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Two *Brassica napus* cultivars differ in gene expression, but not in their response to submergence

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**Abstract**

Heavy rainfall causes flooding of natural ecosystems as well as farmland, negatively affecting plant performance. While the responses of the wild model organism *Arabidopsis thaliana* to such stress conditions is well understood, little is known about the responses of its relative, the important oil crop plant *Brassica napus*. For the first time, we analyzed the molecular response of *Brassica napus* seedlings to full submergence in a natural light–dark cycle. We used two cultivars in this study, a European hybrid cultivar and an Asian flood-tolerant cultivar. Despite their genomic differences, those genotypes showed no major differences in their responses to submergence. The molecular responses to submergence included the induction of defense- and hormone-related pathways and the repression of biosynthetic processes. Furthermore, RNAseq revealed a strong carbohydrate-starvation response under submergence in daylight, which corresponded with a fast depletion of sugars. Consequently, both *B. napus* cultivars exhibited a strong growth repression under water, but there was no indication of a low-oxygen response. The ability of the European hybrid cultivar to form a short-lived leaf gas film neither increased underwater net photosynthesis, underwater dark respiration nor growth during submergence. Due to the high sensitivity of both cultivars, the analysis of other cultivars or related species with higher submergence tolerance is required in order to improve flood tolerance of this crop species. One major target could be the improvement of underwater photosynthesis efficiency in order to enhance submergence survival.

**1 | INTRODUCTION**

During their lifetime, plants are exposed to many different environmental conditions. Changes in light quality and quantity, temperature and water availability frequently occur in nature, and as sessile organisms plants have to adapt to these changing conditions. Also, crop cultures are exposed to these variable conditions, and drastic modifications in their environment might cause substantial losses in yield, affecting food productivity and food quality. Due to the progressing climate change, the frequency and duration of extreme weather events increase, including periods of high- and low water availability (e.g., Blöschl et al., 2019; Kundzewicz et al., 2014; Pekel
et al., 2016; Trenberth et al., 2014). Low water availability over longer periods, often accompanied by high temperatures, leads to drought stress. The other extreme, large amounts of precipitation over a short time period, causes flooding stress. Flooding can be divided into two contrasting situations, (1) only the soil is flooded and hence only the root system is affected, the so-called waterlogging stress and (2) the whole plant is submerged (Sasidharan et al., 2017). Full submergence is often more severe than waterlogging, since also leaves and their photosynthetic processes are directly impaired.

The most obvious challenge in flooded environments is the slow gas diffusion compared to aerial conditions (Armstrong, 1980). The availability of oxygen and carbon dioxide can quickly become limiting when metabolism is high, which is the case in most living tissues (van Dongen & Licausi, 2015). Although plants produce oxygen through photosynthesis, they are also dependent on mitochondrial respiration and its oxygen requirement. Underground organs solely produce energy by glycolysis and mitochondrial respiration, which is also the case in all plant parts during the night.

Two types of survival strategies have been observed to tolerate flooding (summarized in van Veen et al., 2014; Voesenek & Bailey-Serres, 2015): (1) Several plant species avoid oxygen deficiency by multiple mechanisms that favor gas transport, such as development of adventitious roots, formation of aerenchyma in roots and leaves, gas films on leaves as well as elongational growth to restore contact to oxygen-rich air (summarized in Voesenek & Bailey-Serres, 2015; Mustroph et al., 2018; Yamauchi et al., 2018). This strategy is commonly referred to as the low-oxygen escape strategy. (2) Other plant species follow the so-called low-oxygen quiescence strategy, where growth and development are inhibited until floods recede. In this strategy, plants have evolved mechanisms to acclimate their metabolism to low-oxygen availability. This is achieved through the induction of fermentation processes, mainly lactic acid and ethanolic fermentation, and through down-regulation of energy-consuming processes like translation and growth (summarized in van Dongen & Licausi, 2015; Voesenek & Bailey-Serres, 2015). Modifications in gene expression under oxygen deficiency have been especially well studied in the model species Arabidopsis thaliana (e.g., Lee & Bailey-Serres, 2019; Mustroph et al., 2009; van Veen et al., 2016; Yeung et al., 2018).

The major problem for both strategies is the occurrence of an energy crisis (Greenway & Armstrong, 2018). The plants are faced with a challenge so that adaptional responses (1) like elongational growth are energy-requiring, and alternative metabolic pathways (2) like glycolysis in combination with fermentation are less efficient in energy production. Furthermore, under flooding conditions, photosynthesis is restricted due to low light and carbon dioxide availability (summarized in van Dongen & Licausi, 2015; Voesenek & Bailey-Serres, 2015), leading to a reduced production of photosynthates.

Flood-tolerant species like Rumex palustris, Zea nicaraguensis, Nassurtium officinale or the crops Oryza sativa and Colocasia esculenta can tolerate flooding for long periods (weeks to months), while most other crops are highly sensitive to flooding, for example, Z. mays, Gossypium spec., Glycine max or Brassica napus (summarized in Voesenek & Bailey-Serres, 2015; Mustroph, 2018). Many researchers have worked on projects to improve flood tolerance of crop species, especially on maize, barley, and soybean (summarized in Mustroph, 2018). Despite these efforts, true flood-tolerant crop cultivars have not been developed yet.

The important oil plant B. napus has rarely been studied in respect to flood tolerance, and most of those studies have been performed under waterlogging stress (for example, Voesenek et al., 1999; Zou, Tan, et al., 2013; Zou et al., 2014; Xu et al., 2015; Zou et al., 2015; Ploschuk et al., 2018; Wollmer et al., 2018). This tetraploid crop species has been established rather recently, and is therefore genetically relatively narrow (Bus et al., 2011; Chalhoub et al., 2014). Still, many different cultivars exist that differ in multiple parameters, for example, flowering time (e.g., Fletcher et al., 2015; Raman et al., 2013), vernalization requirement (e.g., Wang et al., 2011), or seed oil quality and quantity (e.g., Ecke et al., 1995; Qiu et al., 2006).

It has been shown that differences between cultivars exist in respect to waterlogging tolerance, mainly in Asian cultivars (e.g., Zou et al., 2014; Xu et al., 2015, summarized in Mustroph, 2018). However, the underlying mechanisms of tolerance have not been identified yet, despite comparative analyses at the transcriptional (Zou et al., 2015; Zou, Tan, et al., 2013) and proteomic (Xu et al., 2018) level. These analyses only showed minor differences in gene expression in response to waterlogging between the two cultivars Zhongshuang 9 and GH01 (Zou et al., 2015). It was observed that B. napus, independent of the cultivar, can induce genes coding for enzymes involved in fermentation in waterlogged roots suggesting that the metabolism can be acclimated to low oxygen availability. However, when looking at morphological adaptations to avoid oxygen deficiency within plant organs, B. napus is not able to form aerenchyma as a response to waterlogging (Ploschuk et al., 2018; Voesenek et al., 1999), and adventitious root formation is probably cultivar-dependent (Zou et al., 2014), but detailed studies addressing this topic are still lacking.

Here, we focused on the other flooding variant, full submergence, which can occur especially at the seedling stage, when B. napus plants are still small and water levels quickly rise above the short seedlings. Under these circumstances, avoidance mechanisms of the shoots are required, that is, elongation growth of stems and/or petioles or the formation of leaf gas films. Recently, we identified the molecular response of the flood-tolerant Brassicaceae N. officinale and identified it as a species with a submergence escape response (Müller et al., 2019). The response of the related Brassicaceae B. napus to submergence has not been investigated yet. Even in the model species A. thaliana, metabolic, and other molecular adaptations under submergence are less well understood than under oxygen deficiency, imposed experimentally by nitrogen gassing (Lee et al., 2011; van Veen et al., 2016).

In this study, we characterized the general response to full submergence, including molecular, biochemical, and growth modifications, in the European hybrid cultivar Avatar that had been used before in a study under waterlogging stress (Wollmer et al., 2018). Moreover, we included an Asian semi-winter type, Zhongshuang 9, that had already been characterized in terms of waterlogging
tolerance (e.g., Xu et al., 2016; Zou et al., 2014; Zou, Cong, et al., 2013). Our data suggest that rapeseed seedlings use the quiescence strategy under water and have to cope with a severe carbohydrate starvation, while a hypoxic signature was not seen during submergence in daylight. Considerable differences in gene expression were observed between both cultivars, but the overall responses to submergence were largely independent of the genotype. We detected cultivar-specific difference in the ability to form gas films, but this did not cause differences in the response to submergence or the survival under stress.

2 | MATERIALS AND METHODS

2.1 | Plant material and submergence treatment

Seeds of *Brassica napus* cv. Avatar (winter-type) were obtained from the Bavarian State Research Center for Agriculture. Seeds from cv. Zhongshuang 9 (semi-winter type) were a gift from Xi-Ling Zou (Oil Crops Research Institute, Wuhan, China). Seeds were germinated on moist filter paper for 24 h at 30 °C. Seeds were germinated cv. Zhongshuang 9 (semi-winter type) were a gift from Xi-Ling Zou (Oil Crops Research Institute, Wuhan, China). Seeds were germinated on moist filter paper for 24 h at 30 °C. Subsequently, they were transferred to a sand:soil (1:3) mixture. Plants grew in a growth chamber under short-day conditions (8/16 h light/dark) at around 23°C and 100 µmol photons m⁻² s⁻¹. On the 15th day after imbibition, 2 h after the start of the photoperiod, plants were fully submerged in tap water (equilibrated to room temperature overnight) in the normal short-day rhythm. After different treatment durations, plants were harvested and further analyzed. Control plants were kept in similar boxes, and were well-watered.

2.2 | Determination of growth and survival rates

To estimate whether plants were able to grow under water, 15-day-old plants were submerged for 1 or 2 weeks. Before submergence (t₀) and after stress treatment (tₛ), the hypocotyl length as well as the length of the first and second petiole were measured with a digital caliper. Growth per day was calculated as tₛ – t₀/number of days.

Survival rates were determined by submerging the plants for 12 up to 19 days. Subsequently, plants were carefully removed from the water, and re-growth was observed for another 7 days. Each experiment consisted of eight plants per genotype and time point. Surviving plants were identified through the ability to form new leaves within the recovery period.

2.3 | RNA sequencing

For transcriptome analyses, the first true leaf was harvested from each plant after 24 h of treatment, that is, 2 h after the start of the photoperiod, and immediately frozen in liquid nitrogen. RNA was extracted from the ground plant tissue by use of the ISOLATE II RNA plant kit according to the manual, including a DNase treatment (Bioline, Luckenwalde, Germany). Three independent experiments were performed. RNA was further processed by Eurofins Genomics Europe Shared Services GmbH (Ebersberg). All steps performed have been developed and validated by the company and are based on profound experience. Commercially available kits were used with modified and improved protocols.

Integrity and quantity of the RNA were determined by appropriate methods, that is, measurement of volume and quantity, gel electrophoresis, and fluorimeter measurements. Library preparation incorporated adaptor sequences and indexing compatible for Illumina sequencing technology, using proprietary methods of Eurofins Genomics Europe Shared Services GmbH. The cDNA library preparation was performed following optimized protocols. After first-strand synthesis, the second strand synthesis was performed using dUTP. The ends of the double-stranded cDNA fragments were repaired and dATP ligated to the blunt-ended fragments. Next, the sequencing adapters were ligated to the DNA fragments, and the dUTP containing the second strand was removed. Determination of size distribution and quantification of the sequencing library was accomplished with appropriate methods according to optimized protocols. Sequencing was performed on the Illumina HiSeq 4000 platform with 150 bp paired-end mode.

Fastq files were used for transcript quantification by use of Kallisto (Bray et al., 2016), with 30 bootstrap samples. The reference genome built v5 from Genoscope was used (http://www.genoscope.cns.fr/brassicanapus/), which included 101040 transcripts. The quantification yielded tpm values (transcripts per million) as well as counts. For the cv. Avatar, 79% of the reads could be mapped to the reference transcriptome, while for cv. Zhongshuang 9, only 73–75% of reads could be mapped (Table S1). Lowly expressed genes were removed by using a cut-off of tpm < 12 (sum over 48 samples, 12 samples from this experiment, and another set of 36 samples from an experiment with waterlogging which will be presented elsewhere). A total of 63569 expressed transcripts were retained through this procedure.

The selection of differentially expressed genes, gene ontology (GO) term enrichments as well as gene annotations was performed as described in detail in a study on *N. officinale* (Müller et al., 2019). Data have been deposited at the Gene Expression Omnibus database under the accession GSE140828.

2.4 | RT-qPCR analysis

Aliquots of the RNA subjected to next-generation sequencing were used for cDNA synthesis through RevertAid Reverse Transcriptase (Thermo Fisher Scientific). RT-qPCR was performed in a 10 µl reaction with SsoAdvancedTM Universal SYBR Green Suppermix and the CFX ConnectTM Real-Time PCR Detection System (Bio-Rad, Feldkirchen, Germany). Gene-specific primers are listed in Table S2. Relative mRNA levels were calculated by the 2⁻ΔΔCT x 1000 method and normalized to three reference transcripts (*BnaVIP2, BnaPRPP, BnaSWN*, for gene IDs and primers, see Table S2).
2.5 | Measurement of carbohydrate levels and ADH activities

For measurements of the carbohydrate content, the first true leaf was harvested after different durations of submergence, and of corresponding untreated plants. The plant material was immediately frozen in liquid nitrogen and stored at −80°C until processing. After grinding the plant material in liquid nitrogen, soluble carbohydrates as well as starch were extracted and measured as previously described (Riber et al., 2015). Adenylate levels were determined as described in Mustroph et al. (2006). To determine alcohol dehydrogenase (ADH) activities, the first true leaf was harvested after 24 h of submergence treatment. Enzymes were extracted and ADH activities were measured as described in Gasch et al. (2016).

2.6 | Measurement of gas film parameters

Fifteen-day-old plants of both genotypes were completely submerged in 50 l tanks with artificial floodwater prepared according to Smart and Barko (1985) with a final alkalinity of 2.0 mmol l⁻¹ H⁺ equivalents. Four experimental replicates were established in four separate tanks with O₂ and CO₂ in the floodwater maintained at air-equilibrium by purging with atmospheric air. The water temperature was maintained at 23°C and light was provided by fluorescent tubes at a photon flux of 150 μmol photons m⁻² s⁻¹ in an 8 h light/16 h dark cycle. At the time of submergence, the plants had developed the first set of leaves in addition to the cotyledons. Controls in air were maintained at identical light and temperature conditions.

Leaf surface wettability was assessed by measuring the contact angle of a water droplet of approximately 5 mm³ on the leaf surface (Brewer & Smith, 1997), held flat using double-sided tape. Droplets were applied to the adaxial leaf surface using a custom-made syringe, and photos were taken at ×90 magnification using a horizontally positioned digital microscope camera (Dino-Lite AM4013MZTL, IDCP) and the contact angle was determined by image analysis in ImageJ (Schneider et al., 2012). The wettability of the leaf surfaces could be divided into four classes defined by their contact angle according to Koch and Barthlott (2009): Superhydrophilic (Contact angle <10°), hydrophilic (contact angle 10°–90°), hydrophobic (contact angle 90°–150°) and superhydrophobic (contact angle >150°). This classification is relevant as only superhydrophobic surfaces retain a gas film when submerged in water (Shirtcliffe et al., 2005).

Gas film volume was measured by determining buoyancy of leaf segments (area of 150–700 mm²) before and after gas film removal. The gas films were removed by brushing the leaves with a dilute solution (0.05% v/v) of Triton X-100 (Colmer & Pedersen, 2008; Raskin & Kende, 1983), and the buoyancy of the leaf segments was measured in deionized water using a four-digit balance mounted with a hook underneath. Projected area was measured for each leaf segment using digital scans and image analysis in ImageJ (Schneider et al., 2012). Mean gas film thickness was calculated by dividing gas film volume (mm³) by the two-sided area (mm²).

2.7 | Underwater net photosynthesis and dark respiration

Underwater net photosynthesis was assessed according to the method described by Pedersen et al. (2013). In brief, 1 whole leaf (200–400 mm²) was inserted in a 28 ml glass vial with artificial floodwater (see above). O₂ was initially set to half air equilibrium by mixing 1:1 volumes of air bubbled and N₂ purged artificial floodwater; this procedure served to minimize photorespiration as O₂ accumulates in the vials during incubation (Setter et al., 1989). The water was further prepared with 200 μmol CO₂ l⁻¹ by adjusting pH to 7.35 using HCl; 200 μmol CO₂ l⁻¹ is commonly found in natural floodwaters (Colmer et al., 2011). The vials were mounted on a rotating incubaor at a photon flux of 150 μmol photons m⁻² s⁻¹ at 23°C for 2 h before the O₂ concentration was measured using an O₂ optode (Opto-MR, Unisense, Aarhus, Denmark) connected to an optode meter (Opto-F4 UniAmp, Unisense); vials without tissue served as blanks. After measurements, the projected area of the tissue was measured from digital scans using ImageJ (Schneider et al., 2012) and the underwater net photosynthesis was calculated as net O₂ production per projected area of leaf per time unit (Pₜₚ, μmol O₂ m⁻² s⁻¹).

Underwater dark respiration was assessed using a MicroResp system following the approach of Pedersen and Colmer (2012). Leaf segments of 200–400 mm² were prepared as rectangular segments and positioned individually in a 4 ml glass vial filled with artificial floodwater (see above) which was slightly supersaturated with O₂. The vial was placed in a rack to enable stirring with a glass-coated stir bar and a capillary hole in the lid enables measurements of O₂ consumption with time (MicroResp, Unisense A/S). O₂ was measured using an O₂ optode (Opto-MR, Unisense) connected to an optode meter (Opto-F4 UniAmp, Unisense). The system allowed for eight parallel samples and vials without tissue served as blanks. Measurements were taken at external O₂ concentrations ranging from 22 to 17 kPa and lasted for less than 60 min; the temperature was 2°C. Prior to the measurements, the O₂ optode was calibrated in DI water at air equilibrium as well as at zero O₂ using ascorbate in alkaline solution according to Pedersen et al. (2013). After measurements, the projected area of the tissue was measured from digital scans using ImageJ (Schneider et al., 2012) and the underwater dark respiration rate was calculated as O₂ consumption per projected area of leaf per time unit (Rₑ, μmol O₂ m⁻² s⁻¹).

2.8 | Net photosynthesis in air

Leaf gas exchange measurements were performed during the morning (30 min to 2 h after the start of illumination) in the first fully developed leaf per seedling with a portable LI-6400 infrared gas analyzer with a red/blue light-emitting diode light source and CO₂ injector (LI-COR Inc., Lincoln, Nebraska). Photosynthetic photon flux density (PPFD) was set to 150 μmol m⁻² s⁻¹, CO₂ concentration to 410 ppm, leaf temperature was maintained around 23°C, and relative humidity to chamber conditions at approximately 30%, flow rates were set at 400 μmol s⁻¹ (Evans & Santiago, 2014). Net photosynthesis (A, μmol
m$^{-2}$ s$^{-1}$) was measured and subsequently adjusted to the leaf area. Each leaf was excised after measurements, and the leaf area was measured with a Leaf Area Meter (LI-300 m, LI-COR Inc.).

2.9 Oxygen status within petioles during submergence

Tissue O$_2$ status of the petiole during submergence was measured following the approach of Herzog and Pedersen (2014). In brief, a single plant was positioned horizontally on a stainless-steel mesh with the root hanging freely in a 25 ml beaker with DI water. The petiole of the second fully expanded leaf was gently fixed using pieces of rubber band. Beaker and mesh were transferred to a 9 l glass aquarium to enable complete submergence during the experiment. An O$_2$ microsensor (OX25, Unisense A/S, Denmark) was inserted into the center (350-400 μm) of the petiole halfway between the stem and the lamina using a micromanipulator (MM33, Unisense A/S). The O$_2$ microsensor was connected to a pico amper (pA) meter (fx-6 UniAmp, Unisense A/S, Denmark), and was calibrated prior to each series of experiments in air-purged water (267.8 μmol O$_2$ l$^{-1}$ equivalent to 20.6 kPa pO$_2$ at 23°C) and in an oxygen-free solution (2 g ascorbate in 100 ml 0.1 M NaOH); the signal from the O$_2$ microsensor was logged on a computer every 1 s (Logger, Sensortrace 3.2, Unisense A/S). Positioning of the microsensor was aided using a boom stand stereomicroscope (M3B, Leica Microsystems). All measurements were taken at 23°C in light (150 μmol photons m$^{-2}$ s$^{-1}$; fiber lamp KL1500, Schott AG) or in darkness. During submergence (DI water in air equilibrium in terms of dissolved O$_2$ and CO$_2$), the shoot was completely submerged. The experimental sequence used was shoot submerged in darkness (30 min) → shoot submerged in light (30 min) so that a new quasi-steady-state of tissue oxygen status was reached in each situation.

3 RESULTS

3.1 Transcriptome analysis of Brassica napus cultivars under submergence

Although B. napus is an important oil crop and known to be very sensitive to flooding, very little is known about its molecular and physiological responses to flooding, particularly to complete submergence. In order to build on existing knowledge on different cultivars, we used 15-day-old plants of a waterlogging-tolerant cultivar from Asia, Zhongshuang 9 (e.g., Xu et al., 2016; Zou et al., 2014; Zou, Cong, et al., 2013) and compared its response to submergence with a popular European hybrid cultivar, Avatar (Wollmer et al., 2018). After 24 h of submergence, the first true leaf was harvested, and the transcriptome was obtained.

We identified 63,569 expressed transcripts in the dataset. Since B. napus is a tetraploid species and the Brassica genus underwent a genome triplication (Nikolov & Tsiantis, 2017), a gene from A. thaliana could be present at maximum in six copies in the B. napus genome, explaining the large transcript numbers. Interestingly, while about 79% of the reads could be mapped from samples from the European cultivar Avatar to the reference transcriptome of the cultivar Darmor-bzh (Chalhoub et al., 2014), only about 75% of the reads of the Asian cultivar Zhongshuang 9 could be successfully mapped. This indicates genomic differences between both genotypes. Mapping the reads to another transcriptome from the Asian cultivar Zhongshuang 11 (Sun et al., 2017) did not improve mapping statistics, so we used the reference transcriptome of the cultivar Darmor-bzh for both genotypes.

Submergence for 24 h dramatically changed the gene expression in both genotypes. Total of 7347 and 8344 transcripts were up-regulated by submergence (Signal-log ratio (SLR) > 1, false discovery rate (FDR) < 0.01) in Avatar and Zhongshuang 9, respectively, with an overlap of 4995 transcripts (Table S3, Figure 1); and 7040 and 8991 transcripts were down-regulated (SLR < −1, FDR < 0.01), with an overlap of 4973 transcripts. In summary, about 22 and 29% of the expressed transcriptome were modified in expression.

3.2 Submergence causes up-regulation of starvation-related genes

The functional characterization of differentially expressed genes was done by use of GO enrichment analysis (Table S4). In both genotypes, defense-related GO terms were enriched among up-regulated genes, for example, "response to chitin", "defense response to fungus" or "regulation of defense response". Not surprisingly, hormone-related GO terms were also enriched, for example, "response to ethylene", "abscisic acid-activated signaling pathway" or "response to brassinosteroid". The responses between both genotypes were very similar, and hardly any GO term was only enriched in the cv. Avatar. In contrast, in the cv. Zhongshuang 9 some GO terms were enriched, for example, "cell communication" or "response to nitrate", which did not respond in Avatar. Furthermore, evaluation of the expression data in MapMan suggested a stronger up-regulation of degradation processes in Zhongshuang 9 compared with Avatar, for example, degrada- tion of carbohydrates, amino acids, and nucleotides (Figure S1), while the genes encoding biosynthesis enzymes of these compounds were down-regulated. Accordingly, gluconeogenesis gene expression was also induced.

One example for a highly up-regulated gene involved in degrada- tion was MYO-INOSITOL OXYGENASE 2 (MIOX2), which was present in four annotated transcripts in the B. napus genome. All four transcripts were strongly induced by submergence in both genotypes (Figure S2). This gene is a marker for carbohydrate starvation. Therefore, we com- pared an Arabidopsis dataset of an extended night (Usadel et al., 2008) and a carbon starvation dataset (Cookson et al., 2016) to our data. We found a highly significant proportion of submergence-induced trans- scripts also responding to carbon starvation in Arabidopsis (Table S5, Figure S3). Besides MIOX2, we also identified MIOX4, BETA-
The RNA-seq quantification corresponded well with the levels of the homologs of the 49 Arabidopsis HRGs in the genome contained 154 orthologues of the 49 Arabidopsis HRGs in our dataset, and of this only 40 and 36 transcripts (representing 19 and 16 HRGs in Avatar and Zhongshuang 9, respectively) were identified as significantly up-regulated in our dataset. Among them were the homologs of AT1G19530, a subunit of the DNA polymerase complex, the ethylene sensor ETHYLENE RESPONSE 2 (ETR2), GALACTOSIDASE 4 (BGAL4), BRANCHED-CHAIN AMINO ACID TRANSAMINASE 2 (BCAT-2) as examples of starvation-induced genes.

Interestingly, 127 and 187 transcripts were more strongly induced in Avatar and Zhongshuang 9, respectively, than in the other genotype (comparison of genotype × treatment, Table S3). For example, Zhongshuang 9 induced several genes of the starvation response stronger than Avatar. However, there were no obvious GO terms enriched among the differentially expressed transcripts in this comparison (Table S4). In order to confirm these expression differences for some transcripts, we performed RT-qPCR analysis for four up-regulated genes, three of them starvation markers (MIOX2, BGAL4, EARLY RESPONSIVE TO DEHYDRATION 5), and one gene with a similar expression pattern that was previously not associated with the core starvation response, SULFUR E2 (SUFE2). All four transcripts showed significant induction in Zhongshuang 9, and a lower induction under submergence in Avatar (Figure 2A–D). The relative transcript levels corresponded well with the RNA-seq quantification.

We also looked into the hypoxia core-response genes (HRGs), identified in Arabidopsis (Mustroph et al., 2009). The Brassica napus genome contained 154 orthologues of the 49 Arabidopsis HRGs in our dataset, and of this only 40 and 36 transcripts (representing 19 and 16 HRGs in Avatar and Zhongshuang 9, respectively) were identified as significantly up-regulated in our dataset. Among them were the homologs of ADH enzyme activity. Accordingly, there was no induction of ADH activity in any of the two genotypes at the 24 h-submergence time point (Figure 5), and no significant difference between the

3.3 Sugar content and fermentation activity

We measured the sugar content under submergence in both genotypes, since the observed induction of carbohydrate starvation markers indicated that submergence caused sugar depletion. Indeed, after 24 h of submergence, soluble sugars as well as starch content were dramatically lower in comparison to growth in air, leading to more than 90% decrease in available glucose equivalents (Figure 3). However, there was no significant difference in carbohydrate content between genotypes, neither under control nor under submerged conditions.

To analyze the sugar depletion more deeply, we performed a time-line analysis of soluble sugar and starch contents. Already after 3 h of submergence, there was a significant decrease of carbohydrates in Avatar (Figure 4(A)) and Zhongshuang 9 (Figure 4(B)), although plants were in full growth light during this treatment. This is obvious when looking at the controlled plants that accumulated carbohydrates, and especially starch, during the day, until the 6-h time point. During the night (6–22 h time points), plants under both treatment conditions consumed most of their carbohydrates, and no significant differences between submerged and control plants were visible. However, within the next light period (22–24 h time points), control plants started to accumulate sugars again, which was not the case under submergence in light. Again, we did not observe significant differences between the two genotypes analyzed, indicating that the genome-specific regulation of starvation marker gene expression does not correlate with a difference in the extent of carbon starvation.

In addition, the adenylates ATP and ADP were measured. Surprisingly, their content did hardly change during the treatment (Figure S6). Only after 6 h, the adenylate content decreased significantly in both genotypes compared with control time points, and at no time point there was a difference between the ecotypes.

Since we found no up-regulation of expression of genes coding for fermentative enzymes (ADH and PDC), we also measured the ADH enzyme activity. Accordingly, there was no induction of ADH activity in any of the two genotypes at the 24 h-submergence time point (Figure 5), and no significant difference between the
genotypes, but with a tendency for Zhongshuang 9 to have extremely low ADH activities.

3.4 | Down-regulation of translation and biosynthesis under submergence

Among down-regulated genes, we again observed a common response of the two cultivars. Enriched GO terms were associated with translation, DNA replication, photosynthesis, and cell wall. Many biosynthetic processes were also negatively affected by submergence. Flavonoid and carotenoid biosynthesis were more strongly affected in Avatar, while, for example, pyrimidine ribonucleotide biosynthesis and protein import into the nucleus were more inhibited in Zhongshuang 9. Examples of strongly down-regulated genes in both cultivars were an AMP-dependent synthetase and ligase family protein (AAE2, AT1G77240), and several genes coding for CYP450 monooxygenase family members (Table S3). Again, a highly significant proportion of the down-regulated genes in both genotypes were also negatively regulated by starvation in Arabidopsis (Usadel et al., 2008; Cookson et al., 2016, Table S5).
Evaluation of the MapMan categories revealed down-regulation of photosynthesis and translation (Figure S7). Interestingly, ribosomal proteins appeared to be more down-regulated in Zhongshuang 9 than in Avatar. Further analysis of the data showed that 128 and 414 transcripts were more strongly down-regulated in Avatar and Zhongshuang 9, respectively, than in the other genotype (Table S3, genotype × treatment). GO analysis of this comparison revealed a stronger down-regulation of sulfate assimilation in Avatar, and a stronger down-regulation of cell-wall-associated processes and growth in Zhongshuang 9 (Table S4, genotype × treatment).

We aimed at confirming expression differences between genotypes by RT-qPCR. All four tested genes, the basic helix–loop–helix transcription factor \( \text{HOMOLOG OF BEE2 INTERACTING WITH IBH (HBI1)} \), \( \text{EXPANSIN A6 (EXPA6)} \), \( \text{FASCICLIN-LIKE ARABINOGLUCAN PROTEIN 15 PRECURSOR (FLA15)} \), and \( \text{LIPID TRANSFER PROTEIN 6 (LTP6)} \), were significantly down-regulated after submergence, and in three of the genes, expression levels under control conditions were higher in Zhongshuang 9 than in Avatar at the same time point (Figure 6(A)–(D), Figure S2), explaining the stronger down-regulation in the Asian genotype.

### 3.5 Growth under submergence

The low availability of sugars as well as the down-regulation of biosynthetic processes and translation prompted us to test how much the growth of *Brassica napus* was affected under submergence. For other plant species like *Rumex palustris*, rice or the Brassicaceae *N. officinale*, enhanced elongation of stems or petioles has been described (summarized in Voesenek & Bailey-Serres, 2015; Mustroph, 2018; Müller et al., 2019). Therefore, we determined the growth of the hypocotyl and the petioles of submerged *B. napus* plants within a two-week treatment.

The hypocotyl of the 15-day-old plants hardly grew under control and submerged conditions (Figure 7(A)). A slight increase of less than 1 mm per day was observed, with a tendency for a faster growth under submergence in Zhongshuang 9. On the other hand, petioles of the first and second true leaf grew at about 2–3 mm per day in both genotypes, while their elongation was drastically and significantly lowered under submergence (Figure 7(B), (C)). While under control conditions, the petioles of Zhongshuang 9 grew slightly faster than those of Avatar, there was no significant difference in growth under submergence between both genotypes. Surprisingly, despite drastic down-regulation of growth, we observed the formation of a new small leaf under submergence within the 2 weeks of treatment in both genotypes (Figure S8).
3.6 | Expression differences between both genotypes

When comparing both genotypes, we identified 5272 and 5935 transcripts that were higher expressed in Avatar than in Zhongshuang 9 under control and submerged conditions, respectively (Table S3). On the other hand, Zhongshuang 9 expressed 2887 and 4032 transcripts at a higher level than Avatar. Functional analysis through GO-term enrichment suggested a higher expression of translation-associated genes in Avatar in comparison to Zhongshuang 9, which was especially obvious under submergence, and confirms our MapMan observations mentioned above (Figure S7). On the other hand, Zhongshuang 9 expressed genes involved in defense response more so than Avatar, again more pronounced under submergence. Interestingly, two genes coding for the flower-repressing transcription factor FLOWERING LOCUS C (FLC), BnaC03g04170D and BnaC09g46540D, showed significantly higher expression in winter rape Avatar compared to the semi-winter rape Zhongshuang 9 (Table S3).

Of note, the strong expression differences might not always be associated with different functions, but different isoforms and genes could be expressed in the two genotypes, and the overall expression level of a certain function might not be so different. As previously mentioned, one Arabidopsis gene could have 6 copies in the tetraploid B. napus genome. This would explain why there are little functional differences between the two genotypes under control conditions (Table S4).

3.7 | Differences in gas films do not affect underwater photosynthesis or survival

During our submergence experiments, we repeatedly observed that the genotype Avatar retained a thin leaf gas film upon submergence whereas Zhongshuang 9 did not (Figure S9). This prompted us to assess leaf hydrophobicity by measurements of contact angle according to the approach of Konnerup and Pedersen (2017) since a contact angle exceeding 150° entails superhydrophobic properties (Koch & Barthlott, 2009). Indeed, for leaves that had never been submerged, the contact angle of Avatar exceeded 150° whereas leaves of Zhongshuang 9 had contact angles of 125° showing that leaves of Zhongshuang 9 are not superhydrophobic (Figure 8(B)). However,
upon submergence, the superhydrophobic properties of Avatar were soon lost. In fact, the leaves of both genotypes became hydrophilic within the first 24 h of submergence (Figure 8(B)). The super-hydrophobic properties of leaves of Avatar resulted in retention of leaf gas films with a thickness of approximately 25 μm, but the gas films disappeared as the contact angle declined (Figure 8(A)). Surprisingly, neither underwater net photosynthesis nor dark respiration was affected by the thin leaf gas film that was present for the first 24 h in the genotype Avatar (Figure 8(C), (D)). This observation supports our findings on similar sugar levels (Figure 3) and growth rates (Figure 7) during the stress treatment in both genotypes.

In order to compare the net photosynthesis under water with those normally present in aerated plants of the same age, net photosynthesis was measured under the same illumination conditions as under water. The net photosynthesis was 5.99 and 5.53 μmol CO₂ m⁻² s⁻¹ for Avatar and Zhongshuang, respectively (Figure S10). There were no significant differences between the genotypes. Interestingly, the underwater photosynthetic rate (Figure 8(C)) was less than 20% of the photosynthetic rate in air, pointing to severe restrictions of this process under water.

Subsequently, we tested whether there was a difference in survival under long-term submergence between both cultivars. Previously, Zhongshuang 9 has been described as a waterlogging-tolerant genotype (e.g., Xu et al., 2015; Zou et al., 2014). However, we found no difference in submergence survival between the cultivars at the developmental stage that we analyzed. Both genotypes survived 14 days of submergence quite well, while longer durations between 17 and 19 days caused death of most of the plants (Figure 9).

4 | DISCUSSION

4.1 Submergence in light causes no oxygen deficiency in Brassica napus

Under submergence, gas diffusion between the plant and its surroundings is strongly restricted. Potential gases to be affected are oxygen, carbon dioxide, and the gaseous plant hormone ethylene. Oxygen deficiency is recognized by the subgroup VII of the ethylene response factor (ERF) transcription factor family (Gibbs et al., 2011; Licausi et al., 2011), and causes the induction of the so-called "hypoxia core-response genes" in Arabidopsis (Mustroph et al., 2009). Brassica napus has homologous genes coding for group VII ERFs as well as HRGs (Table S3), which can be induced by oxygen deficiency in the root zone (Zou et al., 2015; Zou, Tan, et al., 2013b). In our dataset, however, the HRGs are hardly affected by the submergence treatment, analyzed 2 h after the onset of light (Figure S4, Table S5). Among the significantly induced HRGs under full submergence are homologs for an YGL motif gene (AT4G27450), the atypical CYS HIS rich thioredoxin 5 (AT5G61440), the ABSCISIC ACID 8'-HYDROXYLASE 3 (AT5G45340), the ethylene sensor ETR2 (AT3G23150), and the PHLOEM PROTEIN 2-A13 (AT3G61060). Most of those submergence-induced genes (despite ABSCISIC ACID 8'-HYDROXYLASE 3 and ETR2) are also induced by carbohydrate starvation caused by artificial darkness in Arabidopsis (Usadel et al., 2008, van Veen et al., 2016, Figure S11).
Other HRGs like ADH (AT1G77120) or the LOB DOMAIN-CONTAINING PROTEIN 41 (AT3G02550) are not induced in our submergence treatment, or under carbon starvation (Figures S4, S11). Those genes are significantly induced by submergence in darkness (van Veen et al., 2016, Figure S11), but not by submergence in light (Figure S4, Müller et al., 2019). In addition to gene expression, we analyzed the activity of the fermentation enzyme ADH, which was not modified under submergence (Figure 5). Those two facts suggest that the leaves, despite under water, had sufficient oxygen, most likely produced through photosynthesis.

In order to further support this hypothesis, we also measured the oxygen status within the petiole tissue of the cultivar Avatar under water in darkness and in light. While a significant drop in internal oxygen content was observed in darkness (12.9 kPa), it increased again under illumination to 18.9 kPa (Figure S5). Oxygen production through photosynthesis under water has been demonstrated before for Arabidopsis leaves (Lee et al., 2011; Vashisht et al., 2011) as well as other plant species (Mori et al., 2019; Müller et al., 2019; Rijnders et al., 2000). For example, petioles of N. officinale contained 14.8 and 23.3 kPa oxygen in darkness and in light under water (Müller et al., 2019), and similar levels were observed for Arabidopsis petioles even after 18 h of submergence (Vashisht et al., 2011). A work on

**Figure 8**  Gas film thickness (A), contact angle (B), underwater net photosynthesis (C) and underwater dark respiration (D) in two genotypes (Avatar, Zhongshuang 9) of *Brassica napus*. Fifteen-day-old plants were submerged in artificial floodwater with O2 and CO2 maintained at air equilibrium in an 8/16 h light/dark cycle at 23 °C. The figure panels show data for submerged individuals (sub) as well as controls in air (con); for contact angle and underwater dark respiration, the 0-day start point indicates controls in air at time of submergence. Values are mean ±SD of four replicates. Different letters show significantly different values (ANOVA and TUKEY HSD test, P < 0.05); n.s., not significant.

**Figure 9**  Survival rates of two genotypes (Avatar, Zhongshuang 9) of *Brassica napus* after submergence. Fifteen-day-old plants were submerged in equilibrated tapwater in an 8/16 h light/dark cycle, for several days. Subsequently, they were taken out of the water and observed for another 7 days. Plants were assigned as surviving when they showed the ability to grow new leaves within the recovery period. Values are mean ±SD of 3–4 replicates (each with 8 plants per time point and genotype). n.s., no significant differences were found between the two genotypes at any time point (T-test, P < 0.05).
Arabidopsis suggests that only at oxygen concentrations of 10% and lower (corresponding to 9.83 kPa in saturated water), the transcription factor AtRAP2.12 starts to accumulate, and induction of a transcriptional response to hypoxia is only observed at even lower oxygen concentrations (Kosmacz et al. 2015). Therefore, leaf tissue of B. napus is not hypoxic under water in light, which is likely due to ongoing photosynthesis (Figure 8(C)), as well as in darkness where oxygen might be taken up from the surrounding water.

4.2 Differences in temporary gas film formation have no impact on photosynthesis or survival under submergence

One strategy to avoid restrictions in gas diffusion under water is the development of leaf gas films (Colmer & Pedersen, 2008; Kurokawa et al., 2018). During our submergence experiments, we observed a difference in the ability to form gas films between both cultivars. While Avatar was able to develop a gas film and maintain it for several hours under submergence, the gas film on Zhongshuang 9 leaves was very weak (Figures 8, S9). Whether this difference is caused by the composition of the cuticle, the surface structure of the leaves, or the number or form of leaf hairs, is not known. There are no obvious GO categories associated with any of those processes that were differentially expressed between genotypes (Table S4). Also, the gene family associated with gas film formation, LGF1 from rice (Kurokawa et al., 2018), whose homologs in Arabidopsis and B. napus are called hydroxysteroid dehydrogenases, was not higher expressed in Avatar (Table S3).

Despite a difference in gas film formation, we could neither detect a significant difference in the photosynthetic rates nor in the dark respiration rates under water (Figure 8). Accordingly, sugar levels, growth rates, and survival were very similar in the two cultivars (Figures 3, 4, 7 and 9). This suggests that gas film formation in the rapeseed cultivar Avatar is not beneficial under submergence, which is not surprising since the superhydrophobic properties are lost within the first 24 h of submergence. In line with this, some studies did not clearly demonstrate a positive effect between gas film formation and submergence survival for non-grass species (Winkel et al., 2016). Whether the ability to form gas films has positive effects on other plant aspects, such as growth, development or under pathogen attack, remains to be elucidated.

4.3 Submergence in light causes carbohydrate starvation and growth retardation in Brassica napus

The comparison of our dataset under submergence with published datasets of stress treatment in Arabidopsis revealed a strong carbon starvation signature (Figure S3, Table S5). Genes known to be induced by extended night or other starvation treatments (Cookson et al., 2008), like MIOX2.12, BGAL4 or ERD5, were strongly induced by our submergence treatment, although plants were in light and had the possibility to photosynthesize under water (Table S3, Figure 2). The same set of genes was induced in Arabidopsis under darkness in air (van Veen et al., 2016, Figure S12), but most of them were not further induced by submergence in darkness (Figure S12). This again suggests that no oxygen deficiency was present under our conditions inside B. napus leaves.

The carbon starvation-responsive gene expression was well correlated with the low sugar content in leaves after 24 h of submergence (Figures 3, 4). In both genotypes, less than 10% of sugars in comparison to aerated control were detectable (Figure 4(A), (B)). This sugar decline already started within the first 3 h of submergence. The fast decline in sugar content could have two reasons. First, photosynthesis and especially the Calvin cycle might be inhibited by low carbon dioxide concentrations within the submerged leaves. Usually, plants grow under carbon dioxide limitation already under control conditions due to low CO₂ concentration in normal air (for example summarized in Ainsworth & Rogers, 2007). Under water, this limitation is further intensified through low gas diffusion under water and most likely through closed stomata. An additional effect of the floodwater could be the lower amount of light penetrating the water, which also negatively affects photosynthesis.

Our data on net photosynthesis under water and in air support this hypothesis. The photosynthetic rate under water (Figure 8(C)) is less than 20% of the rate in air (Figure S10), both based on gas exchange. The lack of 80% of photosynthates normally produced through CO₂ fixation can easily result in severe carbohydrate starvation. However, we cannot exclude cyclic electron transport within chloroplasts under water which could produce ATP (Heber, 2002). Indeed, ATP concentrations did not decrease as much as sugar concentrations within the first 24 h of submergence (Figure S6).

A second reason for sugar depletion could be the enhanced consumption of carbohydrates under water. Although we could not detect elongational growth in B. napus under water (Figure 7), the stress response (including transcription and translation of stress-related genes) is generally more cost-intensive than the maintenance of metabolism under control conditions. Under dark submergence, sugars and starch decreased in Arabidopsis at a similar rate as in darkness in air (Loreti et al., 2018). For submergence in light, no data have been published yet on Arabidopsis leaves to make a comparison of degradation rates.

As a consequence of sugar depletion, submerged plants drastically reduced their biosynthesis, including translation, cell division and DNA replication (Table S4), and reduced the expression of many genes that are also down-regulated under carbon starvation (Usadel et al., 2008, Cookson et al., 2016, Table S3, Table S5, Figure 6). One example of such a gene is HBI1. This transcription factor promotes cell expansion and represses defense responses (Neuser et al., 2019). Under our treatment conditions, HBI1 expression is strongly down-regulated in leaves (Figure 6(C)), suggesting that it is one component of the pathway to downregulate growth of the leaves and petioles (Figure 7(B), (C)). In line with this,
putative target genes for HBI1 are certain expansins that are involved in cell elongation (Fan et al., 2014), whose expressions are also down-regulated under submergence in *B. napus* (e.g., EXPA1, BnaC06g30760D, BnaA07g28080D; EXPA11, BnaCnng55390D; EXPA9, BnaCnng03570D, BnaA03g55050D; EXPB1, BnaC07g00140D, BnaA07g00550D; Table S3). Interestingly, during dark submergence of Arabidopsis, the expression of the transcription factor is not repressed, but under these conditions, the petiole elongation is also not inhibited (van Veen et al., 2016). One could hypothesize that the down-regulation of growth is an active process, rather than a consequence of carbon starvation. This has also been suggested in a study on *Nasturtium*, where petioles showed strong growth inhibition under water, while stems exhibited elongational growth (Müller et al., 2019). In the future, it should be analyzed which factors are contributing to a possible active growth regulation.

### 4.4 Two *Brassica napus* cultivars differ in gene expression, but not in their response to submergence

In our study, we analyzed the gene expression under submergence for two different cultivars, of which one had been described as a waterlogging-tolerant cultivar. We observed many genes that were differentially expressed between the two genotypes, but most of these differences were independent of the treatment (Table S3). Despite the large number of differentially expressed genes (Figure 1), the functions of those genes did not reveal strong differences (Table S4). Avatar showed a higher expression of translation-related categories, while Zhongshuang 9 showed a stronger induction of defense-related genes especially under submergence. Among the enriched categories in Avatar was one flower-related GO term, GO:0048573 (photoperiodism, flowering). Recently, different rapeseed cultivar groups have been analyzed by sequencing at the genome level, and the *FLC* locus was among the ones with multiple single nucleotide polymorphisms (SNPs, Wu et al., 2019). In our dataset, two *FLC* genes were higher expressed in Avatar than in Zhongshuang 9, suggesting repression of flowering in the winter-type rapeseed. However, our gene IDs (BnaC03g04170D, BnaC09g46540D) were different from the gene with SNP enrichment in the other study (BnaA02g12130D).

One reason for the low amount of functional differences could be the fact that *B. napus* is a tetraploid species with multiple genome duplication events (Nikolov & Tsiantis, 2017), and each gene copy could be differentially regulated in different genotypes, with an overall similar expression of a certain gene function. Variation in gene expression between different cultivars has been observed previously (e.g., Havlickova et al., 2018), but no general functional characterization of differential gene expression has been performed yet.

Despite the usage of one presumably waterlogging-tolerant cultivar (Xu et al., 2015; Zou et al., 2014), we did not observe strong differences in the submergence response. In one other study, that compared gene expression differences between tolerant Zhongshuang 9 and sensitive GH01 under waterlogging (Zou et al., 2015; Zou, Tan, et al., 2013), there were also very little gene expression differences described. None of the few expression differences could be associated with higher tolerance so far. In order to evaluate whether true variations in flood tolerance exist within the relatively newly evolved species *B. napus*, experiments with a highly diverse panel of cultivars, including other *Brassica* species, are required.

### 5 Conclusions

In this study, we characterized the submergence response of *B. napus* for the first time at the molecular and physiological level. Even in light, carbohydrates rapidly decreased within the first hours of submergence, causing a drastic carbon starvation response at the transcriptional level. Consequently, biosynthetic processes, translation, and growth were severely affected. With these responses, rapeseed seedlings were able to survive at least 14 days of submergence, presumably with a very low metabolic rate. This suggests a quiescence strategy of this Brassicaceae species, similar to the flood sensitive model plant Arabidopsis. However, the usage of two different cultivars, one of them previously described as waterlogging-tolerant, did not reveal any differences between cultivars in their response to submergence. Nevertheless, this study sheds light on the general genotype-independent response of *B. napus* to submergence and future studies should include other *B. napus* accesses or even other tolerant *Brassica* species.

### Author Contributions

Angelika Mustroph and Ole Pedersen designed research; Philipp R. Wittig, Stefanie Ambros, Leonor Álvarez-Cansino, Bettina Bammer, Dennis Konnerup, Ole Pedersen and Angelika Mustroph, performed experiments; Jana T. Müller and Angelika Mustroph analyzed data; Angelika Mustroph wrote the manuscript. All authors read and commented on the manuscript.

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### Data Availability Statement

The data that support the findings of this study are openly available at the Gene Expression Omnibus database at https://www.ncbi.nlm.nih.gov/geo/ under the accession number GSE140828. The materials that support the findings of this study are available from the corresponding author upon reasonable request.
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**SUPPORTING INFORMATION**
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