Brief Communication

Genetic manipulation of ABI3 confers frost-tolerant seed degreening in canola

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Dear Editor,

Canola (Brassica napus) is an important oilseed crop grown across North America and Eurasia, contributing $26.7 billion to the Canadian economy alone. Canola is primarily grown for its high-quality oil, which is extracted from the seeds and has highly desirable physical properties including a high smoke point, neutral flavour and an unsaturated fatty acid profile beneficial to human health (Lin et al., 2013). Canola oil for human consumption can be compromised by chlorophyll contamination in the seeds, which substantially reduces oil quality by altering colour, odour, taste and shelf life (Endo et al., 1984). During normal seed development, canola embryos are green and turn yellow by losing all their chlorophyll at crop maturity. Exposure of canola crops to frost during the active embryo growth phase results in retention of chlorophyll in seeds at maturity, and this is extracted along with the oil (Figure 1a). Removal of this chlorophyll from contaminated oil is a time-consuming process and uses environmentally hazardous bleaching clays, costing oil processors approximately $30/MT (McClinchey and Kott, 2007). In North America alone, the economic loss due to frost-induced chlorophyll contamination is an estimated $150 million/annum, a penalty charged directly to canola producers (Delmas et al., 2013).

Exposure of canola to sub-lethal frost during active embryonic development can impair the mechanism of chlorophyll catabolism (Bonham-Smith et al., 2006). We have previously elucidated the embryo degreening pathway in Arabidopsis identifying the ABSCISIC ACID INSENSITIVE3 (ABI3) transcription factor as a master regulator of chlorophyll catabolism in developing embryos (Delmas et al., 2013; Nambara et al., 1995). ABI3 was shown to promote degreening through transcriptionally regulating STAY GREEN2 (SGR2), which encodes an active magnesium dechelatase required for chlorophyll degradation (Delmas et al., 2013; Shimoda et al., 2016). Based on the embryo degreening pathway, we hypothesized that overexpression of ABI3 could enhance canola embryo degreening following frost.

To investigate this, through RT-PCR using RNA from canola seed tissue, we amplified the cDNA of the ABI3 homolog, BnABI3 (BnaC03g44820D, C genome of Brassica napus (cv. Westar). The BnABI3 cDNA was subsequently cloned into pCAMBIA1301 vector under the control of the 35S CaMV promoter. Transgenic Brassica napus (cv. Westar) lines overexpressing BnABI3 were generated using Agrobacterium-mediated hypoplastic transformation. We characterized the T2 generation of two transgenic lines (L1 and L2), to test whether ABI3 overexpression could promote embryo degreening under frost conditions, and we subjected ABI3-OX (L1 and L2) and wild-type canola plants (24 DAP) to sub-lethal frost treatment (6 h at −4 °C). Following frost exposure, plants were allowed to mature under normal conditions until seeds were fully mature (52 DAP). When seeds from these plants were examined, significantly higher proportions of green seeds were observed in frost-exposed wild-type seeds, while the overexpressors possessed significantly lower levels of green seeds (Figure 1b). When chlorophyll levels were measured, ABI3-OX lines had significantly reduced chlorophyll levels compared to the wild-type control (Figure 1c). These results provide strong evidence that higher expression of ABI3 can promote seed degreening following frost exposure.

Endogenous ABI3 is a highly seed-specific protein, expression of which is dramatically increased during seed maturity and has

Figure 1 ABI3 overexpression leads to improved embryo degreening following frost exposure and enhanced pod strength. (a) Progression of embryo degreening in canola under normal and frost-exposed conditions in relation to oil quality. (b) Semi-quantitative colour analysis of untreated and frost-exposed seeds from Westar (wild-type) and two independent 35S::BnABI3 lines. (c) Seed chlorophyll concentration (mg/kg) in WT and 35S::BnABI3 lines following frost and non-frosted conditions. Error bars indicate ± SEM (n = 3). (d) Levels of ABI3 mRNA in Westar (WT) and transgenic 35S::BnABI3 line (L2) at 10, 15 and 20 DAP (days after pollination). Error bars indicate ± SEM (n = 3). Significance determined by Student’s t-test (* = P < 0.05, ** = P < 0.01, *** = P < 0.001). (e-g) Levels of SGR2, LEA D-34 and RAB18 mRNA in Westar (WT) and transgenic 35S::BnABI3 at 10, 15 and 20 DAP (days after pollination). Error bars indicate ± SEM (n = 3). Significance determined by Student’s t-test (* = P < 0.05, ** = P < 0.01, *** = P < 0.001). (h) Phenotypic overview of mature Westar (WT) and transgenic 35S::BnABI3 (L1 and L2) seed pods. Scale = 1 cm. Physical parameters of fully mature seed pods from WT and 35S::BnABI3 lines. Error bars indicate ± SEM (n = 7). Significance determined by Student’s t-test. (* = P < 0.05, ** = P < 0.01, *** = P < 0.001) (g, h). (i) Thickness of the pod wall (valve). (j) Diameter of pedicel. (k) Phenotypic overview of replum tissue in Westar (WT) and 35S::BnABI3, Scale = 1 mm. (l) Replum-valve joint area index. (m) Seed oil content from WT and transgenic 35S::BnABI3 seeds. Values reported are means of triplicates taken from 2 biological replicates per line (n = 6). Error bars indicate ± SE of the mean. Significance determined by Student’s t-test (* = P < 0.05, ** = P < 0.01, *** = P < 0.001). Key unsaturated fatty acid profile (18:1 oleic, 18:2 linoleic and 18:3 linolenic acids). (n) Nervonic acid content in WT and transgenic 35S::BnABI3 seeds. (o) ABI3 confers frost-tolerant degreening through hyper-activating seed SGR2 levels (1) and through up-regulating protective genes (2), along with promoting pod strength by enhancing valve thickness (3) and increasing replum-valve junction index (4).
Engineering frost-tolerant seed degreening in canola
been shown to be required for desiccation tolerance, acquisition of dormancy and seed degreening (Delmas et al., 2013). As expected, in both the wild type and ABI3-OX line, ABI3 expression increased from 10 DAP until 20 DAP (Figure 1d). However, ABI3-OX line exhibited significantly higher ABI3 expression across all intervals with ABI3 accumulating in the seeds at a significantly earlier time point than the endogenous ABI3 transcripts in the wild-type control. This likely allows ABI3 to prime the system to respond more forcefully to the natural surge in ABA levels observed in maturing canola, resulting in more complete clearing of chlorophyll from the frost-treated seeds (Delmas et al., 2013). In the ABI3-OX line, increase in ABI3 resulted in increased expression of its downstream transcriptional target SGR2, responsible for seed degreening (Figure 1e). In addition to SGR2 up-regulation, expression of the desiccation and dehydration-protective genes such as 

\[ \text{RAB18 and LEA D-34} \]

were also up-regulated (Figure 1f, g).

We did not observe any morphological or growth anomalies in the ABI3-OX lines following evaluation of these lines over three generations (T2–T4). Agronomic characteristics of yield, plant height, leaf area and flowering date were all found to be unchanged in these lines. One phenotype that stood out in ABI3-OX lines was the development of pods which had increased thickness of pod (valve) walls, leading to an overall increase in pod width (Figure 1h–i). Furthermore, ABI3-OX lines developed pedicels with increased thickness, which could potentially prevent occurrences of pod drop (Figure 1j). These phenotypes are the likely result of misexpression of ABI3 in regions other than the seeds where it is exclusively up-regulated in the wild type. The replum-valve joint area index also increased in ABI3-OX lines (Figure 1k, l), which is a parameter that is correlated with increased tolerance to pod shatter, another major reason for massive yield losses in canola (Hu et al., 2015).

Fatty acid profile of ABI3-OX lines indicated moderate changes (Figure 1k); notably, oleic acid (18:1; omega-9) content was significantly increased in the ABI3-OX lines L1 and L2 by approximately 9% and 13%, respectively (Figure 1m). Higher proportion of oleic acid to di- and tri-unsaturated fatty acids can lead to improvement of oxidative stability of the oil allowing for higher-temperature cooking (Hu et al., 2006). In addition to this, nervonic acid (24:1), which is an elongation of oleic acid and an essential component for brain function (Sargent et al., 1994), was also significantly increased by 32%–48% (but still <0.2% of total oil) (Figure 1n). Concomitant with the increase in oleic acid, both linoleic (18:2, omega-6) and linolenic acid (18:3, omega-9) content (omega-6 and omega-3 fatty acids) were decreased by approximately 20% in both transgenic lines compared to wild type (Figure 1n).

Based on our results, we propose a model in which three potential avenues may promote frost-tolerant degreening in ABI3-OX canola seeds (Figure 1o). Up-regulation of SGR2 expression can positively influence the rate at which chlorophyll can be catabolized. It is likely that ABI3-mediated up-regulation of a variety of stress, seed maturation and molecular chaperone genes, results in seeds that can tolerate sub-lethal frost exposure. Constitutive expression of ABI3 also can alter pod morphology in a manner which may help retain moisture in seeds, which is critical to sustain seed degreening (Bonham-Smith et al., 2006). Increasing thickness of pod valve walls may help reduce moisture loss from silique and seed tissue, which can promote a hydrated environment to facilitate seed degreening (Figure 1o).

We propose two mutually non-exclusive modes of action for ABI3. Misexpression of ABI3 most probably hypersensitizes the tissues that accumulate ABI3 to endogenous ABA accumulation, which, in turn, leads to priming for freezing and desiccation tolerance within the seed. Alternatively, increased expression of ABI3 may directly cause accumulation of ABI3-dependent transcripts required for chlorophyll degradation (SGR2) and freezing/desiccation tolerance (LEA, RAB18) (Figure 1e–g). Nevertheless, we have convincingly demonstrated that hyperaccumulation of ABI3 in seeds and pods leads to a cold tolerance system that efficiently promotes chlorophyll breakdown despite frost exposure. Through manipulating a single gene, we have been able to create a canola germplasm that represents a multi-trait improvement and addresses many of the industry priorities with one modification.

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Conflict of interests

The authors declare no competing financial interests.

Author contributions

M.P., L.S., N.H. and M.J. conducted the experiments. M.P., L.S. and M.A.S. designed the experiments and wrote the manuscript.

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