ng/g DW)	IAA-Leu (ng/g DW)	t-ZOG (ng/g DW)	c-ZOG (ng/g DW)	t-Z (ng/g DW)	c-Z (ng/g DW)	dhZ (ng/g DW)	t-ZR (ng/g DW)	c-ZR (ng/g DW)	dhZR (ng/g DW)	2iP (ng/g DW)	iPA (ng/g DW)	GA1 (ng/g DW)	GA3 (ng/g DW)	GA4 (ng/g DW)	GA7 (ng/g DW)
	Express617	5.98	0.47	1080	9250	2890	45.9	64.8	34.7	473		< 11	5.76		< 28	< 28	< 1	3.19	< 1	< 5	103	< 1	0.86	7	< 59	< 48	< 6	< 60
	1012-98	6.96	0.39	430	5890	1820	116	< 28	30.8	419			23.4		< 22	< 22	< 0.9	2.31	< 1	< 3	74.6	< 3	< 2	1.26	< 81	< 37	< 6	n.d.
1	YE2-DH005	5.54	0.55	489	3760	1040	49.9	< 45	19.5	271	< 6	< 47	< 9	< 3	< 125	< 28	< 2	5.14	< 2	< 5	16.7	< 5	n.d.	1.96	< 588	< 400	< 10	< 1
2	YE2-DH009	5.43	0.55	282	2100	3980	91.3	14	17.2	212	< 5	< 18	< 27	< 3	27.7	39.2	< 2	6.59	< 1	< 6	15.8	< 6	< 7	< 2	< 166	< 42	< 9	< 1
3	YE2-DH026	7.32	0.53	287	1100	827	44.4	< 35	12.6	223	< 7	< 22	< 5	< 3	19.7	< 13	< 9	< 15	< 4	< 1	8.15	< 2	n.d.	< 3	< 568	< 19	< 8	< 1
4	YE2-DH031	4.88	0.50	231	1830	1030	35.2	< 25	14.1	206	< 2	214	< 17	< 3	< 39	< 41	< 3	< 4	< 1	< 6	19.6	< 2	< 4	< 2	< 274	< 137	< 7	< 1
5	YE2-DH036	6.65	0.74	491	1710	1390	88	< 83	18.1	364	< 13	< 55	< 16	< 4	< 39	< 40	< 2	6.65	< 1	< 9	102	< 3	< 0.5	6.38	< 311	< 374	< 10	< 1
6	YE2-DH040	4.78	0.60	600	2480	1130	97.9	< 46	20.3	498	< 9	2240	< 10	< 3	< 15	< 16	< 3	< 5	< 1	< 7	114	< 3	< 2	10.4	< 476	< 95	< 13	< 3
7	YE2-DH041	6.42	0.57	577	2670	2250	62.1	< 30	22.2	150	< 5	< 35	< 8	< 3	< 19	< 20	< 7	< 11	< 1	< 5	72.1	< 4	< 39	3.72	18	< 85	< 8	< 1
8	YE2-DH059	6.58	0.67	631	3160	2420	38.9	37.8	23.4	192	< 10	< 22	< 8	< 3	< 31	34.8	< 5	< 8	< 2	< 3	15.4	< 3	< 2	< 4	< 249	< 240	< 8	< 1
9	YE2-DH063	6.63	0.73	423	1960	1630	67.8	< 41	16.3	269	5.12	< 25	< 14	< 5	< 20	< 20	< 3	< 5	< 2	< 3	22.2	< 4	< 3	< 2	< 778	< 73	< 11	< 2
10	YE2-DH064	6.49	0.70	347	2190	< 3063	91.3	< 68	< 22	553	< 6	< 35	< 33	n.d.	< 71	< 74	< 3	< 5	< 2	< 13	< 27	< 4	n.d.	< 5	< 391	< 19	< 8	< 2
11	YE2-DH066	8.51	0.60	513	2100	2200	74.9	< 24	< 26	307	< 6	< 34	< 14	< 3	< 40	< 42	< 6	< 9	< 5	< 3	< 13	< 4	n.d.	< 5	< 265	< 104	< 9	< 1
12	YE2-DH079	6.06	0.55	454	3110	2330	48.1	< 27	< 21	< 157	< 7	< 89	< 13	< 4	< 108	< 112	< 9	< 14	< 4	< 9	< 15	< 4	< 40	< 3	< 252	< 568	< 9	< 1
13	YE2-DH080	5.46	0.76	666	2070	2590	98.7	40.3	22.4	294	< 13	< 32	< 17	< 4	42.5	< 38	< 4	< 47	< 3	< 7	51.3	n.d.	< 2	< 7	< 254	< 4	< 10	< 1
14	YE2-DH088	7.76	0.60	330	2030	1750	23.9	< 45	19.2	467	< 7	< 28	< 15	< 6	< 344	< 356	< 4	< 7	< 2	< 8	< 20	< 5	< 6	< 2	< 387	< 39	< 11	2.64
15	YE2-DH091	7.96	0.61	293	1550	1050	29.2	< 85	< 14	166	< 4	< 58	< 10	< 4	< 29	< 30	< 6	< 10	< 20	< 7	25.5	< 2	< 3	< 2	< 176	< 94	< 10	< 2
16	YE2-DH094	5.50	0.65	350	1660	737	141	15.4	18.6	193	< 8	66.1	< 12	< 4	< 120	< 124	< 18	< 29	< 6	< 7	< 12	< 2	< 39	< 4	72.6	< 118	< 6	< 2
17	YE2-DH096	6.02	0.57	199	934	1330	36.6	< 51	10.7	149	< 5	< 25	< 11	< 4	< 19	< 20	< 2	< 3	< 2	< 6	24.9	n.d.	< 4	< 4	< 81	< 48	< 11	< 1
18	YE2-DH097	5.03	0.72	327	2390	719	61.9	< 27	18.6	408	< 4	< 45	< 17	< 4	< 24	< 25	< 8	< 14	< 2	< 5	10.7	< 3	< 7	< 3	< 60	< 253	< 11	< 1
19	YE2-DH100	5.63	0.76	417	1220	1560	52.8	28.6	14.7	336	< 8	117	< 17	< 4	< 17	< 18	< 12	< 20	< 6	< 7	30	< 3	n.d.	< 4	< 327	< 68	< 8	< 1
20	YE2-DH112	8.58	0.50	242	2020	1100	32.3	< 34	14.3	289	< 4	< 37	< 19	< 4	< 47	65.8	< 3	< 6	< 3	< 5	15.8	< 11	< 1	< 3	< 734	< 46	< 11	< 1
21	YE2-DH113	4.84	0.60	269	1430	996	49.1	< 31	< 80	170	< 4	< 18	< 20	< 5	< 21	< 21	< 2	< 4	< 2	< 5	23.6	< 3	< 2	1.8	< 116	< 94	< 8	< 1
22	YE2-DH115	4.33	0.84	272	2850	1630	58.8	< 25	11.7	346	3.47	< 42	< 30	< 5	< 156	< 161	< 5	< 8	< 3	< 12	< 23	n.d.	< 40	< 1	< 433	< 123	< 12	< 2
23	YE2-DH121	5.62	0.81	491	3890	1680	120	30.7	24	561	< 6	< 61	< 11	< 5	< 50	< 51	< 10	< 16	< 5	< 9	< 15	< 5	< 37	1.47	< 244	< 100	< 16	< 2
24	YE2-DH130	5.36	0.47	430	3480	1080	68.9	< 23	18.3	270	< 8	< 47	< 39	< 6	< 85	< 88	< 8	< 14	< 12	< 4	< 9	< 5	< 4	2.01	< 271	< 184	< 7	< 1
25	YE2-DH132	7.86	0.50	241	1730	1250	26.7	< 16	< 26	280	< 5	1850	< 16	< 5	< 32	< 33	< 2	< 4	< 2	< 8	18.3	< 4	< 0.1	< 6	< 115	< 71	< 10	< 2
26	YE2-DH137	7.27	0.54	322	1840	4050	66	< 28	< 18	259	< 26	< 27	< 25	< 5	< 55	< 57	< 2	< 4	< 2	< 3	10.1	< 5	< 2	< 4	< 382	< 20	< 8	< 2
27	YE2-DH138	7.48	0.31	298	1850	< 3974	< 143	< 30	< 37	233	< 4	< 32	< 11	< 4	< 60	< 63	< 7	< 11	< 3	1.67	13.7	< 7	< 37	1.96	< 570	< 290	< 12	< 2
28	YE2-DH139	5.49	0.64	483	2420	1910	141	< 27	26.2	217	< 19	20.1	< 17	< 5	< 64	< 66	< 2	5.19	< 4	< 4	31.1	< 7	< 3	< 4	< 130	< 57	< 9	< 1
29	YE2-DH143	6.23	0.62	249	1190	1630	75.3	< 28	< 18	203	< 14	70.7	< 15	< 4	< 21	< 22	< 7	< 11	< 11	< 38	< 29	< 4	< 37	< 5	< 420	< 23	< 11	< 2
30	YE2-DH161	6.17	0.58	655	4320	< 1203	54.8	39.9	29	123	< 6	< 41	< 8	< 4	< 53	62.8	< 6	< 11	< 3	< 4	< 16	< 1	n.d.	< 2	< 154	< 24	< 7	< 1
31	YE2-DH002	4.66	0.32	281	2190	1470	102	< 26	< 29	341	< 9	35.5	< 35	< 5	< 38	< 39	< 9	< 14	< 3	1.41	14.6	< 5	< 3	1.41	< 215	< 32	< 11	< 3
32	YE2-DH008	6.70	0.45	292	2390	2550	66	31.3	19.1	399	< 8	< 44	< 11	< 5	< 97	< 100	< 4	< 6	< 3	< 12	27.4	< 3	< 38	< 6	< 346	< 62	< 11	< 2
33	YE2-DH012	7.94	0.44	453	3250	2030	110	< 48	< 32	217	< 10	< 27	< 13	< 5	< 57	< 59	< 3	< 6	< 2	< 9	< 20	< 6	< 4	< 4	< 475	< 129	< 12	< 2
34	YE2-DH013	5.77	0.48	295	762	< 1957	92.3	< 10	11.6	278	< 9	< 35	< 9	< 5	< 18	< 18	< 5	< 8	< 4	< 8	12.2	< 5	n.d.	< 3	< 562	< 41	< 10	< 2
35	YE2-DH024	7.46	0.35	258	2000	1230	57.6	< 61	15.1	189	< 11	< 39	< 28	< 4	< 53	< 54	< 3	< 6	< 2	< 18	12.4	< 15	n.d.	n.d.	< 335	< 298	< 12	< 2
36	YE2-DH028	7.30	0.45	508	3720	2890	103	< 136	25.5	347	6.43	< 79	< 19	n.d.	< 55	< 57	< 7	< 11	< 4	< 4	28.3	< 6	< 6	< 5	< 236	< 118	< 12	< 6
37	YE2-DH042	5.00	0.26	738	3500	4040	192	< 71	35.1	355	< 25	< 129	< 17	< 12	< 223	< 230	< 13	< 22	< 7	< 10	45.5	< 4	n.d.	< 10	< 971	< 283	< 14	< 3
38	YE2-DH043	8.04	0.33	492	5690	2140	193	< 14	26.6	325	< 5	< 43	< 13	< 4	< 32	< 33	< 4	< 7	< 2	< 2	51.7	< 2	< 39	3.55	< 448	< 87	< 10	< 2
39	YE2-DH046	6.27	0.52	423	2370	851	66.4	< 31	19	230	< 14	< 24	< 18	< 3	< 44	52.2	< 11	< 18	n.d.	< 6	9.26	< 2	< 38	< 1	< 431	< 67	< 11	< 1
40	YE2-DH051	9.04	0.44	159	1060	868	64.1	< 13	9.51	250	< 4	8340	40.8	n.d.	< 11	< 11	< 3	< 6	< 2	< 2	25.8	< 3	< 39	1.54	< 189	< 19	< 10	< 2
41	YE2-DH054	5.95	0.45	153	1200	606	68.4	< 10	12.3	252	< 5	< 48	< 16	< 5	< 15	25.9	< 2	3.73	< 2	< 3	8.98	n.d.	< 2	< 2	< 569	< 89	< 10	< 1
42	YE2-DH058	6.18	0.46	231	601	< 1098	< 50	< 17	9.39	315	< 2	< 39	< 9	< 4	< 12	< 13	< 4	< 6	< 1	< 4	22.7	< 2	< 39	< 5	< 437	< 111	< 8	< 2
43	YE2-DH090	5.74	0.47	275	2230	891	59.3	6.35	14.9	336	< 7	< 24	< 20	< 4	< 12	38.2	< 4	< 7	< 0.8	< 6	24.9	< 4	< 39	< 3	< 123	< 11	< 11	< 1
44	YE2-DH052	6.89	0.47	479	2870	1230	62.4	< 30	23.7	444	< 5	< 26	< 10	< 5	< 31	37.4	< 2	4.99	< 1	< 3	66.3	0.72	< 38	3.78	< 497	< 31	< 9	< 8
45	YE2-DH097	8.10	0.40	207	614	< 446	< 135	< 12	8.69	201	< 3	< 28	< 11	< 3	< 9	< 10	< 6	< 13	< 2	n.d.	32.5	n.d.	< 39	1.86	< 337	< 200	< 9	< 2
46	YE2-DH069	5.12	0.31	148	506	419	< 66	< 38	10.8	197	< 5	67.7	9.33	< 3	10.5	< 10	< 1	< 2	< 1	< 3	26.9	n.d.	< 0.7	< 5	< 120	< 57	< 13	2.61
47	YE2-DH076	6.38	0.41	362	2490	1400	54.2	37.6	15	263	< 9	< 13	< 23	< 5	< 37	50.3	< 3	< 4	< 2	n.d.	12.2	< 2	< 40	< 2	< 265	< 55	< 8	< 2
48	YE2-DH104	6.14	0.52	157	1400	< 740	28.7	< 25	10.5	139	< 14	< 22	< 10	< 3	< 38	< 39	< 6	< 10	< 4	< 4	16.9	< 5	< 38	< 3	< 365	< 145	< 9	< 1
49	YE2-DH117	9.06	0.36	333	1180	896	225	< 39	17.2	584	< 7	< 41	< 39	< 6	< 21	< 22	< 3	< 6	< 2	< 9	110	< 2	< 4	4.82	< 416	< 260	< 11	< 2
50	YE2-DH119	7.12	0.38	176	1770	533	72.6	< 48	10.3	406	< 7	< 31	< 18	< 4	< 75	< 77	< 4	< 6	< 1	< 7	33.8	< 7	< 6	3	< 287	< 353	< 14	< 2
51	YE2-DH126	8.38	0.28	547	3220	2650	175	< 21	< 40	381	< 3	< 1539	170	< 6	< 57	< 59	< 5	< 8	< 5	< 3	51.6	< 3	< 38	2.45	< 631	< 12	< 10	< 2
52	YE2-DH128	7.00	0.41	291	2020	1230	57.4	< 20	16.2	429	< 12	< 36	< 23	< 4	< 29	63.8	< 4	< 7	< 2	< 3	10.3	< 6	< 39	< 2	< 265	< 112	< 12	< 2
53	YE2-DH133	5.57	0.70	315	1850	1530	71.2	< 62	< 17	273	< 9	< 26	< 26	< 4	< 26	38.1	< 3	< 4	2.05	n.d.	18.4	< 11	< 17	1.93	< 634	< 95	< 11	< 1
54	YE2-DH135	7.24	0.52	384	1230	840	89.5	< 58	13	242	7.65	< 20	< 14	< 4	< 11	< 12	< 1	2.87	< 2	< 3	68	n.d.	4.14	< 283	< 33	< 9	4.61	
55	YE2-DH136	7.51	0.37	1130	6660	3430	279	37.1	53.1	483	< 8	52.3	< 8	< 5	< 13	28.2	< 3	8.75	2.37	< 2	78.9	< 4	< 2	4.8	< 247	< 630	< 17	4.45
56	YE2-DH148	6.56	0.47	473	2450	1340	28.7	< 28	21.8	334	< 10	< 20	< 15	< 4	< 86	< 89	< 2	4.82	< 1	< 5	45.3	< 4	< 0.5	2.66	< 416	< 60	< 10	< 3
57	YE2-DH157	6.01	0.39	524	2850	2190	58.6	51	< 49	448	< 4	< 34	< 17	< 3	< 96	< 100	< 6	< 9	< 2	< 3	10.6	n.d.	< 39	1.88	< 495	< 284	< 14	< 1
58	YE2-DH158	5.95	0.35	392	2470	1610	< 171	< 12	21.5	199	< 6	< 20	< 13	< 4	25.6	< 15	< 2	< 4	< 0.9	< 6	27.3	0.62	n.d.	1.93	< 214	< 219	< 11	< 2
59	YE2-DH163	5.36	0.51	338	1850	1180	94.3	20.6	< 32	264	< 9	< 30	< 15	< 5	< 11	< 11	< 2	< 4	< 2	< 2	< 9	0.56	< 38	< 2	114	< 84	< 10	< 2

#	Parental lines/Timepoints	ABA metabolism (ng/g DW)						Auxins (ng/g DW)			Cytokinins (ng/g DW)										Gibberellins (ng/g DW)			
		ABA	DPA	ABAGE	PA	7'OH-ABA	n-PA	IAA	IAA-Asp	IAA-Glu	t-ZOG	c-ZOG	t-Z	c-Z	dhZ	t-ZR	c-ZR	dhZR	2iP	iPA	GA3	GA1	GA7	GA4
1	E617-0DAS	49.4		53.1	45	5.47		68.3		1.79							0.74							4.07
2	1012-98-0DAS	47.2		46	19.1		1.49	593	26100	1100							3.34					44.3		
3	E617-1DAS	12.4		22.4	90.9	7.82		42.2	48.5							1.13	3.23							5.49
4	1012-98-1DAS	69.7			7.74	5.43		390	23400	383							30.3					27.6		
5	E617-2DAS	10.5		27.1	106	3.99		42.8	21.3								3.31							5.32
6	1012-98-2DAS	41.7	132	44.7	95.5	5.3		306	26700	487							19.3					63.3		
7	E617-3DAS	25		27.7	21.6	2.43		42.3	53.9								6.06							3.99
8	1012-98-3DAS	38.3		30.1	144			158	24100	502							17							
9	E617-4DAS	220	822	34.2	94.7		6.6	325	34.6								75.3					30.7		36.3
10	1012-98-4DAS	25.3			61.9			225	25700	753							28.6							
11	E617-5DAS	910	4610	181	133	14.8	27.5	1230	126	6.73							230	1.78	1.06	19.2				6.21
12	1012-98-5DAS	95.7		84.7	21.1			289	26700	338	17.4						13.9							5.71
13	E617-6DAS	156	442	57.7	138		6.54	247								0.12	85.7		0.56	4.86				10
14	1012-98-6DAS	288	804	173	65.9		8.71	416	28800	373							64							
15	E617-7DAS	330	1170	151	20.1	4.46	6.67	659	10.7	3.75							183	1.04		9.94				9.45
16	1012-98-7DAS	208	677	212	33.7	4.15		513	32000	138							68.7							
17	E617-8DAS	998	4400	366	61.2	11.8	28.5	900	19.8	6.04				4.3			430			27.9				11.4
18	1012-98-8DAS	671	2800	486	86.8	5.82	14.7	526	5140	24.5				5.08			224			13		23.9		
19	E617-10DAS	1610	6630	959	66.4	43.2	29.6	980	18.6	7.35				5.59			406		2.58	35.5				
20	1012-98-10DAS	834	4990	1020	121	10.3	29.4	622	1490	22.1				6.41			250			13.7				
21	E617-12DAS	1080	9250	2890	45.9	64.8	34.7	473		5.76				3.19			103		0.86	7				
22	1012-98-12DAS	430	5890	1820	116		30.8	419	454	23.4				2.31			74.6			1.26				

Supplementary Table S3. Pairwise identities (%) in genomic/coding regions (above diagonal) and deduced protein sequences (below diagonal) from *ARF10* orthologues in *A. thaliana* (*At*), *B. rapa* (*Bra*), *B. oleracea* (*Bol*) natural *B. napus* (*Bna*) line Express 617 (E617) and resynthesised *B. napus* line 1012-98, respectively. Locus/allele nomenclature follows the convention for *Brassica* spp. described by Østergaard and King (2008): [Species – 3 letter code][genome – A or C][gene – ARF10].[locus].[allele – in this case E617 or 1012-98].

Locus/Allele	*At.ARF10*	*BraA.ARF10*	*BnaA.ARF10.a.E617*	*BnaA.ARF10.b.E617*	*BnaA.ARF10.b.1012-98*	*BnaAC.ARF10.a.1012-98*	*BolC.ARF10*	*BnaC.ARF10.a.E617*	*BnaC.ARF10.b.E617*	*BnaC.ARF10.a.1012-98*	*BnaC.ARF10.b.1012-98*	*BnaC.ARF10.c.1012-98*	*BnaC.ARF10.d.1012-98*
At.ARF10		85/88	85/88	85/88	85/88	85/88	85/88	85/88	85/88	85/88	85/88	85/88	85/88
BraA.ARF10	91		99/99	99/99	99/99	98/98	97/97	97/97	97/97	97/97	97/97	97/97	97/97
BnaA.ARF10.a.E617	95	99		100/100	100/100	98/99	97/97	97/97	97/97	97/97	97/97	97/97	97/97
BnaA.ARF10.b.E617	95	99	100		100/100	98/99	97/97	97/98	97/98	97/98	97/97	97/98	97/97
BnaA.ARF10.b.1012-98	95	99	100	100		98/99	97/97	97/98	97/98	97/98	97/97	97/98	97/97
BnaAC.ARF10.a.1012-98	95	98	99	100	100		98/98	99/99	99/98	99/99	99/98	99/98	99/98
BolC.ARF10	91	98	98	98	98	98		100/100	99/99	99/100	99/99	99/99	99/99
BnaC.ARF10.a.E617	95	98	99	99	99	100	99		100/100	100/100	100/100	100/100	100/100
BnaC.ARF10.b.E617	95	98	99	99	99	100	99	100		100/100	100/100	100/100	100/100
BnaC.ARF10.a.1012-98	95	98	99	99	99	100	99	100	100		100/100	100/100	100/100
BnaC.ARF10.b.1012-98	95	98	99	99	99	100	99	100	100	100		100/100	100/100
BnaC.ARF10.c.1012-98	95	98	99	99	99	100	99	100	100	100	100		100/100
BnaC.ARF10.d.1012-98	90	98	99	99	99	99	99	100	100	100	100	100	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Section 2
Section 3
Section 4
Section 5
Section 6
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BnaA.ARF10.b.1012-98
BnaA.ARF10.b.E617
BnaAC.ARF10.a.1012-98
BolC.ARF10
BnaC.ARF10.a.E617
BnaC.ARF10.b.E617
BnaC.ARF10.a.1012-98
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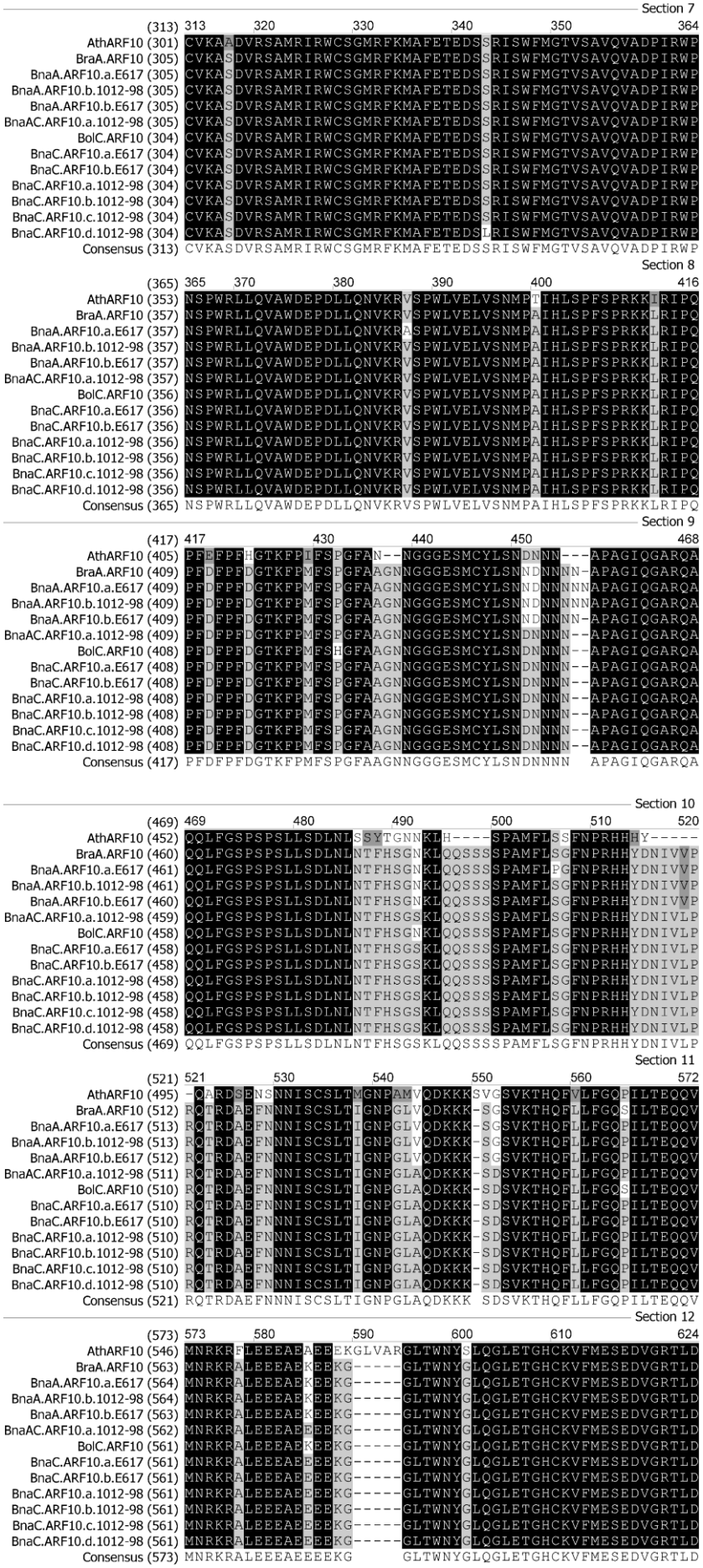
BnaC.ARF10.d.1012-98
Consensus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BnaA.ARF10.a.E617 (305) CVKASDVRSAMRIRWCSGMRFKMAFETEDSSRISWFMGTVSAVQVADPIRWP
BnaA.ARF10.b.1012-98 (305) CVKASDVRSAMRIRWCSGMRFKMAFETEDSSRISWFMGTVSAVQVADPIRWP
BnaA.ARF10.b.E617 (305) CVKASDVRSAMRIRWCSGMRFKMAFETEDSSRISWFMGTVSAVQVADPIRWP
BnaAC.ARF10.a.1012-98 (305) CVKASDVRSAMRIRWCSGMRFKMAFETEDSSRISWFMGTVSAVQVADPIRWP
BolC.ARF10 (304) CVKASDVRSAMRIRWCSGMRFKMAFETEDSSRISWFMGTVSAVQVADPIRWP
BnaC.ARF10.a.E617 (304) CVKASDVRSAMRIRWCSGMRFKMAFETEDSSRISWFMGTVSAVQVADPIRWP
BnaC.ARF10.b.E617 (304) CVKASDVRSAMRIRWCSGMRFKMAFETEDSSRISWFMGTVSAVQVADPIRWP
BnaC.ARF10.a.1012-98 (304) CVKASDVRSAMRIRWCSGMRFKMAFETEDSSRISWFMGTVSAVQVADPIRWP
BnaC.ARF10.b.1012-98 (304) CVKASDVRSAMRIRWCSGMRFKMAFETEDSSRISWFMGTVSAVQVADPIRWP
BnaC.ARF10.c.1012-98 (304) CVKASDVRSAMRIRWCSGMRFKMAFETEDSSRISWFMGTVSAVQVADPIRWP
BnaC.ARF10.d.1012-98 (304) CVKASDVRSAMRIRWCSGMRFKMAFETEDSLRISWFMGTVSAVQVADPIRWP
Consensus (313) CVKASDVRSAMRIRWCSGMRFKMAFETEDSSRISWFMGTVSAVQVADPIRWP
Section 8
(365) 365 370 380 390 400 416
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BraA.ARF10 (357) NSPWRLLQVAWDEPDLLQNVKRVSPWLVELVSNMPAIHLSPFSPRKKLRIPQ
BnaA.ARF10.a.E617 (357) NSPWRLLQVAWDEPDLLQNVKRASPWLVELVSNMPAIHLSPFSPRKKLRIPQ
BnaA.ARF10.b.1012-98 (357) NSPWRLLQVAWDEPDLLQNVKRVSPWLVELVSNMPAIHLSPFSPRKKLRIPQ
BnaA.ARF10.b.E617 (357) NSPWRLLQVAWDEPDLLQNVKRVSPWLVELVSNMPAIHLSPFSPRKKLRIPQ
BnaAC.ARF10.a.1012-98 (357) NSPWRLLQVAWDEPDLLQNVKRVSPWLVELVSNMPAIHLSPFSPRKKLRIPQ
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BnaC.ARF10.b.E617 (356) NSPWRLLQVAWDEPDLLQNVKRVSPWLVELVSNMPAIHLSPFSPRKKLRIPQ
BnaC.ARF10.a.1012-98 (356) NSPWRLLQVAWDEPDLLQNVKRVSPWLVELVSNMPAIHLSPFSPRKKLRIPQ
BnaC.ARF10.b.1012-98 (356) NSPWRLLQVAWDEPDLLQNVKRVSPWLVELVSNMPAIHLSPFSPRKKLRIPQ
BnaC.ARF10.c.1012-98 (356) NSPWRLLQVAWDEPDLLQNVKRVSPWLVELVSNMPAIHLSPFSPRKKLRIPQ
BnaC.ARF10.d.1012-98 (356) NSPWRLLQVAWDEPDLLQNVKRVSPWLVELVSNMPAIHLSPFSPRKKLRIPQ
Consensus (365) NSPWRLLQVAWDEPDLLQNVKRVSPWLVELVSNMPAIHLSPFSPRKKLRIPQ
Section 9
(417) 417 430 440 450 468
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BraA.ARF10 (409) PFDFPFDGTKFPMFSPGFAAGNNGGGESMCYLSNNDNNNN-APAGIQGARQA
BnaA.ARF10.a.E617 (409) PFDFPFDGTKFPMFSPGFAAGNNGGGESMCYLSNNDNNNNAPAGIQGARQA
BnaA.ARF10.b.1012-98 (409) PFDFPFDGTKFPMFSPGFAAGNNGGGESMCYLSNNDNNNNAPAGIQGARQA
BnaA.ARF10.b.E617 (409) PFDFPFDGTKFPMFSPGFAAGNNGGGESMCYLSNNDNNNN-APAGIQGARQA
BnaAC.ARF10.a.1012-98 (409) PFDFPFDGTKFPMFSPGFAAGNNGGGESMCYLSNDNNNN--APAGIQGARQA
BolC.ARF10 (408) PFDFPFDGTKFPMFSHGFAAGNNGGGESMCYLSNDNNNN--APAGIQGARQA
BnaC.ARF10.a.E617 (408) PFDFPFDGTKFPMFSPGFAAGNNGGGESMCYLSNDNNNN--APAGIQGARQA
BnaC.ARF10.b.E617 (408) PFDFPFDGTKFPMFSPGFAAGNNGGGESMCYLSNDNNNN--APAGIQGARQA
BnaC.ARF10.a.1012-98 (408) PFDFPFDGTKFPMFSPGFAAGNNGGGESMCYLSNDNNNN--APAGIQGARQA
BnaC.ARF10.b.1012-98 (408) PFDFPFDGTKFPMFSPGFAAGNNGGGESMCYLSNDNNNN--APAGIQGARQA
BnaC.ARF10.c.1012-98 (408) PFDFPFDGTKFPMFSPGFAAGNNGGGESMCYLSNDNNNN--APAGIQGARQA
BnaC.ARF10.d.1012-98 (408) PFDFPFDGTKFPMFSPGFAAGNNGGGESMCYLSNDNNNN--APAGIQGARQA
Consensus (417) PFDFPFDGTKFPMFSPGFAAGNNGGGESMCYLSNDNNNN APAGIQGARQA
Section 10
(469) 469 480 490 500 510 520
AthARF10 (452) QQLFGSPSPSLLSDLNLSSYTGNNKLH----SPAMFLSSFNPRHHHY-----
BraA.ARF10 (460) QQLFGSPSPSLLSDLNLNTFHSGNKLQQSSSSPAMFLSGFNPRHHYDNIVVP
BnaA.ARF10.a.E617 (461) QQLFGSPSPSLLSDLNLNTFHSGNKLQQSSSSPAMFLPGFNPRHHYDNIVVP
BnaA.ARF10.b.1012-98 (461) QQLFGSPSPSLLSDLNLNTFHSGNKLQQSSSSPAMFLSGFNPRHHYDNIVVP
BnaA.ARF10.b.E617 (460) QQLFGSPSPSLLSDLNLNTFHSGNKLQQSSSSPAMFLSGFNPRHHYDNIVVP
BnaAC.ARF10.a.1012-98 (459) QQLFGSPSPSLLSDLNLNTFHSGSKLQQSSSSPAMFLSGFNPRHHYDNIVLP
BolC.ARF10 (458) QQLFGSPSPSLLSDLNLNTFHSGNKLQQSSSSPAMFLSGFNPRHHYDNIVLP
BnaC.ARF10.a.E617 (458) QQLFGSPSPSLLSDLNLNTFHSGSKLQQSSSSPAMFLSGFNPRHHYDNIVLP
BnaC.ARF10.b.E617 (458) QQLFGSPSPSLLSDLNLNTFHSGSKLQQSSSSPAMFLSGFNPRHHYDNIVLP
BnaC.ARF10.a.1012-98 (458) QQLFGSPSPSLLSDLNLNTFHSGSKLQQSSSSPAMFLSGFNPRHHYDNIVLP
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Section 11
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BraA.ARF10 (512) RQTRDAEFNNNISCSLTIGNPGLVQDKKK-SGSVKTHQFLLFGQSILTEQQV
BnaA.ARF10.a.E617 (513) RQTRDAEFNNNISCSLTIGNPGLVQDKKK-SGSVKTHQFLLFGQPILTEQQV
BnaA.ARF10.b.1012-98 (513) RQTRDAEFNNNISCSLTIGNPGLVQDKKK-SGSVKTHQFLLFGQPILTEQQV
BnaA.ARF10.b.E617 (512) RQTRDAEFNNNISCSLTIGNPGLVQDKKK-SGSVKTHQFLLFGQPILTEQQV
BnaAC.ARF10.a.1012-98 (511) RQTRDAEFNNNISCSLTIGNPGLAQDKKK-SDSVKTHQFLLFGQPILTEQQV
BolC.ARF10 (510) RQTRDAEFNNNISCSLTIGNPGLAQDKKK-SDSVKTHQFLLFGQSILTEQQV
BnaC.ARF10.a.E617 (510) RQTRDAEFNNNISCSLTIGNPGLAQDKKK-SDSVKTHQFLLFGQPILTEQQV
BnaC.ARF10.b.E617 (510) RQTRDAEFNNNISCSLTIGNPGLAQDKKK-SDSVKTHQFLLFGQPILTEQQV
BnaC.ARF10.a.1012-98 (510) RQTRDAEFNNNISCSLTIGNPGLAQDKKK-SDSVKTHQFLLFGQPILTEQQV
BnaC.ARF10.b.1012-98 (510) RQTRDAEFNNNISCSLTIGNPGLAQDKKK-SDSVKTHQFLLFGQPILTEQQV
BnaC.ARF10.c.1012-98 (510) RQTRDAEFNNNISCSLTIGNPGLAQDKKK-SDSVKTHQFLLFGQPILTEQQV
BnaC.ARF10.d.1012-98 (510) RQTRDAEFNNNISCSLTIGNPGLAQDKKK-SDSVKTHQFLLFGQPILTEQQV
Consensus (521) RQTRDAEFNNNISCSLTIGNPGLAQDKKK SDSVKTHQFLLFGQPILTEQQV
Section 12
(573) 573 580 590 600 610 624
AthARF10 (546) MNRKRFLEEEAEAEEEKGLVARGLTWNYSLQGLETGHCKVFMESEDVGRTLD
BraA.ARF10 (563) MNRKRALEEEAEKEEKG-----GLTWNYGLQGLETGHCKVFMESEDVGRTLD
BnaA.ARF10.a.E617 (564) MNRKRALEEEAEKEEKG-----GLTWNYGLQGLETGHCKVFMESEDVGRTLD
BnaA.ARF10.b.1012-98 (564) MNRKRALEEEAEKEEKG-----GLTWNYGLQGLETGHCKVFMESEDVGRTLD
BnaA.ARF10.b.E617 (563) MNRKRALEEEAEKEEKG-----GLTWNYGLQGLETGHCKVFMESEDVGRTLD
BnaAC.ARF10.a.1012-98 (562) MNRKRALEEEAEEEEKG-----GLTWNYGLQGLETGHCKVFMESEDVGRTLD
BolC.ARF10 (561) MNRKRALEEEAEKEEKG-----GLTWNYGLQGLETGHCKVFMESEDVGRTLD
BnaC.ARF10.a.E617 (561) MNRKRALEEEAEEEEKG-----GLTWNYGLQGLETGHCKVFMESEDVGRTLD
BnaC.ARF10.b.E617 (561) MNRKRALEEEAEEEEKG-----GLTWNYGLQGLETGHCKVFMESEDVGRTLD
BnaC.ARF10.a.1012-98 (561) MNRKRALEEEAEEEEKG-----GLTWNYGLQGLETGHCKVFMESEDVGRTLD
BnaC.ARF10.b.1012-98 (561) MNRKRALEEEAEEEEKG-----GLTWNYGLQGLETGHCKVFMESEDVGRTLD
BnaC.ARF10.c.1012-98 (561) MNRKRALEEEAEEEEKG-----GLTWNYGLQGLETGHCKVFMESEDVGRTLD
BnaC.ARF10.d.1012-98 (561) MNRKRALEEEAEEEEKG-----GLTWNYGLQGLETGHCKVFMESEDVGRTLD
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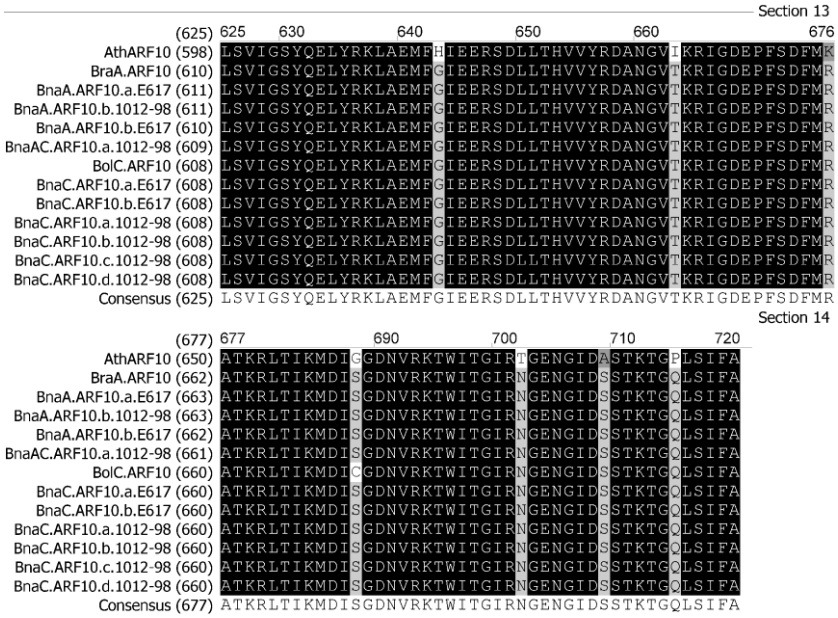
Section 13
(625) 625 630 640 650 660 676
AthARF10 (598) LSVIGSYQELYRKLAEMEHIEERSDLLTHVVYRDANGVIKRIGDEPFSDFMK
BraA.ARF10 (610) LSVIGSYQELYRKLAEMEGIEERSDLLTHVVYRDANGVTKRIGDEPFSDFMR
BnaA.ARF10.a.E617 (611) LSVIGSYQELYRKLAEMEGIEERSDLLTHVVYRDANGVTKRIGDEPFSDFMR
BnaA.ARF10.b.1012-98 (611) LSVIGSYQELYRKLAEMEGIEERSDLLTHVVYRDANGVTKRIGDEPFSDFMR
BnaA.ARF10.b.E617 (610) LSVIGSYQELYRKLAEMEGIEERSDLLTHVVYRDANGVTKRIGDEPFSDFMR
BnaAC.ARF10.a.1012-98 (609) LSVIGSYQELYRKLAEMEGIEERSDLLTHVVYRDANGVTKRIGDEPFSDFMR
BolC.ARF10 (608) LSVIGSYQELYRKLAEMEGIEERSDLLTHVVYRDANGVTKRIGDEPFSDFMR
BnaC.ARF10.a.E617 (608) LSVIGSYQELYRKLAEMEGIEERSDLLTHVVYRDANGVTKRIGDEPFSDFMR
BnaC.ARF10.b.E617 (608) LSVIGSYQELYRKLAEMEGIEERSDLLTHVVYRDANGVTKRIGDEPFSDFMR
BnaC.ARF10.a.1012-98 (608) LSVIGSYQELYRKLAEMEGIEERSDLLTHVVYRDANGVTKRIGDEPFSDFMR
BnaC.ARF10.b.1012-98 (608) LSVIGSYQELYRKLAEMEGIEERSDLLTHVVYRDANGVTKRIGDEPFSDFMR
BnaC.ARF10.c.1012-98 (608) LSVIGSYQELYRKLAEMEGIEERSDLLTHVVYRDANGVTKRIGDEPFSDFMR
BnaC.ARF10.d.1012-98 (608) LSVIGSYQELYRKLAEMEGIEERSDLLTHVVYRDANGVTKRIGDEPFSDFMR
Consensus (625) LSVIGSYQELYRKLAEMFGIEERSDLLTHVVYRDANGVTKRIGDEPFSDFMR
Section 14
(677) 677 690 700 710 720
AthARF10 (650) ATKRLTIKMDISGDNVRKTWITGIRTGENGIDASTKTGPLSIFA
BraA.ARF10 (662) ATKRLTIKMDISGDNVRKTWITGIRNGENGIDSSTKTGQLSIFA
BnaA.ARF10.a.E617 (663) ATKRLTIKMDISGDNVRKTWITGIRNGENGIDSSTKTGQLSIFA
BnaA.ARF10.b.1012-98 (663) ATKRLTIKMDISGDNVRKTWITGIRNGENGIDSSTKTGQLSIFA
BnaA.ARF10.b.E617 (662) ATKRLTIKMDISGDNVRKTWITGIRNGENGIDSSTKTGQLSIFA
BnaAC.ARF10.a.1012-98 (661) ATKRLTIKMDISGDNVRKTWITGIRNGENGIDSSTKTGQLSIFA
BolC.ARF10 (660) ATKRLTIKMDICGDNVRKTWITGIRNGENGIDSSTKTGQLSIFA
BnaC.ARF10.a.E617 (660) ATKRLTIKMDISGDNVRKTWITGIRNGENGIDSSTKTGQLSIFA
BnaC.ARF10.b.E617 (660) ATKRLTIKMDISGDNVRKTWITGIRNGENGIDSSTKTGQLSIFA
BnaC.ARF10.a.1012-98 (660) ATKRLTIKMDISGDNVRKTWITGIRNGENGIDSSTKTGQLSIFA
BnaC.ARF10.b.1012-98 (660) ATKRLTIKMDISGDNVRKTWITGIRNGENGIDSSTKTGQLSIFA
BnaC.ARF10.c.1012-98 (660) ATKRLTIKMDISGDNVRKTWITGIRNGENGIDSSTKTGQLSIFA
BnaC.ARF10.d.1012-98 (660) ATKRLTIKMDISGDNVRKTWITGIRNGENGIDSSTKTGQLSIFA
Consensus (677) ATKRLTIKMDISGDNVRKTWITGIRNGENGIDSSTKTGQLSIFA

Appendix II: Supplementary materials from the following publication

Nguyen, T.C.T., C. Obermeier, W. Friedt, S.R. Abrams, and R.J. Snowdon, 2018: Quantitative trait locus analysis of seed germination, seedling vigour and seedling-regulated hormones in *Brassica napus*. Plant Breeding **137**, 388-401.

Supplementary Table S1.

Internal standards used for hormone quantification by HPLC-ESI-MS/MS.

Metabolite	Abbreviation	Source
Abscisic acid	ABA	Sigma-Aldrich, St. Louis, MO, USA
Indole-3-acetic acid	IAA	
Indole-3-acetic acid aspartate	IAA-asp	
Zeatin	Z	
Zeatin riboside	ZR	
Isopentenyl adenosine	iPA	
Isopentenyl adenine	2iP	
Dihydrozeatin	dhZ	Olchemim Ltd., Olomouc, Czech Republic
Dihydrozeatin riboside	dhZR	
Zeatin-O-glucoside	Z-O-Glu	
Duterated forms	d3-DPA	NRC-PBI, Saskatoon, SK, Canada
	d5-ABA-GE	
	d3-PA	
	d4-70-OHABA	
	d3-neoPA	
	d4-ABA	
	d3-IAA-Asp	
	d3-IAA-Glu	
	d5-IAA	Cambridge Isotope Laboratories, Andover, MA, USA
	d3-dhZ	Olchemim Ltd., Olomouc, Czech Republic
	d3-dhZR	
	d5-Z-O-Glu	
	d6-iPA	
	d6-2iP	
Recovery standards	d6-ABA	NRC-PBI, Saskatoon, SK, Canada
	d2-ABA-GE	

Supplement Table S2

Statistical analysis of germination rate (GR), shoot length (SL) and seedling fresh weight (SFW) at 7 and 14 DAS

Genotype	**GR**	**SL7**	**SL14**	**SFW7**	**SFW14**
Express 617	88.67 ± 2.96	3.72 ± 0.22	5.98 ± 0.08	0.12 ± 0.01	0.47 ± 0.05
1012-98	27.33 ± 5.36	4.60 ± 0.13	6.96 ± 0.38	0.10 ± 0.01	0.39 ± 0.06
p-value	0.00056***	0.02666*	0.06542ns	0.23020ns	0.34099ns

*, *** indicate significant at 0.05 and 0.001 probability level, respectively whereas ns indicate insignificant
Values are presented as averages ± SEMs with Student's t-test

Declaration

I declare that the dissertation here submitted is entirely my own work, written without any illegitimate help by any third party and solely with materials as indicated in the dissertation. I have indicated in the text where I have used texts from already published sources, either word for word or in substance, and where I have made statements based on oral information given to me. At all times during the investigations carried out by me and described in the dissertation, I have followed the principles of good scientific practice as defined in the “Statutes of the Justus Liebig University Gießen for the Safeguarding of Good Scientific Practice”.

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