Pollen Grain Number and Viability Contribute to Variation in Effective Ovule Number Among Oilseed Rape Genotypes

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Abstract

While no significant differences in initial ovule number were found among oilseed rape genotypes, there was a large variation in effective ovule number (EON), which determines the final seeds per silique (SPS), a critical component of yield. In this study, we selected 18 oilseed rape genotypes with contrasting nitrogen utilization efficiency (NUE) to unravel the main factors responsible for different EON and determine the critical period of EON formation under both a field and a pot experiments from 2016-2018. The high NUE genotypes displayed significantly higher NUE by 14.3%, along with greater yield per plant (29.4%) and SPS (21.1%) than the low NUE genotypes. The greater productivity of the high NUE genotypes was associated with 44.1% higher pollen grain number, 23.5% greater pollen viability, and 39.3% lower ovule abortion rate, compared to the low NUE genotypes. In addition, the heart stage was the critical ovule development period for delineating the variability of EON among contrasting NUE oilseed rape genotypes, when the high NUE genotypes displayed higher silique net photosynthetic rate, surface area, biomass, and RNA expression levels. Taken together, this study indicated the pollen grain number, pollen viability and ovule abortion rate contributed to the final variation in EON and the heart stage was the critical period of determining the EON differences among contrasting NUE genotypes. Increasing pollen grain number and pollen viability, and decreasing ovule abortion rate before heart stage should be the prerequisite for breeders to improve yield and NUE of oilseed rape genotypes.

Introduction

Oilseed rape (*Brassica napus* L.) is the world's second largest crop source of vegetable oil following soybean, with increasing significance in the international market. Yield, one of the most important traits in oilseed rape breeding, is the product of seeds per silique (SPS), siliques per plant (SPP) and thousand seed weight (TSW) (Ma et al. 2015). As with other crops, yield components are critical to yield formation and often compensate each other, depending on genotypes and interaction with environment (Yang et al. 2017a; Shi et al. 2015). Additionally, the three yield components (SPS, SPP and TSW) of oilseed rape display different degree of negative correlation, which indicates that the final seed yield can be improved by increasing the individual yield components (such as SPS) (Yang et al. 2017b). SPS, which is usually negatively correlated with the other two yield components (SPP and TSW) for competition among carbohydrate sinks, has received much attention (Zhang et al. 2012; Yang et al. 2016; Wang et al. 2016b; Yang et al. 2017a; Gardner and Latta 2007). On the other hand, in the study conducted by He et al. (2017), the SPS was positively correlated with nitrogen utilization efficiency (NUE; seed dry weight / shoot N accumulation), which indicated that SPS can be used as a simple and applicable indicator for the evaluation of NUE in oilseed rape.

During the pollination process, pollen grains have to reach and germinate on the stigma, and then form pollen tubes to accurately reach the female gametes. After that, fertilization is initialized once the male gametes meet the female gametophyte within the ovule (Palanivelu and Tsukamoto 2012). Thus, successful reproduction relies on the pollen grains number, grain viability and female ovule number (Gao et al. 2017; Li et al. 2015). Following fertilization, the developmental process from embryogenesis to final seed can be divided into five distinct stages: pre-embryo, globular, heart, torpedo and maturation (Andriotis et al. 2010; Tan et al. 2011; Hehenberger et al. 2012). At the same time, the fertilized ovules may fail at any one of the stages due to the internal and external environmental conditions, and the remaining ovules (effective ovule number, EON) eventually develop into viable seeds (SPS). Therefore, to some degree, the EON determines the SPS. While no significant difference in the initial ovule number was observed among the diverse oilseed rape genotypes (Li et al. 2015; Yang et al. 2016), large variations (from 5 to 35) in the SPS have been reported (Yang et al. 2016). Based on this, we hypothesized that there is a critical period from the pre-embryo to maturation that determines the variation in EON among contrasting NUE genotypes.

Biologically, the EON of oilseed rape genotypes is primarily determined by the number of flowers initiated, the number of ovules per placenta, the proportion of ovules to be fertilized (the proportion of fertile ovules $\times$ the proportion of fertile ovules to be fertilized), the proportion of fertilized ovules to develop into seeds as well as the frequency of embryonic abortions (the proportion of fertilized ovules to develop into seeds) (Jeong et al. 2012; Yang et al. 2016; Yang et al. 2017b). Embryonic abortion, which occurs at different developmental stages within the fruit, has been shown to be non-random and based on the genotype of the embryo and environmental factors as well as their interaction (Calvino 2014), such as pollen grain germination (Wang et al. 2014), silique photosynthesis (Khan et al. 2018; Labra et al. 2017), nutrient accumulation (Li et al. 2020) and the number of flowers (Wang et al. 2011; Brookes et al. 2010). Although the developmental processes from the ovule fertilization to seed maturity is well known among oilseed rape genotypes, there is a paucity of knowledge that pinpointed the main factors that determine the EON, especially in pollen indices. Thus, establishment of the link between EON and pollen indices will provide basic information for breeders to take steps to reduce ovule abortion and increase yield of oilseed rape genotypes.

It has been reported that the EON is strongly and positively correlated with NUE, and nitrogen utilization-efficient genotypes had higher EON than the inefficient genotypes (He et al. 2017). Therefore, 18 highly pure rapeseed lines with contrasting NUE and broad genetic background were used to systematically dissect the main causes and critical period of the natural variation of EON in rapeseed under both a field and a pot experiments from 2016–2017 and 2017–2018. In detail, the main objectives of the current study were to: i) unravel the main factors that restrict EON, and ii) determine the critical period of EON formation. It was hoped that our results would be helpful for the researchers that work in breeding for high yielding and NUE oilseed genotypes for sustainable agriculture in the future.

Materials And Methods

Plant materials

The results from a previous study, in which 50 different oilseed rape genotypes were examined under both pot and field experiments, allowed us to categorize the tested genotypes into four groups, based on their NUE values: Nt-responder, Nt-nonresponder, Nt-efficient, and Nt-inefficient. Nt-responder referred to genotypes with an NUE value above the mean at high N (0.3 g N kg^{-1} dry soil or 180 kg N ha^{-1}), while genotypes with a NUE value below the

mean were categorized into the Nt-nonresponder group. At the low N supply (0.1 g N kg^{-1} dry soil or 0 kg N ha^{-1}), genotypes displayed a NUtE value above the mean were called Nt-efficient, and Nt-inefficient genotypes were those genotypes with a NUtE value below the mean (He et al. 2017). According to the rank of responses reported previously, we chose 18 genotypes with distinctive NUtE for this study. These included 5 Nt-responder, 5 Nt-nonresponder, 4 Nt-efficient and 4 Nt-inefficient and are described in Table S1.

Experimental design

In this study, a field and a pot experiments were conducted at Yangling district, Shaanxi province, P.R. China ($34^{\circ} 24' \text{ N}$, $108^{\circ} 08' \text{ E}$) from 2016–2018. In the field trial, the soil was with a pH of 7.65, containing 13.7 g kg^{-1} organic matter, 1.19 g of total N kg^{-1} , 24.7 mg of available N kg^{-1} , 15.7 mg of Olsen-P kg^{-1} and 76.9 mg of available K kg^{-1} . In the pot experiment, the soil had the following properties: 9.21 g kg^{-1} organic matter, 0.92 g of total N kg^{-1} , 22.0 mg of available N kg^{-1} , 10.7 mg of Olsen-P kg^{-1} and 73.6 mg of available K kg^{-1} , and a pH of 7.5.

Our previous study indicated that the Nt-responder and Nt-nonresponder genotypes were expressed only under high N supply conditions, while the Nt-efficient and Nt-inefficient were expressed under low N supply conditions. Therefore, the 10 high N genotypes were planted with high N level (150 kg N ha^{-1} in the field experiment and 0.30 g of N kg^{-1} dry soil in the pot experiment), while the 8 low N genotypes were planted with low N level (0 kg N ha^{-1} in field experiment and 0.10 g of N kg^{-1} dry soil in pot experiment). In the field experiment, the high or low plot of 10 m in length $\times$ 2 m wide or 8 m length $\times$ 2 m width, consisted of forty or thirty-two rows of oilseed rape with row spacing of 50 cm . Each experiment was arranged in a randomized complete block design with four replicates. In both experiments, sufficient phosphorus as superphosphate (P_2O_5 135 kg ha^{-1} and 0.20 g kg^{-1} dry soil) and potassium (K_2O 150 kg ha^{-1} and 0.30 g kg^{-1} dry soil) were supplied.

Observations and measurements

In order to monitor the dynamics of silique development, flowering and fertilization process of the genotypes were categorized into: pre-embryo (1–4 days after flowering), globular (5–8 days after flowering), heart (9–14 days after flowering), torpedo (15–22 days after flowering), and maturation (23–30 days after flowering) stages (Andriotis et al. 2010; Tan et al. 2011; Hehenberger et al. 2012). For accurate monitoring, fully-opened flowers on the main inflorescence of each plant were marked with colored strings each day. In the pot experiment, five marked siliques of each plant at each developmental stage were chosen for the determination of net photosynthetic rate, silique length, width, surface area, EON, biomass and RNA expression levels.

At the flowering stage, we removed the petals from the sampled buds with tweezers, and recorded the stamen and anther number. Then, the pollen grain number and pollen viability were measured according to Lankinen et al. (2018): Pollen grains were sprinkled on a microscope slide in Hoekstra medium with 16% sucrose. The microscope slides were placed in a dark constant temperature incubator with a temperature of 25°C for 3 h. Pollen germination was terminated by adding 100% glycerol. Pollen grain number and germination rate (pollen viability) were determined under a light microscope (Axioplan 2; Zeiss) as the percentage of germinated pollen grains from 100 pollen grains in a randomly chosen area with the following equation: Pollen viability = the total number of germinated pollen grains / the total number of pollen grains $\times$ 100%. The initiation ovule number and abortion rate were determined according to Wang et al. (2011): Ovule abortion rate = the aborted ovule number / the initial ovule number.

Measurement of agronomic traits

At maturation stage, plant height was measured from the base of the stem to the tip of the main inflorescence (Sun et al. 2016). The point of measurement of stem diameter was set at 10 cm from the base of the main stem (Ohashi et al. 2006). First, valid branch height was measured as the height from the base of the stem to the effective primary branches at the bottom of the main stem. Then, the number of first valid branches was measured according to Xu et al. (2014a).

Measurement of yield and N use efficiency

Number of siliques per plant was measured as the number of effective pods on the main inflorescence, branch inflorescence and the whole plant (Shi et al. 2015). Seed weight of each plant was measured by weighing 500 fully developed seeds with four replications; the weight of 500 seeds was then converted to 1,000-seed weight for easy comparison with other studies (Fan et al. 2010). Seed yield per plant was measured as the average dry weight of seeds of the four randomly selected plants from each genotype (Shi et al. 2011). Each of the ground seed and straw samples was weighed, and then digested and determined for N concentration by following the Kjeldahl procedure. Seed and shoot (seed + straw) N accumulations were calculated as the product of dry matter by the respective N concentration. According to He et al. (2017), N utilization efficiency (NUtE) and N uptake efficiency (NUpE) were estimated with the following equations: NUtE = seed yield / shoot N accumulation; NUpE = shoot N accumulation / N supply.

Measurement of siliques indices

At each stage (pre-embryo, globular, heart, torpedo, and maturation), silique net photosynthetic rate was measured on the marked siliques between 09:00 and 11:00, using a Portable Photosynthesis System (Li-6400, LI-COR, Lincoln, NE, USA). Afterwards, the marked siliques were removed from the main inflorescence. Half of each of the sampled siliques was rapidly frozen in liquid nitrogen, and stored at -80°C for later RNA transcript analysis, and the other half was used for the determination of surface area (Hua et al. 2012) and normal ovule numbers which were counted under a light microscope. The sampled siliques were oven-dried at 105°C for 30 min and then at 70°C until a constant dry weight was reached and then were weighed.

Quantitative RT-PCR analysis

Silique samples of the 18 diverse NtE genotypes were frozen in liquid nitrogen immediately after collection and stored at -80°C. Approximately 100 mg of tissue was ground in liquid nitrogen and total RNA was extracted using an EZNATM Plant RNA Kit (Omega Bio-Tek Inc., Norcross, GA, USA). The sample was used for cDNA synthesis using TransScript® First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Quantitative PCR analyses were performed in an QuantStudio® Design and Analysis (QuantStudio 5, Life Technologies, CA, USA), using a TransStart Tip Green qPCR SuperMix (TransGen Biotech, China). All of the primers used in the analysis are listed in Table S2. The low NtE genotypes (Nt-nonresponder and Nt-inefficient) were used as references (Nt-nonresponder for Nt-responder and Nt-inefficient for Nt-efficient, respectively). Data were expressed as the mean of four biological replicates $\pm$ SD.

Statistical Analysis

All the data from both the field and pot experiments, were subjected to analysis of variance. Estimates of correlation coefficients and tests of significance, were performed using the SPSS version 17.0. Principal component analysis was performed using the Canoco 5.0 software. When the analysis showed significance, mean comparisons were made according to the least-significant difference test at $P \leq 0.05$ ($LSD_{0.05}$). All the figures were created using Origin 9.0 software, Microsoft Excel and PowerPoint 2016.

Results

Stamen and ovule characteristics

Our results showed that the stamens of the contrasting NtE genotypes were identical and normal in the field and pot experiments (Fig. 1A-D), and no significant differences in anther and filament number were found (Fig. 1E-H, Supplementary Fig. 1). On average, the high NtE (Nt-responder and Nt-efficient) genotypes showed 44.1% (52.7% and 33.1%) greater pollen grain number and 23.5% (21.2% and 26.2%) higher pollen viability than the low NtE (Nt-nonresponder and Nt-inefficient) genotypes (Fig. 1I-P). However, there were no significant differences in the initial ovule numbers among the high and low NtE genotypes under both the field and pot experiments (Fig. 2A-H).

Silique physical indices

We also observed the silique net photosynthetic rate of Nt-efficient genotypes was greater than the Nt-inefficient genotypes from the heart to maturation stages (Fig. 3B), and silique surface area for the high NtE genotypes was higher than for the low NtE genotypes from the heart to torpedo stages. The same trend was found in the silique length and width (Supplementary Fig. 2). Finally, higher effective ovule number and silique biomass were recorded for the high NtE than for the low NtE genotypes from the heart to maturation stages (Fig. 3E-H).

Silique RNA transcript levels

In order to understand the observed differences in silique development, 18 of the contrasting NtE genotypes were selected for measurement of the expression profile of silique development related-genes (*BnARF18*, *BnaC9.SMG7b*, *BnCLV3* and *BnDA1*). Our results found that there was no significant difference in the expression level of the four genes among the high and low NtE genotypes at the globular stage (Fig. 4). However, from the heart to torpedo stages, the RNA expression levels were higher by 15-40 fold in *BnARF18*, 5-21 fold in *BnaC9.SMG7b*, 5-15 fold in *BnCLV3* and 4-18 fold in *BnDA1* for the high NtE than for the low NtE genotypes.

Ovule abortion rate

Differences in ovule abortion rate among the contrasting NtE genotypes also existed in both field and pot experiments, with 39.3% (45.4% in Nt-responder and 34.3% in Nt-efficient) lower ovule abortion rate for the high NtE than for the low NtE genotypes (Fig. 5A-H).

Agronomic traits, yield and NUE

No significant difference of agronomic traits (plant height, stem diameter, first valid branch height and number of first valid branches) among the contrasting NtE genotypes was recorded under field or pot experiments (Table S3). However, the number of siliques per plant, seeds per silique and yield per plant were respectively 12.4%, 21.1% and 29.4% greater for the high NtE genotypes than for the low NtE genotypes. In contrast, the low NtE genotypes displayed 14.3% higher 1000-seed weight than the high NtE genotypes in both experiments (Table 1). Compared to the low NtE, the high NtE genotypes showed 14.3% and 27.7% larger NtE and NUpE, respectively (Fig. 6).

Correlations between effective ovule number and the agronomic traits

Principal component analysis showed that the first two principal components explained about 66%-83% of the total variation (Fig. 7A-B). The first principal component was mainly spanned by traits of EON, anther number, filament number, pollen grain number, pollen viability, initial ovule number, stem diameter, first valid branch height, 1000-seed weight, yield per plant, and NtE on the positive side of the scale, while ovule abortion rate spanned on the negative side. The vectors indicated NtE, the yield per plant, pollen grain number and pollen viability exhibited obviously positive correlations with EON, while there was a strong and negative correlation between ovule abortion rate and EON in both the field and the pot experiments.

Using the path coefficient analysis, we illustrated the direct and indirect effects of the three traits (pollen grain number, pollen viability and ovule abortion rate) on EON and their interrelationships (Fig. 7C-D). The pollen grain number and pollen viability displayed a positive direct effect on EON, while the ovule

abortion rate displayed a negative direct effect on EON. A positive indirect effect of pollen grain number (or pollen viability) was evident on EON via the pollen viability (pollen grain number), however a negative direct effect occurred between EON and ovule abortion rate.

Discussion

Our study showed that although no significant differences in anther number and initial ovule number between the contrasting NuTE genotypes were found, the high NuTE genotypes displayed the greater pollen grain number, higher pollen viability and lower ovule abortion rate, leading in larger EON, yield and NuTE, compared to the low NuTE genotypes. After flowering, the fertilized ovules went through proembryo, globular, heart, torpedo and maturation stages. The results of silique net photosynthetic rate, surface area, biomass, and RNA expression levels revealed that there was no significant difference of EON from the proembryo to globular stages, but the high NuTE oilseed rape genotypes displayed higher EON than the low NuTE genotypes at the heart stage, until the maturation stage. We suggested that the crop physiologists and plant breeders should take effective measures to increase the pollen number and vigor, and decrease ovule abortion rate before the heart stage, so as to boost EON, seed yield and NUE. A simplified model (Fig. 8) was drawn to illustrate the mechanistic understanding of the main reasons and critical period of EON formation among the contrasting NuTE oilseed rape genotypes.

Greater pollen grain number, higher pollen viability and lower ovule abortion rate contribute to the larger EON in the high NuTE genotypes

The main biological processes that determine SPS are basically known in oilseed rape (Yang et al. 2017b), however the pollen indices and ovule abortion rate responsible for its natural variation among contrasting NuTE genotypes as well as their relative contributions are poorly known. The specific aspect of present study was to report that the high NuTE genotypes produced greater seed yields and higher NuTE (Table 1 and Fig. 6). These high NuTE genotypes displayed a larger EON, which was associated with greater pollen grain number, higher pollen viability, and lower ovule abortion rate, compared to the low NuTE genotypes. The process from the initial ovule to final seeds is affected by many factors, such as pollen sterility (Yang et al. 2016), floral bud quantity (Luo et al. 2018), inflorescence position (Wang et al. 2014), planting density (Khan et al. 2018), shading (Labra et al. 2017) and silique photosynthesis (Wang et al. 2016a). In this study, we explored some factors that may have caused variation in EON among the contrasting NuTE oilseed rape genotypes. After ovule fertilization, the ovule abortion is frequently associated with seed yield (Mena-Ali and Rocha 2005). In agreement with this study, we observed a negative correlation between EON and the ovule abortion rate (Fig. 7C-D), and the ovule abortion rate of the high NuTE genotypes was significantly lower than the low NuTE genotypes (Fig. 5), resulting in greater seed yield and NuTE (Table 1 and Fig. 6). This suggested that breeding towards high NuTE genotypes can be a relatively quick trait-based strategy to reduce ovule abortion and improve seed yield and NuTE. Isoda et al. (2010) demonstrated the lower supply of photosynthates can cause more pod abortion, thereby reducing seed yields. Therefore, further increase in potential yield will likely rely on improving photosynthesis (Zhu et al. 2018). According to our results (Fig. 3A-B), the greater silique net photosynthetic rate of the high NuTE genotypes than that of the low NuTE genotypes leads to higher photoassimilates supply to the developing pods, and ultimately greater crop yield and NuTE (Table 1 and Fig. 6). Additionally, the majority of reproductive abortion occurs early in development (Coello and Martinez-Barajas 2016), and negative correlations between EON and the abortion rate were recorded, suggesting ovule abortion rate may have acted as the “upstream” of EON. Therefore, implementation of strategies targeted on reducing ovule abortion rate, such as sufficient supply of water (Gusmao et al. 2012), appropriate temperature (Zinn et al. 2010) and light (Wang et al. 2011) during the early ovule developmental period, may increase EON, yield and NuTE in oilseed rape genotypes.

It is generally believed that there were no significant differences in initial ovule number among different oilseed rape genotypes (Yang et al. 2016; Li et al. 2015). These previous studies are fully in agreement with our present results (Fig. 2), indicating the initial ovule number could not contribute to EON variation among oilseed rape genotypes. While no significant difference in the number of anthers among the high and low NuTE genotypes was found (Fig. 1A-H), the high NuTE genotypes exhibited 44.1% greater pollen grain number and 23.5% higher pollen viability than the low NuTE genotypes (Fig. 1I-P). It suggested that the gametophytic competition is intensified and the chance of fertilization yielding vigorous zygotes increases when larger pollen grain number and viability are selected in breeding programs (Zhang et al. 2010). Chen et al. (2014) reported that the decreased pollen viability resulted in lower grain yield in rice lines, and lower seed-set in sorghum was due to decreased pollen grain number and viability. Similar result was observed in *Pyrus pyrifolia*, where the higher number of pollen grains improved pollen germination rate, seed viability, fruit yield and fruit quality (Zhang et al. 2010). The present study demonstrated significant and positive correlations of EON with both the pollen grain number and pollen viability (Fig. 7C-D). It indicated that the greater pollen grain number and pollen viability in the high NuTE genotypes led to the higher EON, and the latter ultimately resulted in greater seed yield and NuTE (Table 1 and Fig. 6). In oilseed rape plants, ovules fail to develop into mature seeds because of fewer viable pollens available to fertilize all the ovules, subsequently resulting in aborted ovules (Fu et al. 2018; Dai et al. 2018). That means the low viable pollen grain number and low pollen viability could therefore, be the “upstream” cause of ovule abortion, and those two pollen indices ultimately regulate the variation in EON by controlling ovule abortion rate. Pollen grains are produced within the anther and released to the environment after maturation (Selinski and Scheibe 2014). During pollen development, nitrogen is essential to ensure efficient growth. Inadequate N supply significantly reduced pollen grain number and pollen viability in maize (Zheng et al. 2017; Soto et al. 2010). Nikolic et al. (2012) demonstrated a significant correlation between the plant N accumulation and grain yield in wheat. In our study, we found 19.2% greater shoot N accumulation at pre-flowering for the high NuTE than for the low NuTE genotypes (data not shown). Accordingly, we speculated that the pre-flowering shoot N accumulation in the high NuTE genotypes may have served as the N reserve that allowed the plant to produce larger pollen grain number with higher pollen viability, leading to less ovule abortion, greater seed yield and larger NuTE, compared to the low NuTE oilseed rape genotypes.

Larger effective ovule number for the high NuTE than for the low NuTE genotypes at the heart stage

Because of the complex conditions, especially germplasm resources, the EON formation of diverse oilseed rape genotypes showed great variation during the reproductive stage, the most important process in the life cycle of plants (Yang et al. 2016; Pereira et al. 2014). In the present study, we first tested the anther and filament number, as well as initial ovule number, and found there was no significant difference among contrasting NuTE genotypes (Fig. 2).

Based on this, we surmised that the critical period for EON variation occurred after fertilization during one or more stages of the pre-embryo, globular, heart, torpedo or maturation (Andriotis et al. 2010; Tan et al. 2011; Hehenberger et al. 2012). Our results clearly identified the heart stage as the critical stage at which the difference in dynamic change of EON formation became significant among the contrasting NuTE genotypes and remained until the maturation (Fig. 3–4). Xu et al. (2014b) and Yang et al. (2016) respectively claimed the globular and torpedo stage as the critical period for EON formation among diverse oilseed rape genotypes, which was inconsistent with our results. Our fine turning procedure with more frequent sampling in the current study allowed us to pinpoint the critical stage which may explain the inconsistency with the literature. The rapeseed silique is one of the main photosynthetic organs in oilseed rape, and it also serves as an important sink organ that stores carbohydrates for filling the developing seeds (Wang et al. 2016a; Chen et al. 2018). In this study, the net photosynthetic rate and biomass of the silique were both greater for the high NuTE than for the low NuTE genotypes at the heart stage (Fig. 3A-B and 3G-H), leading to an increase in silique biomass accumulation and sustained photosynthesis which are the major physiological determinants of EON increases (Makino 2011). It was expected that plants with greater silique surface area would have higher silique photosynthesis and thus would increase seed yield (Kebede et al. 2014). Compared to the low NuTE oilseed rape genotypes, our results showed significantly larger silique surface area, silique length and width in high NuTE genotypes at heart stage, implying that the heart stage is the critical period of EON development among contrasting NuTE oilseed rape genotypes. This was consistent with the results of silique net photosynthetic ratio, biomass and EON.

Fertilized ovules development is a complex and important process in the development of flowering plants, and it is indispensable for the next generation production (Gao et al. 2017; Wei et al. 2015). The silique is the main organ in which fertilized ovules are formed and filled, and this is closely correlated to the growth conditions of plants and is all complex quantitative trait controlled by polygenes (Wang et al. 2016b). Recent literatures have reported the identification of genes that regulate fertilized ovules or silique development in oilseed rape plants. For example, *BnARF18* gene simultaneously regulated silique development and seed weight (Liu et al. 2015). *BnaC9.SMG7b* gene was found to alter fruit development and seed formation with regard to seed morphology (Li et al. 2015). The mutation of *BnCLV3* produced more multilocular siliques with a significantly higher number of seeds per silique and a higher seed weight (Yang et al. 2018), and *BnDA1* controlled the seeds weight and organ size (Wang et al. 2017). Here, we selected the above four silique development genes to understand the relationship between the EON development and the expression level of genes obtained from the high and low NuTE genotypes. Our results clearly showed higher silique RNA transcript levels of the high NuTE than of the low NuTE genotypes from the heart to torpedo stages (Fig. 4). It supported the conjecture that green siliques of oilseed rape functioned as a photosynthesis center, and may contribute assimilates and nutrients into the silique biomass and developing seeds, generating larger seeds (Wang et al. 2016a; Hua et al. 2012; Bennett et al. 2011). This may be one of the reasons that the silique indices of the high NuTE genotypes were higher than the low NuTE genotypes, and further research on selection the silique development related-genes may be one of the prerequisites to enhance EON, and ultimately higher yield and NuTE (Table 1 and Fig. 6). Taken together, the significant differences in silique development related-genes transcript levels among the high and low NuTE genotypes were found at the heart stage, which led to the larger silique surface area, greater biomass and higher EON, accompanied with higher silique net photosynthetic rate for the high NuTE genotypes, and difference in the dynamic silique indices maintained until maturation. With these results as the supporting evidence, it was concluded that the heart stage is the critical period of determining the EON among contrasting NuTE genotypes in silique development.

Conclusion

Compared to the low NuTE oilseed rape genotypes, the high NuTE genotypes produced a larger number of pollen grains with higher pollen viability before flowering, and displayed a lower ovule abortion rate after ovule fertilization. At the heart stage, the high NuTE genotypes formed a larger EON than the low NuTE genotypes, and the dynamic difference in EON between the genotypes was maintained until the maturation. Consequently, the high NuTE genotypes produced more SPS and greater yield with larger NuTE than the low NuTE genotypes. With this evidence, we suggest that breeding programs on oilseed rape improvement to achieve higher NuTE and seed yield should be targeted at increasing pollen grain number and pollen viability, and decreasing the ovule abortion rate before the heart stage.

Declarations

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Conflict of interest The authors declare no competing financial interests.

Author contributions XG conducted the two experiments, and analyzed the data and wrote the manuscript; YX and YN participated in pot experiment; XW and BC provided the experimental materials; BM, MN and YG drafted the work and revised the manuscript critically. All authors read and approved the final manuscript.

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Tables

Table 1 Means of yield and yield components of contrasting NUtE oilseed rape genotypes in the field and pot-culture experiments.

	Siliques per plant		Seeds per silique		1000-seed weight (g)		Yield per plant (g)	
	Field experiment	Pot experiment	Field experiment	Pot experiment	Field experiment	Pot experiment	Field experiment	Pot experiment
Nt-responder	310.1 a	242.9 a	17.8 a	22.1 a	5.02 b	4.00 a	30.7 a	26.3 a
Nt-nonresponder	266.2 b	207.3 b	15.4 b	17.5 b	5.81 a	4.13 a	24.7 b	21.7 b
Nt-efficiency	252.2 a	210.6 a	16.8 a	19.1 a	4.71 a	3.36 b	21.8 a	21.0 a
Nt-inefficiency	221.2 b	209.3 a	14.9 b	14.8 b	5.05 a	4.56 a	14.1 b	16.6 b

Means of each trait between Nt-responder and Nt-nonresponder or between Nt-efficient and Nt-inefficient genotypes in each experiment followed by different letters indicate significant differences according to the ANOVA-protected LSD_{0.05} test.

Figures

Figure 1

Stamen traits under field and pot experiments. (A-D) the stamen phenotype among the high and low NUtE genotypes; anther number (E-H), pollen number (I-L) and pollen viability (M-P) of the contrasting NUtE genotypes. Each bar represents a mean plus SE of four biological replicates, and different letters above the bars within a figure indicate significant differences according to the ANOVA-protected LSD0.05 test.

Figure 2

Initial ovule number under field and pot experiments. (A-D) the initial ovule phenotype under light microscopy; (E-H) the initial ovule numbers of contrasting NUtE genotypes. Each bar represents a mean plus SE of four biological replicates, and different letters above the bars indicate significant differences according to the ANOVA-protected LSD0.05 test.

Figure 3

Silique net photosynthetic rate (A-B), silique surface area (C-D), effective ovule number (E-F) and silique biomass (G-H) among contrasting NtE genotypes from pre-embryo, globular, heart, torpedo and maturation stages. * indicates significant difference among the contrasting NtE genotypes ($P < 0.05$).

Figure 4

BnARF18 (A-B), BnLCR (C-D), BnaC9.SMG7b (E-F) and BnDA1 (G-H) expression levels at the globular, heart and torpedo stages. The low NtE genotypes (Nt-nonresponder and Nt-inefficient) were used as references (Nt-nonresponder for Nt-responder and Nt-inefficient for Nt-efficient, respectively). Data are expressed as the means of four biological replicates, and * indicates significant differences between the contrasting NtE genotypes (P < 0.05).

Figure 5
Ovule development phenotypes of contrasting NUtE genotypes under field and pot experiment. (A-D) the image of ovule abortion at maturation phase; (E-H) the mean of the ovule abortion rate of contrasting NUtE oilseed rape genotypes. Each bar represents the mean of four biological replicates, and different letters above the bars indicate significant differences according to the ANOVA-protected LSD0.05 test.

Figure 6
NUtE (A-D) and NUpE (E-H) of the contrasting NUtE genotypes under field and pot experiments. Each bar represents the mean and standard error of the mean of four biological replicates, and different letters above the bar indicate significant differences according to the ANOVA-protected LSD0.05 test.

Figure 8

A simplified model for the main reasons and critical period for the difference in the effective ovule numbers among contrasting NUtE oilseed rape genotypes. Compared to the low efficient genotypes, the high NUtE genotypes produced more pollen grain number, with higher pollen viability and less ovule abortion rate. After flowering, the EON of contrasting NUtE oilseed rape genotypes went through five distinct stages (proembryo, globular, heart, torpedo and maturation). There was no significant difference in EON from the proembryo to globular stages, but the high NUtE oilseed rape genotypes displayed higher EON than the low NUtE genotypes at the heart stage, and the difference in EON maintained until the maturation stage. Therefore, with higher pollen number and pollen viability, and lower ovule abortion rate, the high NUtE genotypes produced higher EON, yield and NUtE than the low NUtE genotypes.

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