Depletion of cellular brassinolide decreases embryo production and disrupts the architecture of the apical meristems in *Brassica napus* microspore-derived embryos

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Abstract

Exogenous applications of brassinolide (BL) increased the number and quality of microspore-derived embryos (MDEs) whereas treatments with brassinazole (BrZ), a BL biosynthetic inhibitor, had the opposite effect. At the optimal concentration \(4 \times 10^{-6} \text{ M}\) BrZ decreased both embryo yield and conversion to less than half the value of control embryos. Metabolic studies revealed that BL levels had profound effects on glutathione and ascorbate metabolism by altering the amounts of their reduced forms (ASC and GSH) and oxidized forms [dehydroascorbate (DHA), ascorbate free radicals (AFRs), and GSSG]. Applications of BL switched the glutathione and ascorbate pools towards the oxidized forms, thereby lowering the ASC/ASC+DHA+AFR and GSH/GSH+GSSG ratios. These changes were ascribed to the ability of BL to increase the activity of ascorbate peroxidase (APX) and decrease that of glutathione reductase (GR). This trend was reversed in a BL-depleted environment, effected by BrZ applications. These metabolic alterations were associated with changes in embryo structure and performance. BL-treated MDEs developed zygotic-like shoot apical meristems (SAMs) whereas embryos treated with BrZ developed abnormal meristems. In the presence of BrZ, embryos either lacked a visible SAM, or formed SAMs in which the meristematic cells showed signs of differentiation, such as vacuolation and storage product accumulation. These abnormalities were accompanied by the lack or misexpression of three meristem marker genes isolated from *Brassica napus* (denoted as *BnSTM*, *BnCLV1*, and *BnZLL-I*) homologous to the *Arabidopsis* **SHOOTMERISTEMLESS** (STM), **CLAVATA 1** (CLV1), and **ZWILLE** (ZLL). The expression of *BnSTM* and *BnCLV1* increased after a few days in cultures in embryos treated with BL whereas an opposite tendency was observed with applications of BrZ. Compared with control embryos where these two genes exhibited abnormal localization patterns, *BnSTM* and *BnCLV1* always localized throughout the subapical domains of BL-treated embryos in a zygotic-like fashion. Expression of both genes was often lost in the SAM of BrZ-treated embryos. The results suggest that maintenance of cellular BL levels is required to modulate the ascorbate and glutathione redox status during embryogenesis to ensure proper development of the embryos and formation of functional apical meristems.

Key words: *Brassica napus*, brassinazole, brassinolide, meristem marker genes, meristems, microspore-derived embryos.

Introduction

The ability of plant cells to regenerate embryos in culture, first reported by Levine (1947), has been extensively exploited for propagating angiosperm and gymnosperm species (see Dunstan *et al.*, 1995; Thorpe and Stasolla, 2001). One way in which embryos are produced in culture is through gametophytic androgenesis, in which the developmental fate of
microspores (immature pollen grains) can be re-routed towards an embryogenic pathway. This process is advantageous in that the endogenous genetic variation can be fixed with the recovery of double haploid plants after chromosome doubling by colchicine treatments (Yao et al., 1997). Androgenesis has been applied to a variety of species, including *Brassica napus*, and successfully implemented in breeding programmes. Production of *Brassica* microspore-derived embryos (MDEs) has been facilitated by several optimizations of culture and media conditions (Ferrie et al., 2005; Belmonte et al., 2006; Stasolla et al., 2008) and has become a suitable model system to study embryogenesis. Through this system, a large number of synchronized embryos can be produced in a short period of time, thereby allowing the selection of stage-specific embryos to be used for physiological and molecular studies. This is especially important for studying early embryogeny, as immature embryos produced *in vivo* are embedded in the maternal tissue and difficult to dissect. Comparative studies between *in vivo* and *in vitro* systems are possible due to the similar morphogenic events underlying the development of *B. napus* MDEs and zygotic embryos (Yeung et al., 1996). In addition, the genetic information available in *Arabidopsis* can be used in *Brassica* given the high similarities shared between the two species. Therefore, *B. napus* MDE culture has been used in many studies investigating structural, physiological, and molecular events during embryogenesis (Yeung, 2002; Boutiler et al., 2005; Belmonte et al., 2006; Joosen et al., 2007; Malik et al., 2007).

Physiological studies have shown that a key regulator of embryo development is represented by the glutathione and ascorbate redox status, as defined by the balance between the respective reduced forms [reduced glutathione (GSH) and reduced ascorbate (ASC)] and oxidized forms [oxidized glutathione (GSSG), dehydroascorbate (DHA), and ascorbate free radicals (AFRs)]. A switch of the redox status towards a more reduced environment (high GSH/GSH+GSSG and ASC/ASC+DHA+AFR ratios) is observed during the early days in culture whereas a more oxidized environment (low GSH/GSH+GSSG and ASC/ASC+DHA+AFR ratios) occurs during the late stages of development (Stasolla and Yeung, 2001; Belmonte et al., 2006; Belmonte and Stasolla, 2007). Experimental manipulations of these ratios improve the yield and quality of MDEs (Belmonte et al., 2006; Stasolla et al., 2008). Recent work has shown that the imposition of an oxidized environment enhances the structure of *B. napus* MDEs and favours the formation of functional shoot apical meristems (SAMS). These improvements would ensure the successful regeneration of the embryos into vigorous plants (Stasolla et al., 2008).

Steroids are a group of growth-promoting natural products ubiquitous to both plants and animals involved in several aspects of development (reviewed by Bishop and Koncz, 2002). Plants are able to synthesize a variety of sterols and steroids, including brassinosteroids (BRs) (Clouse, 1996). Interest in these compounds emerged from the pioneering work of Mitchell et al. (1970) showing that extract of rape pollen containing BRs possessed growth-promoting properties. Since then, BRs have been associated with a variety of cellular responses, with elongation and proliferation being the most characterized. Their applications in several systems induced cell elongation and proliferation through auxin- and cytokinin-independent pathways (Adam and Marquardt, 1986; Mandava, 1988; Azpiroz et al., 1998). Additional BR-induced responses include tracheary element differentiation (Yamamoto et al., 1997), activation of proton pumps, growth of pollen tube, leaf bending (Mandava, 1988), resistance to abiotic stress (Brosa, 1999; Clouse, 2001), and induction of ethylene synthesis (Schlagnhauger and Arteca, 1991). In recent years the discovery and characterization of mutants have further established the key role played by BRs during plant growth and development (Clouse and Sasse, 1998). Given their emerging role as phytohormones it is not surprising that they have been exploited by tissue culturists to stimulate *in vitro* growth and morphogenesis. Besides promoting cell division of protoplast cultures (Nakajima et al., 1996; Oh and Clouse, 1998), exogenous applications of brassinolide (BL), the most active form of BRs, induced adventitious shoot regeneration from hypocotyl segments of *Brassica oleracea* (Sasaki, 2002). A similar beneficial effect was also reported by Pullman et al. (2003) who observed an improvement in embryogenic tissue initiation when exogenous BL was added to loblolly pine and rice cell cultures. A more recent study (Ferrie et al., 2005) revealed that two forms of BRs, 24-epibrassinolide and BL, enhanced the yield of MDEs from several *Brassica* species possibly by protecting microspores from the imposed heat stress required for the initiation of the embryogenic programme.

Besides this speculation, to date there is no clear information on the requirement for BRs, and BL in particular, during embryogenesis. One way to gain a better understanding of the function of this phytohormone during embry development is to manipulate its endogenous level experimentally and assess embryo yield and quality. This approach has been rendered possible by the work of Asami et al. (2000) who, through the screening of BR biosynthesis inhibitors, identified brassinazole (BrZ) as the most potent inhibitor. The specificity of this compound was further demonstrated in two independent studies in which BrZ inhibition of secondary xylem development in *Lepidium sativum* and stem elongation in *Arabidopsis* were reversed by exogenous applications of BL (Asami et al., 2000; Nagata et al., 2001). Here BrZ is used to assess the role played by BL during *B. napus* microspore-derived embryogenesis. It is proposed that maintenance of cellular BL levels is required to modulate the ascorbate and glutathione redox status during embryogenesis and ensure proper development of the embryos and formation of functional apical meristems.

### Materials and methods

*Brassica napus* embryo development: BrZ and BL treatments

Growth of *B. napus* cv Topaz DH4079 plants, isolation of microspores and induction of MDE development were carried out...
Effects of brassinolide on Brassica napus embryogenesis in vitro

Effects of BL and BrZ on embryo yield and conversion

Inclusions of BL in the culture medium increased MDE yield at any concentration used (Fig. 1A). At the optimal level (10^{-7} M), BL increased the number of embryos produced >5-fold. Embryo production was significantly reduced by BrZ especially when applied at a concentration of 4×10^{-6} M (Fig. 1B). To eliminate the possibility that this effect was due to the toxicity of the inhibitor rather than to its ability to repress the synthesis of BL, BR was applied (at its optimal concentration) in conjunction with BrZ. The combined applications of these two compounds reversed the inhibitory effect of BrZ and increased the percentage of embryo yield to control values (Fig. 1B). External analyses of the embryos clearly showed that BrZ reduced the length of at least three independent experiments.

Fig. 1. Number of MDEs developed in the presence of different levels of (A) brassinolide (BL) and (B) brassinazole (BrZ). Treatments were applied at day 0 (see Materials and methods for details) and the embryos were counted at day 35. Values are expressed as percentages relative to control and are means ± SE of at least three independent experiments. * indicates values that are statistically significantly different (P ≤ 0.05) from control. (C) External morphology of embryos treated as in B.
of the embryonal axis in a dosage response fashion (Fig. 1C).

Conversion frequency (formation of visible root and shoot systems at germination) after treatments with BrZ and BL was measured in embryos harvested at different days during development (day 15, 20, 25, and 35). The conversion frequency of control embryos was highest at day 15 and declined during the following harvesting days (Fig. 2A). Differences between treatments were observed after day 15, when applications of BL markedly increased the post-embryonic performance of the MDEs. After day 20, BL, at any concentration used, more than doubled the conversion frequency of the embryos compared with control (Fig. 2A). Due to the limiting amount of BrZ available, the effect of this inhibitor was tested in embryos harvested at day 25, when the beneficial effect of BL became apparent. Embryo conversion decreased with increasing levels of BrZ (Fig. 2B), and this inhibition was reversed by the combined inclusion of BL+BrZ.

Effects of BL and BrZ applications on glutathione and ascorbate metabolism

Ascorbate and glutathione production in plant cells is tightly linked through the activity of common enzymes (Fig. 3). The level of cellular GSH in control embryos was highest at day 15 and it declined during the following days in culture (Fig. 4A). In these embryos, cellular GSSG was always very low. Overall, applications of BL increased the total glutathione pool and this was mainly the result of increasing levels of the oxidized form GSSG. The highest levels of GSH were measured in BrZ-treated embryos which were almost depleted of GSSG. When BL was applied with BrZ, a small but consistent increase in GSSG was observed (Fig. 4A). Different profiles in the glutathione redox state (GSH/GSH+GSSG ratio) were observed during MDE development (Fig. 4B). In BrZ-treated embryos this ratio remained high throughout the culture period whereas a switch of the glutathione pool towards an oxidized state was observed in the presence of BL. Intermediate levels of the GSH/GSH+GSSG ratio were observed for control embryos and embryos treated with BL+BrZ (Fig. 4B).

The total ascorbate level of control embryos increased during development, reaching a maximum value at day 25 before declining during the following days (Fig. 5A). Within this pool ASC was always the predominant form. An overall enrichment in DHA+AFR was observed in BL-treated embryos and this contributed to the enlargement of the total ascorbate pool. An opposite trend was noticed for embryos cultured with BrZ which, compared with the other treatments, displayed the lowest total ascorbate level after day 15. This trend was, however, reverted if BL was added to BrZ (Fig. 5A). Different patterns in the ascorbate redox state (ASC/ASC+DHA+AFR ratio) were observed during MDE development (Fig. 5B). In the presence of BrZ the ascorbate pool remained in a reduced state throughout the developmental programme, whereas it switched slowly towards an oxidized state in embryos treated with BL (Fig. 5B). Intermediate values were observed for control embryos and embryos cultured with BL+BrZ.

Among the enzymes involved in glutathione and ascorbate metabolism (Fig. 3), the activity of GR fluctuated during development in all treatments (Fig. 6). The highest activity of this enzyme was measured in the presence of BrZ after 25 d in culture. Overall BL treatments reduced the activity of GR. The profiles of APX, the enzyme converting
reduced ASC to its oxidized form AFR (Fig. 3), did not change throughout development regardless of the treatments. The lowest activity of this enzyme was observed in BrZ-treated embryos at all days in culture whereas the highest activity values were measured in embryos treated with BL. No significant differences in activity profiles were observed for DHAR among treatments. The activity of the other ascorbate recycling enzyme, AFRR, slowly declined during maturation in control embryos, as well as in embryos treated with BrZ and BL+BRZ. At the end of the developmental programme the highest activities of this enzyme were measured in embryos treated with different levels of BL (Fig. 6).

Structural analyses of MDEs treated with BL or BrZ

Major structural differences among treatments were observed within the apical poles of the embryos: the SAM and the root apical meristem (RAM). At the end of the culture period the SAM of control embryos was composed of a few layers of cytoplasmic cells subtended by elongated and vacuolated cells (Fig. 7A). Formation of intercellular spaces, which disrupted the integrity of the subapical cells, was observed in the majority of the SAMs analysed. Compared with their control counterparts, the apical poles of BL-treated embryos were more organized; they were mainly dome-shaped and composed of a larger number of cytoplasmic cells (Fig. 7B). Two extreme major structural abnormalities were observed in the SAMs of BrZ-treated embryos. In ~70% of the abnormal embryos the apical poles lacked a visible SAM (Fig. 7C) whereas in other cases (~30%) SAMs were present but were devoid of the characteristic cytoplasmic cells occupying the apical layers. Pronounced vacuolation and accumulation of storage products were observed within these SAMs (Fig. 7D). The RAM of control embryos was always very defined, with a group of large cells subtending the procambial region (Fig. 7E). Such an arrangement, also visible in BL-treated embryos (data not shown), was lost in embryos cultured with BrZ (Fig. 7F). Minor structural alterations as a result of BrZ treatments were also observed in the cotyledons. Compared with control (Fig. 7G), the cotyledons of BrZ-treated embryos displayed a corrugated epidermal layer (Fig. 7H) and were often characterized by small cellular protrusions (Fig. 7I). Cotyledons of embryos cultured in the presence of BL were similar to those of control embryos (data not shown).

The effect of BrZ on SAM development was further investigated by applying the inhibitor at specific days in culture throughout the developmental programme (Table 1) or during specific windows of time in the initial phases of development (Table 2). The negative effects of BrZ on SAM development only occurred if the inhibitor was included in the medium within the first 15 d in culture (Table 1) and the most deleterious results were observed if BrZ was applied between day 10 and day 15 (Table 2).

Expression and localization patterns of SAM marker genes

The relative expression of the three SAM marker genes \textit{BnSTM}, \textit{BnCLV1}, and \textit{BnZLL-1} was measured during MDE development (Fig. 8). To facilitate the interpretation of the data, the expression of the genes was based on comparisons with transcript levels at day 5 (set at 1) for each treatment. This was possible since no statistically significant differences were observed in the transcript levels of embryos collected at day 5 regardless of the treatment. The expression of \textit{BnSTM} increased during development in control embryos (Fig. 8). A similar, but more pronounced trend was observed in the presence of BL (10^{-7} M, which was the optimal concentration for improving embryogenesis, Fig. 1a). Applications of BrZ repressed the expression of this gene; this repression was relieved in BL+BrZ-treated embryos. The expression profiles of \textit{BnCLV1} in the different treatments mimicked those observed for \textit{BnSTM}. The expression of this gene increased in the presence of BL whereas it decreased when BrZ was applied to the culture medium. This inhibitory effect was reversed when BL was added to the inhibitor (BL+BrZ). No major differences in expression pattern among treatments were observed for \textit{BnZLL-1} (Fig. 8).

In globular-stage control embryos, \textit{BnSTM} was mainly localized in the apical pole (Fig. 9A) although a delocalized...
expression was often observed (Fig. 9A2), and retained in early cotyledonary embryos (Fig. 9A3). In fully developed control embryos BnSTM transcripts were present in the subapical layers of the SAM, although some pockets of cells often remained unstained (Fig. 9A4). In the presence of BrZ, BnSTM was still localized in the apical pole of globular embryos, but confined to a smaller pocket of cells (Fig. 9A5). Upon further development a very faint signal was visible in the apical pole of middle cotyledonary embryos (Fig. 9A6). At the end of the culture period, BrZ-treated embryos lacking a SAM did not stain for BnSTM (Fig. 9A7), whereas in those forming abnormal SAMs only a few cells retained its expression (Fig. 9A8). In BL-treated embryos BnSTM signal was very strong and always restricted to the subapical cells in the central domain of the SAM throughout development (Fig. 9A9–11). This localization pattern coincided with that observed in zygotic embryos (data not shown). No signal was detected in sections hybridized with a sense riboprobe (Fig. 9A12).

In globular-stage control embryos, expression of BnCLV1 was first localized below the third layer of cells (Fig. 9B1), before extending to the upper subapical layers in early

**Fig. 4.** (A) Endogenous levels of reduced (GSH) and oxidized (GSSG) glutathione in MDEs cultured in the presence of brassinolide (BL) and/or brassinazole (BrZ). Embryos were harvested on different days during development and processed for analyses. (B) Glutathione redox state expressed as the ratio between GSH and total glutathione (GSH+GSSG) of embryos treated as in A. Values are means ± SE of at least three independent experiments.
(Fig 9B2) and middle cotyledonary (Fig. 9B3) embryos. At the end of the culture period the signal was confined in a few subapical cells within the SAM (Fig. 9B4). As for the control, \textit{BnCLV1} expression was observed in the subapical cells of BrZ-treated globular embryos (Fig. 9B5). A very faint signal was also retained in early cotyledonary embryos (Fig. 9B6). At the end of the culture period \textit{BnCLV1} expression occupied the central domain of a few embryos (<10%) (Fig. 9B7). No signal was observed in the remaining embryos (Fig. 9B8). BL treatments extended \textit{BnCLV1} expression to the second layer of cells of globular embryos (Fig. 9B9) and to the whole subapical region of the SAM in early cotyledonary embryos (Fig. 9B10). A very strong signal was visible throughout the meristematic region of middle (Fig. 9B11) and late (Fig. 9B12) cotyledonary embryos. This localization pattern mimicked that of zygotic embryos (data not shown). The expression domain of embryos treated with BL+BrZ was similar to that described for control embryos, whereas no signal was detected when a sense probe was used (data not shown).

\textit{BnZLL} was mainly localized within the procambial and subapical cells of control embryos at the globular stage of
development (Fig. 9C). Expression of this gene was retained mainly in the vascular tissue of middle (Fig. 9C2) and late (Fig. 9C3) cotyledonary embryos. In the latter, a faint signal, often extended to the base of the cotyledons, was present in the subapical cells of the SAM (Fig. 9C3). A similar expression pattern was also observed in embryos treated with BL+BrZ (data not shown). In BrZ-treated embryos, BnZLL expression included the procambial domain of globular embryos (Fig. 9C4) and the vascular cells of middle cotyledonary embryos (Fig. 9C5). At the end of the culture period no expression was detected within the SAM (Fig. 9C6). The localization pattern of BnZLL in developing BL-treated embryos (Fig. 9C7–9) was similar to that described for their control counterpart. However, similar to zygotic embryos (data not shown), the expression of this gene was always observed in a pocket of meristematic cells occupying the central domain of the SAM and never in lateral regions of the apical pole (Fig. 9C8, 9). No signal was detected using a sense probe (Fig. 9C10).

Discussion

BL is required for MDE yield, quality, and conversion

In order to evaluate the role played by BRs during embryogenesis B. napus MDEs were cultured in an environment enriched or depleted of BL. Brassica napus MDE development is greatly affected by BL, with high levels
promoting embryo formation and conversion, and low levels, generated by BrZ applications, repressing both processes (Figs 1, 2). This inhibitor, the specificity of which was demonstrated by the reversion tests, that is BL+BrZ applications (Figs 1, 2), and other studies (Nagata et al., 2001), is a triazole derivative able to reduce the supply of...
Table 2. Effect of brassinazole (Brz) on the percentage of Brassica napus microspore-derived embryos lacking a visible shoot apical meristem (SAM) (see Fig. 7C) or with extensive SAM abnormalities (lack of cytoplasmic cells, formation of vacuoles with storage product accumulation in apical and subapical cells, as well as large intercellular spaces) (see Fig. 7D).

Cultures were treated at the days indicated with brassinazole (BrZ) alone or in conjunction with brassinolide (BL) and observed at day 35 after being fixed and sectioned (see Materials and methods for details). All data are means ±SE.

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<th>Treatments</th>
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<td>Control</td>
<td>13±3</td>
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<td>BrZ (4×10^{-6} M)</td>
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<td>BrZ (4×10^{-6} M) + BL (10^{-7} M)</td>
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cellular BL by targeting a P450-cytochrome which catalyses the oxidation of cathasterone to teasterone (Asami et al., 2000). Previous tissue culture studies (Pullman et al., 2003; Ferrie et al., 2005) documented the beneficial effects of BL during embryogenesis although its mode of action remains elusive. A possible role for this phytohormone was proposed by Ferrie et al. (2005) who speculated that BL protects B. napus MDEs from the initial heat shock treatment applied at the beginning of the culture period to re-route the gametophytic pathway of the microspores towards an embryogenic fate. This notion is very plausible as BRs increased thermotolerance of tomato and B. napus seedlings (Dhaubhadel et al., 1999), as well as drought tolerance and cold stress in A. thaliana (Kagale et al., 2007). The BR protective role was exercised through the activation of both structural (heat shock proteins) and regulatory (transcription factors) elements (Kagale et al., 2007). As an extension of these findings the present study suggests that maintenance of cellular BL during early embryogenesis is needed to induce specific changes in ascorbate and glutathione metabolism required for proper embryo development and apical meristem formation.

**BL enhancement of embryo development correlates with alterations in glutathione and ascorbate metabolism**

Besides participating in defence reactions against oxidative stress, the glutathione/ascorbate redox systems are key regulators of embryogenesis. Specific fluctuations in the balance between reduced (ASC and GSH) and oxidized (DHA+AFR and GSSG) forms accompany embryo development in vitro (Arrigoni et al., 1992; Belmonte et al., 2005). Total ascorbate and glutathione pools are generally high in immature seeds, to support proliferation of the growing embryo, before declining steadily during the late phases of seed maturation. Most importantly, as the embryo develops, both pools are depleted of their reduced forms (GSH and ASC) and enriched in their oxidized forms (GSSG and DHA+AFR) (Arrigoni et al., 1992; Belmonte et al., 2005). These metabolic fluctuations, resulting in low GSH/GSH+GSSG and ASC/ASC+DHA+AFR ratios, represent a switch controlling proper histodifferentiation and development. Exemplification of this notion comes from in vitro experiments where the imposition of an oxidized environment improves the number and quality of embryos in both gymnosperm and angiosperm species (Belmonte et al., 2005; Belmonte and Stasolla, 2007). In spruce (Picea glauca) an imposed oxidized environment almost doubled the number of somatic embryos and their post-embryonic performance (Belmonte et al., 2005). A more pronounced response was observed in B. napus MDEs when the GSH/GSSG ratio was experimentally lowered to values close to 0.6 using a biosynthetic inhibitor of GSH. Under this favourable environment the conversion frequency of the embryos almost tripled (Belmonte and Stasolla, 2007).

The beneficial effects of BL on embryo yield and quality might be due its ability to switch the glutathione and ascorbate pool towards an oxidized state. Compared with control embryos the endogenous level of GSSG increased in BL treatments (Fig. 4A), possibly due to the lower activity of GR measured in these embryos (Fig. 6). The activity of this enzyme which decreased during embryo development in vivo (Belmonte et al., 2005) catalyses the reduction of GSSG to GSH (Fig. 3). Increasing levels of GSSG in BL-treated embryos switched the glutathione pool towards an oxidized state (low GSH/GSH+GSSG) to values close to 0.6 (Fig. 4B). These values are similar to those observed in zygotic embryos, and almost identical to those of MDEs in which the glutathione pool was experimentally shifted towards an oxidized state to improve embryo conversion (Fig. 1 in Stasolla et al., 2008). In the presence of a depleted BL environment, effected by BrZ applications, the glutathione pool is maintained in a reduced state, which is unfavourable to embryo development and conversion (Figs 1, 2; Belmonte et al., 2005). However, if in BrZ-treated embryos the glutathione pool is forcibly switched towards a more oxidized state [by inclusions of buthionine sulfoximine (0.1 M)], a GSH inhibitor able to lower the GSH/GSSG+GSH ratio in MDEs (Stasolla et al., 2008)] both embryo number and conversion frequency increased to almost control values (data not shown). This observation confirms the notion that high levels of cellular BL favour embryogenesis through the imposition of an oxidized environment.

Similar metabolic changes to those described for glutathione were also measured for ascorbate. Inclusion of BL in
the culture medium increased the production of DHA and AFR, thereby switching the redox state towards an oxidized environment. An opposite trend was observed when BL was experimentally depleted by BrZ inclusion (Fig. 5). These metabolic fluctuations can be explained by the activity of APX, the recycling enzyme converting ASC to AFR (Fig. 3), which was high in BL-treated embryos and low in the other treatments, especially in the presence of BrZ (Fig. 6). Besides reducing ASC, thereby lowering the ASC/ASC+DHA+AFR ratio, higher APX activity is required in detoxification processes (by decreasing the level of \( \text{H}_2\text{O}_2 \) generated during active cellular proliferation), and in the regulation of cell wall plasticity (by reducing the endogenous \( \text{H}_2\text{O}_2 \) utilized by peroxidases in cross-linking cell wall polymers) (De Gara et al., 1996). Maintenance of cell wall plasticity was shown to be critical for proper SAM function in vitro (Stasolla and Yeung, 2007). Another important difference in ascorbate metabolism among treatments is the increased level of AFRR, the ASC-recycling enzyme, which at the end of the developmental period is highest in BL-

**Fig. 8.** Semi-quantitative RT-PCR analysis of three SAM marker genes isolated from *Brassica napus* and designated as *BnSTM* (*SHOOTMERISTEMLESS*), *BnCLV1* (*CLAVATA 1*), and *BnZLL-1* (*ZWILLE*). The expression level of these genes was measured in control embryos and embryos treated with brassinolide (BL) and/or brassinazole (BrZ) on different days in culture. The relative expression is based on comparisons with transcript levels of day 5 embryos, which are set at 1. The values represent the average of at least three independent experiments. Asterisks above each bar indicate values which are significantly different from control values (\( P < 0.05 \)) at the same sampling time.
Fig. 9. RNA in situ hybridization studies of BnSTM (A), BnCLV1 (B), and BnZLL-1 (C). Two BnSTM localization patterns were observed in globular-stage control embryos. BnSTM was either localized in the subapical region of the embryos (A1) or delocalized within two distinct areas of the apical pole (A2). This latter pattern was often retained in early cotyledonary embryos (A3). In fully developed embryos BnSTM expression was observed in almost all the subapical cells of the SAM, although pockets of cells (arrows) often remained unstained (A4). In the presence of brassinazole only a few cells in late globular embryos expressed BnSTM (A5). A faint apical staining was retained in early cotyledonary embryos (A6) but it was almost completely lost in fully developed embryos characterized by the absence of a visible SAM (A7) or in an embryo with poorly organized SAMs (A8). In the presence of brassinolide the localization pattern of BnSTM was always
treated embryos and lowest in the BrZ-treated counterparts. The high level of this enzyme in the presence of BL might represent a strategy for generating ASC from the oxidized forms (which are also present at high levels in BL-treated embryos) at the onset of germination, prior to the reactivation of the ASC de novo biosynthetic pathway. This recycling activity during the initial phases of post-embryonic growth was observed both in vivo (De Gara et al., 1997) and in vitro (Stasolla and Yeung, 2001) where it is required to provide ASC quickly in support of tissue growth and meristem reactivation (Stasolla and Yeung, 2006). The inability to recycle ASC promptly at germination may be one of the causes responsible for the low conversion frequency observed in the presence of BrZ. This notion is in line with other studies showing that an experimental reduction in ASC production at the onset of germination compromises the reactivation of the meristems (Stasolla and Yeung, 2006).

The BL-induced changes in ascorbate and glutathione metabolism would also explain the structural differences of the apical meristems observed among treatments. Previous studies (Belmonte et al., 2005; Belmonte and Stasolla, 2007; Stasolla et al., 2008) revealed that the beneficial effect of an imposed oxidized environment during embryogenesis is mainly due to structural improvements of the apical meristems. The architecture of the SAM in BL-treated embryos closely resembles that of SAMs observed in MDEs cultured under a low GSH/GSH+GSSG ratio (compare Fig. 7B in this study with Fig. 2F in Stasolla et al., 2008). Similarly, the abnormal apical poles produced in a BL-depleted environment share striking features with those of MDEs cultured in a highly reduced environment (MB and CS, unpublished results). These abnormalities, which include the absence of a visible SAM (Fig. 7C), or SAMs in which the meristematic cells start differentiating, as revealed by the formation of vacuoles and accumulation of storage products (Fig. 7D), might contribute to the poor conversion frequency observed in BrZ-treated embryos. The additional alterations in RAM structure (Fig. 7F), as well as growth morphology (Fig. 1C) and cellular organization (Fig. 7H, I) in these embryos further support the requirement for high BL levels for proper tissue patterning. This idea is in agreement with the work of Jang et al. (2000) who revealed severe defects in embryonic patterning and meristem programming in Arabidopsis fackel mutants showing abnormal sterol composition and lowered BR levels. Abnormal cell patterning during root meristem formation was also observed in plants with a mutated BIM1 gene, which is involved in BR signalling (Chandler et al. 2009).

BL requirement for proper SAM formation is specific to the early embryogenic phase, especially between days 10 and 15 (Tables 1, 2), corresponding to the late globular–heart stage of development. This observation confirms the notion proposed by Ramesar-Fortner and Yeung (2006) that the fate of the meristematic cells is not determined, but rather influenced by the physiological conditions of embryonic environment. This influence can be exercised only within a short window of time (prior to day 15 in the present system), after which the fate of the meristematic cells becomes more determined and their identity cannot be altered. The negative effect of BrZ on SAM development is attenuated significantly if the inhibitor is applied after day 15 (Table 2).

The BL level affects the expression of SAM marker genes

The requirement of BL for SAM formation was further investigated by measuring expression levels and localization patterns of three SAM marker genes isolated from B. napus and homologous to Arabidopsis genes involved in apical

specific to the central subapical region of globular embryos, which always exhibited a very strong signal (A0). This pattern persisted in early cotyledonary (A10) and fully developed (A11) embryos, where a very strong BnSTM signal was observed throughout the subapical region of the SAM. No staining was detected when sense RNA was used (A12). In control embryos the expression of BnCLV1 was observed in globular embryos, where it was localized below the third layers of cells of the embryo proper (B1). In early cotyledonary (B2) and cotyledonary (B3) embryos the expression of this gene extended to the second layer of cells of the SAM, and remained localized to the central region of the apical pole. At the completion of the developmental programme BnCLV1 signal was observed in the subapical domain of the SAM, although not in all cells (B4). brassinazole-treated globular embryos exhibited a control-like localization pattern (B5). However, the signal was attenuated in early cotyledonary embryos (B6). At the completion of the developmental programme the expression pattern of this gene was not consistent among embryos. A visible, but faint signal was still retained in a few embryos (<10%) (B7), whereas it was almost completely lost in the others (B8). In globular embryos treated with brassinazole BnCLV1 expression was observed in the apical domain, including the second layer of cells (B9). During the subsequent phases of development, i.e. early cotyledonary (B10), middle cotyledonary (B11), and late cotyledonary (B12), a very strong signal was observed throughout the subapical region of the SAM, and included cells of deeper layers. In globular-stage control embryos the expression of BnZLL was mainly localized in the procambial cells and apical pole (C1). In early cotyledonary embryos a very faint signal was also localized in the subapical cells of the shoot apical meristem (C2). This localization pattern was retained in fully developed control embryos, although the localization of this gene within the apical pole was more delocalized and extended to cells at the base of the cotyledon (arrow) (C3). As for control, in globular BrZ-treated embryos BnZLL transcripts were localized within the procambial region and apical pole (C4). In early cotyledonary (C5) and late cotyledonary (C6) embryos no signal was detected in the cells of the apical meristem. Although the expression pattern of globular BL-treated embryos was similar to that of control (C7), BnZLL expression in the presence of BL was localized to a cluster of cells occupying the central domain of the apical meristem in both early (C8) and late (C9) cotyledonary embryos. No signal was observed using a sense probe (C10).
meristem development and maintenance. Availability of BL affects the expression of both \textit{BnSTM} and \textit{BnCLV1}. High BL levels increased the transcripts of both genes after day 5, while low levels had an opposite effect (Fig. 8). In \textit{Arabidopsis}, expression of \textit{STM} is restricted to the SAM, where it is required to maintain the meristematic cells in an undifferentiated state (Long et al., 1996). Genetic studies revealed that mutations in the \textit{stm} alleles compromise the formation of functional SAMs (Endrizzi et al., 1996). The delocalized expression of this gene observed during development of SAM in control embryos is a clear indication that culture conditions are not optimal for proper meristem development of SAM in control embryos is a clear indication that culture conditions are not optimal for proper meristem formation. Pockets of cells within the apical pole of control embryos not expressing \textit{BnSTM} (Fig. 9A4) might lack the ‘meristematic identity’ conferred by this gene (Long et al., 1996). This would compromise their reactivation at the onset of germination. In BL-treated embryos \textit{BnSTM} expression is extended throughout the apical pole of developing embryos (Fig. 9A10, 11), denoting enhanced SAM functionality and meristem fate acquisition. These zygotic-like conditions are not met in embryos depleted of BL where the \textit{BnSTM} signal is always weak and in some instances completely lost (Fig. 9A?, 8).

Compared with their control counterparts, \textit{BnCLV1} expression was extended in BL-treated embryos from the early stages of development and encompassed the subapical domain of the SAM (Fig. 9B). In \textit{Arabidopsis}, \textit{CLV1}, which encodes a putative receptor kinase, is also expressed in the subapical cells of SAM (in a pattern similar to that observed in the present study) where together with WUSCHEL it is involved in feedback loop mechanisms maintaining a central reservoir of stem cells. Misexpression of this gene negatively affects the architecture of SAM and results in the production of an abnormal number of lateral organs (reviewed by Carles and Fletcher, 2003). The restricted localization pattern of this gene in BrZ-treated developing embryos (Fig. 9B5, 7), as well as the absence of a clear signal in many mature embryos (Fig. 9B8), further supports the role of BL for inducing proper SAM formation.

The expression pattern of \textit{BnZLL-1} was confined to the vascular tissue and to a lesser extent to the apical pole of the developing MDEs (Fig. 9C), as also observed for the \textit{Arabidopsis AtZLL} (Long, 1996). The role of this gene in \textit{Arabidopsis} is to maintain an undifferentiated group of cells in the SAM during late embryogenesis, possibly by specifying proper \textit{STM} expression (Moussian et al., 1998). Mature \textit{zll} embryos have disrupted SAMs in which the stem cells differentiate and fail to generate new organs during post-embryonic growth (Moussian et al., 1998). Although no differences in \textit{BnZLL-1} transcript levels were observed among treatments (Fig. 8), the expression of this gene was always localized to a subapical pocket of central cells in developing BL-treated MDEs (Fig. 9C8, 9) whereas it was delocalized in their control counterparts (Fig. 9C4), and completely lost in embryos cultured with BrZ (Fig. 9C6). The absence of \textit{BnZLL-1} expression following BrZ treatments indicates that the molecular mechanisms required for the establishment of a functional SAM are compromised in an environment depleted of BL.

In conclusion, the present data indicate that BL plays a key role during \textit{B. napus} microspore-derived embryogenesis where it improves embryo yield and quality. High levels of cellular BL affect both glutathione and ascorbate pools by increasing the contributions of the oxidized forms. The imposed oxidized environment favours normal embryo development and apical meristem formation possibly through the proper expression and localization of meristem genes. All these events are compromised in a BL-depleted environment characterized by a reduced redox state, abnormal meristem development, and poor post-embryonic performance.

**Supplementary data**

Supplementary data are available at JXB online.

**Figure S1.** Sample of semi-quantitative RT-PCR of \textit{BnSTM}, \textit{BnCLV1}, and \textit{BnZLL-1} in developing \textit{Brassica napus} microspore-derived embryos (MDEs) cultured in the presence of brassinolide (BL) and/or brassinazole (Brz). C, control or untreated MDEs.

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**References**


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