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ORIGINAL ARTICLE

Effects of altered expression of *LEAFY COTYLEDON1* and *FUSCA3* on microspore-derived embryogenesis of *Brassica napus* L.

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Abstract *Brassica napus* (*Bn*) microspore-derived embryogenesis has become a model system to study basic aspects of plant development. Recognized transcription factors governing embryogenesis include: *FUSCA3* (*FUS3*), a member of the plant-specific B3-domain family, and *LEAFY COTYLEDON1* (*LEC1*), a member of the HAP3 subunit of the CCAAT binding factor family. The effects of altered expression of both genes were investigated during microspore-derived embryogenesis in established *B. napus* lines over-expressing or down-regulating *BnLEC1*, as well as in tilling lines where *BnFUS3* was mutated. While over-expression of *BnLEC1* decreases the yield of microspore-derived embryos (MDEs) without affecting their ability to regenerate plants, suppression of *BnLEC1* or *BnFUS3* reduced both embryo number and regeneration frequency. Embryos produced by these lines showed structural abnormalities accompanied by alterations in the expression of several embryogenesis-marker genes. Oil accumulation was also altered in the transgenic MDEs. Total oil content was increased in MDEs over-expressing *BnLEC1* and decreased in those suppressing *BnLEC1* or *BnFUS3*. Mutation of *BnFUS3* also resulted in a small but significant increase in linoleic (C18:2) acid. Together this study demonstrates the crucial role of *BnLEC1* and *BnFUS3* during *in vitro* embryogenesis.

1. Introduction

Embryogenesis is one of the most important events in the life cycle of plants. The process begins with the double fertilization marking the formation of the zygote and the endosperm. During embryogenesis, the zygote divides producing characteristic embryogenic stages (globular, heart-shaped, and torpedo-shaped) that are accompanied by profound molecular, physiological, and metabolic changes [55]. During the middle-late stages of embryogenesis, the embryos accumulate storage products and undergo desiccation prior to entering a dormant period [20,3]. Most of these events are also observed during *in vitro* embryogenesis, where embryos can be produced without fertilization. *In vitro* produced embryos proceed through a similar developmental pathway characteristic of seed embryos and are therefore utilized as a model system [37]. Studies using

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in vitro embryos have some advantages: the embryos develop in the absence of maternal tissue, can be produced in high numbers and in a synchronous fashion, and can be easily harvested. Due to the development and optimization of propagation protocols [17,49], investigations on *in vitro* plant embryogenesis have grown exponentially over the past few years.

In vitro embryogenesis can be executed through different methods, with gametophytic embryogenesis being routinely used in many species. Gametophytic embryogenesis involves the utilization of microspores (or immature pollen grains) and precise culture treatments to induce embryo formation, i.e. microspore-derived embryos (MDEs, Fig. 1). The process uses several types of stress which repress the gametophytic pathway in favor of the embryogenic pathway [9,26,36,41,48,54,57].

Microspore-derived embryogenesis is largely used to propagate *Brassica napus* L. (canola), an economically important species used for oil production. Oilseed rape (canola/rapeseed) oil is the third most important vegetable oil in the world [52]. The production of canola oil relies on the genetic potential of canola cultivars to produce high seed yield and high seed oil content. The quality of canola seed oil is determined by the fatty acid (FA) composition. The process of FA biosynthesis during seed maturation is genetically controlled, and requires the synchronization of several biochemical pathways. Fatty acids and triacylglycerols (TAGs) accumulate during embryo and seed maturation [1,5], making this stage crucial when attempting to increase seed oil content. Independent studies have shown that FA biosynthesis is controlled by the expression of several transcription factors, including *LEAFY COTYLEDON1* (*LEC1*), *LEAFY COTYLEDON2* (*LEC2*), *FUSCA3* (*FUS3*), *WRINKLED1* (*WRI1*), and *ABSCISIC ACID INSENSITIVE3* (*ABI3*), which interact to regulate different phases of embryo development and seed maturation [18,32,46]. Many of these genes have critical roles during embryo development [29]. *LEC1* is expressed throughout the entire process of embryogenesis, from the initial to the late developmental stages [56], while *FUS3* is responsible for inducing the maturation phase [8,39]. The *LEC1* protein has the HAP3 subunit of the CCAAT binding factor that allows *LEC1* to be a specific transcriptional regulator of downstream genes containing the CCAAT recognition domain.

FUS3, encoding a B3 protein that accumulates mainly during seed maturation, binds to the RY element CATGCA found in the promoters of several genes [8,39]. Current literature indicates that ectopic expression of *LEC1* is sufficient to induce somatic embryogenesis from vegetative tissue; thus, suggesting a role in regulation of embryogenic competence [29,46,5]. *Arabidopsis* plants with a null *lec1* allele produced abnormal embryos characterized by small hypocotyls and cotyledons [32]. *Arabidopsis fus3* and *lec1* mutant plants also show a decrease in protein and lipid accumulation during seed development [32,21]. As synthesis and storage of oil is linked to several stages of embryo and seed development, it has been suggested that the genetic regulation of embryo morphogenesis and maturation influences oil production. In our previous studies, we demonstrated that over-expression of *BnLEC1* increases seed oil accumulation in *B. napus*, while suppression of *BnLEC1* or *BnFUS3* decreases oil content [11,12]. While these studies suggest a clear involvement of these genes during *in vivo* embryogenesis, little information is available regarding *in vitro* embryogenesis.

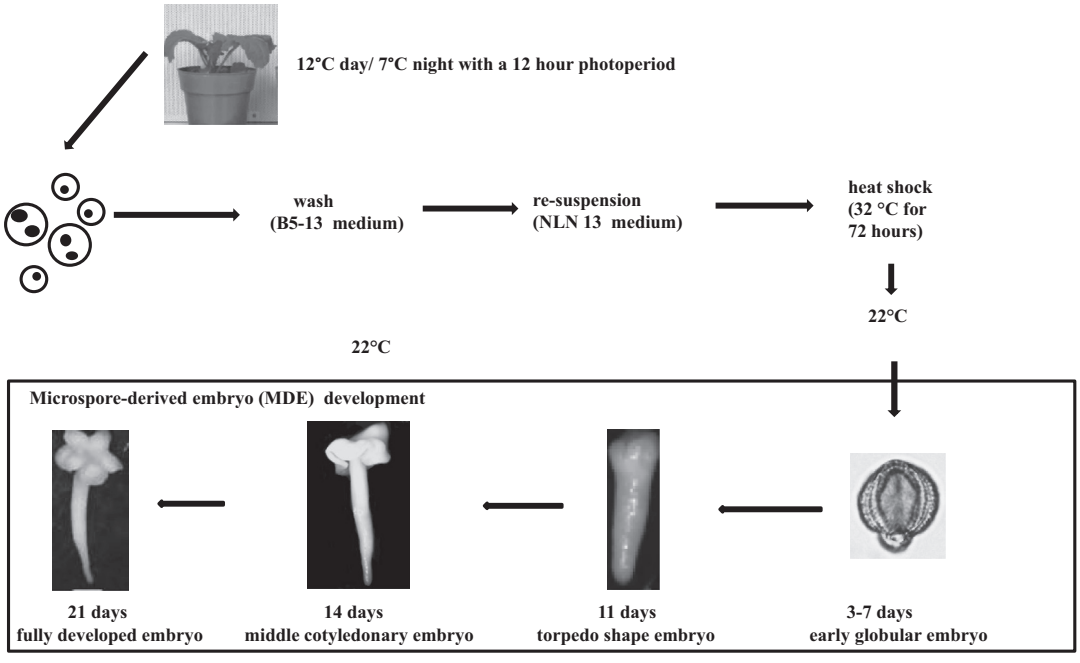

Figure 1 Schematic diagram of microspore-derived embryo development in *Brassica napus*. Plants with young buds were grown in cabinets set at 12 °C day/7 °C night with a 12 h photoperiod. Flower buds (2-3 mm in length) were harvested, sterilized, and ground in a mortar with half strength B5-13 medium supplemented with 13% (w/v) sucrose. The homogenate was centrifuged at 750 rpm (g) at 4 °C for 3 min and this process was repeated three times. The microspore-containing pellet was thereafter re-suspended and diluted in NLN-13 medium with 13% sucrose (pH 5.8) to a concentration of 10,000 microspores/ml. Embryo development was triggered after an initial heat shock treatment at 32 °C for 72 h. Embryos were subsequently incubated at 22 °C on a shaker set at 80 rpm. The number below each developmental stage of MDEs shows the days in culture.