

# Generative Development of Winter Rape (*Brassica napus* L.) – The Role of Vernalization

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## ABSTRACT

Winter rape (*Brassica napus* L.), a bright yellow-flowering member of the *Brassicaceae*, is the single most important oilcrop, recognized as an alternative to cereals over the wide range of temperate agricultural regions. Thus, its adaptation to local climatic conditions is of special importance. The timing of flowering is a crucial determinant of the adaptation to the environment. Vernalization, i.e. the exposure of plants to a prolonged period of low temperatures is, beside photoperiodism, considered as most specific primary factor inducing and controlling generative development. Ecological, physiological and molecular aspects of vernalization of winter rape are dealt with in this review.

**Keywords:** electrochemical signals of plant development, flowering, low temperature effects, thermoinduction, phytohormones in flowering

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## INTRODUCTION

Generative development of rape (*Brassica napus*) involves a change in plant architecture, as a result of cell division, expansion and differentiation (Newbigin *et al.* 1995). Reproductive meristems are initiated from previously existing apical meristems, i.e. from cells that produce vegetative organs and which are reprogrammed to generative morphogenesis (Newbigin *et al.* 1995; Denis *et al.* 1996). The flowering state is inherited through successive mitotic division. This shift occurs in response to primary environmental signals such as photoperiod and low temperatures as well as to inner factors, e.g. hormones, transmitted from other plant parts (Peeters *et al.* 1991). Day-length is perceived by four known types of photoreceptors, each of which is sensitive to specific wavelengths. Activation of a photoreceptor by light initiates a signal transduction pathway that results in specific changes in gene expression, which participate in the network controlling the onset of reproductive development (Newbigin *et al.* 1995). Low temperature stimulation (vernalization) of meristem differentiation has been documented in numerous plant species and studied at the physiological level (Michaels and Amasino 2000). Vernalization is a process by which the plant perceives and summates exposure to long periods of low temperature which subse-

quently leads to the induction of generative development (Bernier *et al.* 1981). The cells at the shoot apex were suggested as the site of perception of the low temperature signal. However, Wellensiek (1964) and in greater detail Metzger (1988) provided evidence that dividing cells in any part of the plant can respond to vernalization. Once dividing cells have been exposed to cold temperature, they acquire a “vernalized state” which is mitotically stable and maintained via an epigenetic mechanism (e.g. Sung and Amasino 2004). The vernalizing effect is reversible in some species by subsequent exposure to high temperatures, usually in the range from 25° to 40°C, although a temperature as low as 18°C may be effective (Bernier *et al.* 1981). Also, low temperatures of vernalization requirement may, at least partly, be substituted by short days and/or high irradiance (Krekule 1987).

The annual change in photoperiod and the period of vernalization are considered to be the most specific or “primary” environmental factors controlling flowering induction (Bernier and Périlleux 2005; Montgomery 2006). Less predictable climatic factors, such as ambient temperature, light integral (day-length × irradiance) and water availability, are usually viewed as “secondary” factors that can only modulate the effects of primary ones. However, the promotion of flowering by a “primary” factor can also be re-

duced by “secondary” factors (Bernier and Périlleux 2005) triggering flowering.

Numerous grafting studies have suggested the long distance transport of a florigenic stimulus, mostly in photoperiodic plants (Zeevaert 1958; Filek *et al.* 1988; Thomas and Vince-Prue 1997; Filek *et al.* 2003). Originally referred to as florigen by Chailakhyan (1936), subsequent work has shown that, in terms of a single substance, gibberellins (namely GA<sub>5</sub>) (King and Evans 2003) and the transcript of the *FT* gene (Huang *et al.* 2005) best fit such a role. However, Bernier (1988) has advanced the concept of a multi-component system of chemical signals/factors which control flowering, which includes phytohormones, nutrients and other physiologically active substances. It is assumed that they act sequentially within shoot-root-shoot signalling loops. Among candidates for such a role are cytokinins (Macháčková *et al.* 1993, 1996; Havelange *et al.* 2000; Schmölling 2002), sucrose (Périlleux and Bernier 1997; Bernier *et al.* 2002) and nitrate (Corbesier *et al.* 2001).

The use of molecular approaches and the availability of *Arabidopsis* developmental mutants have brought new insights into the control of the transition to the reproductive stage. A dense hierarchically ordered network of interacting controlling pathways has been proposed (e.g. Mouradov *et al.* 2002; Boss *et al.* 2004). However, the links between molecular events and physiological evidence is not yet fully understood.

In this review the term canola is often used. It refers to those varieties of *B. napus* that meet specific standards on the levels of erucic acid and glucosinolates.

## ENVIRONMENTAL CONTROL OF WINTER RAPE GENERATIVE DEVELOPMENT

Oilseed crops are the second most valuable commodity in agricultural trading and rapeseed (*B. napus*) is the single most important winter oilcrop, globally recognised as an alternative to cereals in the winter-spring growing season of most temperate agricultural regions (Miralles *et al.* 2001). Traditionally rape has been grown in the northern Great Plains of the United States and Canada, and northern Europe. An important management factor in the production of this crop is the planting date (Adamsen and Coffelt 2005), critical in cold climates due to the potential for frost damage both early and late in the season (Kondra 1977; Adamsen and Coffelt 2005). Developmental traits of winter rape have been analyzed under field conditions in terms of photoperiodic and temperature requirements (Mendham and Salisbury 1995; Miralles *et al.* 2001). It was observed that photoperiod and temperature affect some plant organs differently as the number of leaf primordia and the rate of leaf emergence determine time of flowering. Winter rape is sown at the beginning of autumn, usually in August and/or September. The juvenile growth phase lasts from emergence through cessation of growth in winter and comprises stem elongation and the start of flowering. To withstand subsequent periods of temperatures below freezing, individuals should reach the 6-8 true-leaf stage (rosette), have a root head diameter of >5 mm and a shoot length of <20 cm (Schröder and Makowski 1996; Diepenbrock 2000). The beginning of generative development already occurs before or during winter and usually takes place from early November to mid-December (Tittonel *et al.* 1982; Tittonel 1988). Prolonged growth of plants at temperatures close to 0°C results in the modification of their growth pattern and the adjustment of cellular metabolism to low temperature conditions, and results in increased resistance of leaf cells to extracellular freezing (Kubacka-Zębalska and Kacperska 1999; Stefanowska *et al.* 1999). Low temperature and low light intensity during the winter cause a significant loss of foliage and, thus, of stored nitrogen as well as reduced area of leaf (Colnenne *et al.* 1998). In autumn, early sowing and a high N-regime accelerate plant development and elongate stem length resulting in increased susceptibility to frost damage (Rathke *et al.* 2006). The vernalization requirement

ensures that flower development of autumn sown rape is delayed until the following spring when photoperiods and temperatures increase (Habekotté 1997). After vernalization, temperature is the main determinant of development rate. Re-growth of rape starts in early spring when temperature continually exceeds 5°C, however temperatures above 27°C can inhibit flowering at that time (Morrison and Stewart 2002). In *Arabidopsis thaliana*, another member of the *Brassicaceae* family, devernization responses have been demonstrated, including the ability to be re-vernalized after devernization, suggesting the involvement of thermolabile and thermostable intermediate signals in the vernalization process (Napp-Zinn 1969; Dahanayake and Galwey 1998). Because the leaf is a major source of photosynthesis until full flowering, it is important that the rates of leaf emergence and expansion should be high. Grosse *et al.* (1992) found correlations between the duration of the leaf area until flowering and seed yield. Such environmental factors as water logging, drought and pollution can also affect generative development in rape. However, most of these factors act as important constraints on yield formation, i.e. in the final stages of generative development, long after vernalization and photoperiodic requirements for flowering have been fulfilled.

## WINTER RAPE DEVELOPMENT UNDER CONTROLLED CONDITIONS

In recent years various models have been proposed to describe the influence of the main parameters i.e. temperature and photoperiod on phenological development in rape and other *Brassica* species, both under controlled and field conditions (e.g. Nanda *et al.* 1996). Such models may be used for practical purposes or as a module in a crop growth model. One of the most extensive analyses of genotypic and environmental control of time to flowering has been provided by Robertson *et al.* (2002) in 21 varieties of canola (*B. napus*). Based on phytotron and field experiments they stated that the number of days to flowering in all genotypes was reduced in response to vernalization and long days, due to a reduced duration between sowing and bud visibility. The vernalization response was saturated with *c.* 25 days at 3°C. The photoperiod response occurred between 10.8 and 16.3 h and the plants responded to photoperiod from emergence. Later flowering genotypes had model parameters that indicated a greater response to vernalization and photoperiod than early-flowering ones. They developed a simulation model which also incorporated base and optimum temperatures for development (0°C and 20°C respectively) and were able to predict days to flowering with a mean deviation of *c.* 5 days.

In another set of experiments using cultivars with higher vernalization requirements winter rape was cultured in growth chambers before vernalization at temperatures from 22°C (Netzer *et al.* 1986) to 17°C (Filek *et al.* 2006). The plants reached the stage of 5-6 rosette leaves (Fig. 1A). Irradiance usually mimicked the light condition characteristic for autumn. Flower initiation was stimulated by vernalization at temperatures between 2°C and 12°C (Markowski *et al.* 1986; Andrew *et al.* 1991; Habekotté 1997). Eight weeks seems to be the optimal time of vernalization under controlled conditions for rape cultivars grown in Europe (Markowski and Ryka 1983). During this period physiological changes in meristematic tissue of growth apices and initiation of flower buds appear which make possible the subsequent differentiation of generative organs in warmer temperatures (Markowski *et al.* 1986). Two weeks of cooling is too short a time for flowering of Polish winter rape cultivars to be initiated under room temperatures of 17-22°C (Skoczowski and Filek 1994; Filek *et al.* 2006); 3-weeks vernalization stimulated flowering in only about 10% plants but a drastic increase in flowering was observed when 34-56 days of cooling was used (Skoczowski and Filek 1994). Tittonel and Palleau (1991) demonstrated that vernalization not only influenced the rate of development until flower initiation,



Fig. 1 (A) Vegetative plants of winter rape (*Brassica napus* L.). (B) Flowering plants (after 8 weeks vernalization at 2°C and 7 weeks growth at 17°C).

but also the rate of flower development after initiation. As indicated by e.g. Robertson *et al.* (2002), Netzer *et al.* (1986) and Filek *et al.* (2003) winter rape is responsive to long days and under most experimental conditions the strict distinction between vernalization and photoperiodic flowering response is rather difficult to draw. Markowski and Ryka (1983) indicated that the complete vernalization process in rape only occurs in plants grown in light condition, whereas winter wheat seedlings were vernalized successfully in darkness (Filek *et al.* 2002) (Fig. 1B).

## PHYSIOLOGICAL ASPECTS OF VERNALIZATION IN RAPE

### Changes in the metabolism of lipids and other substances and in the state of membranes

Numerous studies on vernalization have attempted to ascribe developmental functions to metabolic changes which have been observed during the low temperatures treatment. A major problem is whether they reflect processes linked to vernalization or are due to rather non-specific effects of low temperatures and eventually involved in building up the

frost resistance. This was also the case with winter rape. The focus was on changes in lipid membrane composition, in content of soluble sugars and growth regulators as well as in enzyme activities (Zhou and Leul 1998).

Much attention was given to changes in lipid metabolism which has been considered as an adaptation of membranes to low temperature stress (Smoleńska and Kuiper 1977; Kuiper 1985). These observations became a basis for the assumption that the state of cellular membranes might play a crucial role in the process of vernalization. Markowski *et al.* (1986) and Skoczowski and Filek (1986) reported an increase of unsaturated fatty acids and especially  $\alpha$ -linolenic acid in membrane lipids during vernalization. However, Markowski and Filek (1986) suggested that these changes in fatty acid composition are merely a non-specific reaction of the plants grown under low temperatures. Further studies, including subcellular fractionation, indicated that in all fractions a substantial rise of unsaturation of lipids took place following low temperature treatment. This was observed after 45 days of low temperature, which induced flowering in more than 50% of plants (Skoczowski and Filek 1994). It may be that changes in membrane lipids, observed during vernalization, represent the effect of two processes: the first connected with adaptation of membranes to low temperatures (acclimation), and the second, in which induction of generative development stimulate changes in lipid content.

Experiments involving grafting of the upper part of non-vernalized winter rape on vernalized stocks showed that leaves can retain a fatty acid composition characteristic of vegetative plants, whereas after grafting the shoot apex acquires the ability for generative development (Filek *et al.* 1988). These observations corroborated the suggestion of distinct autonomy of leaves in winter rape with regard to the apical meristem.

It should be noted that in addition to temperature, mineral nutrients, especially sulphur, are a major factor affecting changes in lipids (McGrath *et al.* 1996). The application of sulphur in *Brassicaceae* increased the lipid content, led to an increase of fatty acid unsaturation after flowering, and reduced the erucic acid content in rapeseed (Ahmad and Abdin 2000). There are other, possibly indirect, interactions between plant nutritional status and cool temperatures of vernalization, for example in autumn, early sowing and a high N-regime accelerate plant development and elongate stem resulting in higher susceptibility to frost damage (Rathke *et al.* 2006).

Exposure of plants to low temperature may also result in the increased generation of reactive oxygen species (ROS) (Baeka and Skinner 2003). The ROS may attack plant cellular components or may deliver signals for detecting the changed environment, or both (Fridovich 1991). To protect from oxidative stress, plants have evolved very efficient antioxidant systems to scavenge ROS (Allen 1995). Increased expression of antioxidant systems in winter rape might afford protection from ROS generated as a result of cold stress (Zhou and Leul 1998). However, after vernalization, a decrease in antioxidative enzymes activities (superoxide dismutase and catalase) in generative apices was observed (our data, in preparation). This suggests that changes in redox activity might be connected, directly or indirectly, with developmental stages and reflects their progress due to vernalization.

### Soluble sugars as both source of energy and signalling molecules

The level of soluble sugars has also been postulated to regulate the progression from the vegetative to generative phase, flowering and senescence (Bernier *et al.* 1993). In studies on *Arabidopsis* a positive correlation was found between greater export of carbohydrates from the leaves and increased flower induction (Corbesier *et al.* 1998). Moreover, there are several cases in which sugar plays a role as a signalling molecule regulating a variety of genes (Koch 1996).

Thus, there may be a dual role of high sucrose content, mobilising photosynthates for translocation to buds to support differentiation of floral organs, and triggering gene expression leading to a signalling chain that induces flowering (Kataoka *et al.* 2004). In *Brassicaceae* the total sugar content increased at all the stages leading to seed development (Fazli *et al.* 2005). However, understanding the response to sugar is complicated by the fact that plants have multiple sugar-response pathways and that the molecules actually being sensed are not known in all cases (Gibson 2005). Detailed studies to elucidate the role of soluble sugars in winter rape are still lacking.

### Role of phytohormones in vernalization

In plants with a rosette stage of development and a vernalization response, one of the earliest detectable events following chilling treatment is a rapid increase in the level of endogenous gibberellins (Hillman 1969; Zeevaart 1983). Zanewich and Rood (1995) investigated the effect of a chilling treatment (which fulfilled the vernalization requirement) on the content, composition and metabolism of gibberellins in apical segments of winter rape (canola). They found that relative to non vernalized plants the content of GA<sub>19</sub> and GA<sub>20</sub> was elevated 12- and 24-fold, respectively, at the end of vernalization (the GA<sub>19</sub> and GA<sub>20</sub> levels were at the end of vernalization ca. 11 ng/gDW and ca. 1.2 ng/gDW in vernalized plants, respectively, while only ca. 1 ng and 0.1 ng/gDW in control plants, respectively). To a lesser extent the level of GA<sub>1</sub>, GA<sub>3</sub> and GA<sub>8</sub> was also increased. Vernalized plants showed an increased conversion of GA<sub>20</sub> to GA<sub>1</sub>-like metabolites and a reduced conversion of GA<sub>1</sub> or GA<sub>20</sub> to polar metabolites (putative glucosyl conjugates). The authors concluded that vernalization affects the content and metabolism of GAs, suggesting that GAs serve as probable regulatory intermediates between chilling treatment and subsequent stem growth. In spring rape the effect of applied GA<sub>3</sub> (10 or 40 µg/ml) was similar to that of inductive long days Dahanayake and Galwey (1998). Further, the use of GA biosynthesis inhibitors on some *Brassicaceae* plants has revealed that some GAs are not involved in floral induction in these species, but are required for stem elongation (Talon and Zeevaart 1990). Some experimental data from the early sixties indicates that stem elongation (shooting) may be experimentally separated from floral induction as two autonomous processes, although this has not been clearly confirmed either by physiological or genetic approaches. Treatment of mutants defective in GA biosynthesis with authentic labelled or unlabelled GAs has demonstrated that certain C<sub>19</sub>-GAs such as GA<sub>1</sub>, GA<sub>3</sub>, GA<sub>4</sub> and GA<sub>5</sub> have biological activity *per se* (Spray *et al.* 1996; Stokes *et al.* 2003). However, as suggested by Lange (1998), GAs with relatively low or negligible activity in common bioassays may be physiologically active in some plant species, at specific development stages and/or in certain tissues. Studies using exogenous applications of a wide range of authentic GAs (in concentration of 25 µg - a single application to the leaf) have revealed that the extent of biological activity of GAs in advancing flowering may depend on the structural features of the GA molecules, as well as the plant species (Evans *et al.* 1990; Hisamatsu *et al.* 2000). In species like *Lolium temulentum*, gibberellins such as GA<sub>5</sub>/GA<sub>6</sub> seem to fit with the concept of florigen and may substitute for the long-day induction (King *et al.* 2001; King and Evans 2003).

The promotion of *in vitro* flowering by exogenous cytokinins (20 µM benzyladenine, isopentenyladenine or zeatin) is well documented in many plant species (reviewed in Taylor and van Staden 2006). In general, the experimental evidence indicates that cytokinins mediate the growth changes within the apical meristem, particularly by elevating the rate of mitosis, which accompany the onset of reproductive state (Bernier *et al.* 1993; Jacquard *et al.* 2002). Cytokinins are synthesized predominantly in the roots with subsequent transport to the shoots (Hoad 1995). Numerous studies have

demonstrated an increase in cytokinin supply to the apical meristem at the floral induction in long-day as well as in short-day plants (Macháčková *et al.* 1993; Kinet *et al.* 1994; Macháčková *et al.* 1996; Corbesier *et al.* 2003). In winter rape plants an increase of cytokinin level during generative development and apparently induced by vernalization was observed (our data – in preparation). This increase was detected in both the apical part of shoots and in the juvenile leaves, although the changes were especially pronounced in shoot apices. Induction of flowering by grafting of non-vernalized winter rape on vernalized stocks was also correlated with an increase of the total cytokinin content. Induction of flowering by grafting of non-vernalized winter rape onto vernalized stocks was also correlated with an elevation of the total cytokinin content. Such preliminary data indicate the possible involvement of cytokinins in the transition of winter rape to generative phase due to vernalization.

Surprisingly in the short-day plant *Chenopodium rubrum* L., the promotive effect of root removal on flowering has been demonstrated, provided the treatment is performed after photoperiodic induction (Krekule and Přivratský 1976; Vondráková *et al.* 1998). A similar effect of de-rooting on shoot apex development was observed in winter rape (Filek *et al.* 2006). *In vitro* generative development of isolated apical parts was observed only in tissues cut-off from completely vernalized plants. Endogenous cytokinin content declined in the later stages of plant development, starting at the onset of flowering (the total level of cytokinins decreased from 473 pmol/g FW to 97 pmol in epiphytic bromeliad) (Mercier and Endres 1999).

### Electrochemical signals in flower initiation

Although the transition to reproductive development has been mostly approached from the aspect of chemical signals (florigen) and the metabolic changes which they control, there is experimental evidence of electrogenic flower initiation. In these cases “florigen” is viewed as a frequency-coded electric signal (e.g. Wagner *et al.* 1998). Such ideas provided the basis for experiments aiming to enhance or perturb environmental signals by means of an electric current. Such an approach was successfully carried out in photoperiodic plants (Adamec *et al.* 1989; Macháčková *et al.* 1990). In winter rape attaching the cathode to the apical part of the shoot and the anode to the roots (giving negative polarity) significantly inhibited the percentage of flowering plants when non-vernalized scions were grafted onto vernalized stocks (Filek *et al.* 2003). The reverse polarity markedly enhanced flowering under short days and gave only slightly promotion under long days. Moreover, when *in vitro* cultured winter rape plants had direct electric current applied to apical parts of the stems, with the anode attached to the apical part and the cathode to the medium, generative differentiation of the apical meristem was stimulated (Filek *et al.* 2006). Additionally, this effect was observed in the apices which were cut from plants grown in incomplete vernalization conditions (14 days at 5°C). Thus, it is clear that in winter rape negative polarity may at least partly nullify the developmental state achieved by vernalization, while positive polarity can partly substitute chilling requirement and long day photoperiod. However, as already pointed out, a clear-cut distinction between vernalization and photoperiodic effects is rather difficult to make. Although vernalization is usually considered to act at apical meristems and thus not involving mobile signals the existence of graft-transmissible mobile component of vernalization as suggested in pea e.g. by Murfet (1985) should be mentioned. On the basis of our experiments it was suggested that transport of more negatively charged chemicals is involved in the switch from the vegetative to the generative phase. A detailed analysis of correlations between patterns of transport, distribution of flowering factors and electric current developmental effects might become an efficient tool in studying flowering control (Filek *et al.* 2006).

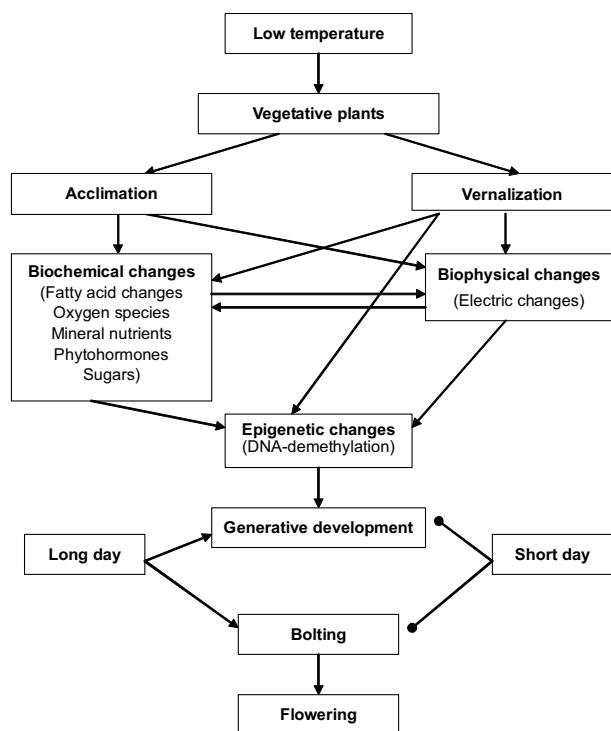
Signal pathways of generative development (*Brassica napus*)

Fig. 2 Diagram of signal pathways of generative development in rape (*Brassica napus* L.). arrow, activation; asterisk, inhibition.

## Genetic and genomic studies of vernalization

Due to its small genome, short vegetation period and existence of diversity of late- and early flowering ecotypes with relative simple acquisition of mutants, *Arabidopsis* has been used extensively to reveal a complex genetic network of flowering time control. Winter annual ecotypes exhibit very late flowering phenotypes but the flowering time is substantially shortened by chilling treatment (vernalization). Extensive genetic studies have led to the identification of more than 80 flowering time genes that have been classified into 4 independent genetic pathways: photoperiodic, autonomous, vernalization and gibberellin-dependent. Among these *CONSTANS* (*CO*) and *FLOWERING LOCUS C* (*FLC*) act as central regulators. *CO* mediates long-day pathway, whereas *FLC* mediates autonomous and vernalization pathway (Lee *et al.* 1994). Vernalization promotes the flowering of winter annuals by the epigenetic down-regulation of *FLC* (e.g. Bastow *et al.* 2004; Amasino 2006). Genetic analysis has led to identification of three genes that mediate vernalization response (*VRN 1,2,3*). It was shown that during vernalization the *FLC* chromatin undergoes a series of histone modification (deacetylation, methylation) which represent the molecular basis of vernalization (Bastow *et al.* 2004; Sung and Amasino 2004; Amasino 2006). Such mechanism of *FLC* repression that ultimately results in stable repressed state and its extension by mitosis is considered as epigenetic nature of vernalization.

In *Brassica* plants, vernalization-responsive flowering time loci segregate as two major quantitative trait loci (QTLs) were collinear with the regions of the *Arabidopsis* genome in which *FRI* and *FLC* were located (Osborn *et al.* 1997). This suggested that the same genes are important in both species. The observation that prolonged growth at low temperatures results in reduced genomic DNA methylation was the base of hypothesis that vernalization involves the activation, by demethylation, of gene(s) that are essential in the promotion of flowering. Burn *et al.* (1983) and Finne-

gan and Kovac (2000) observed that DNA demethylation, induced either by treatment with 5-azacytidine or by a *METHYLTRANSFERASE I* (*MET1*), promoted flowering in vernalization-responsive *Arabidopsis* ecotypes and mutants. The presence of methylcytosine in sequences of DNA would alter the binding of transcriptional factors or some other proteins to DNA or, as it has been shown in animals, would attract proteins known as methyl-binding proteins (Ballestar and Wolffe 2001). Thus, high methylcytosine contents are frequently associated with silent genes. The extent and pattern of methylation of genomic DNA are species and tissue specific, which implies that the pattern of methylation is faithfully inherited in all cells of common lineage within a tissue (Christman *et al.* 1995). Although not all genes are regulated by methylation, the hypomethylation at specific sites or in specific regions in a number of genes is correlated with active transcription (Wada *et al.* 2003). Methylation at specific sites in vernalization response-genes would be diluted by successive cycles of DNA replication, accounting for the requirement for cell-division during vernalization process, and the observed correlation between the duration of the cold treatment and the extent to which flowering is promoted (Finnegan *et al.* 1998; Causevic *et al.* 2005). It could be that changes in the methylation patterns of specific genes, which are established during growth in the cold, are maintained through mitotic cell divisions, but reset in progeny (Sheldon *et al.* 1999, 2000).

We have observed an increased number of demethylated loci in vernalized shoot apices of rape in comparison with vegetative, non-vernalized ones (Filek *et al.* 2006a). In shoot apices of vegetative and generative grafts the same number of demethylation events occurred. Thus, it was suggested, that most of demethylation events can be ascribed to vernalization and not to mobile signals of generative stage. In the juvenile leaves of winter rape methylation and demethylation events occurred with similar frequency in both generative and vegetative phase of development. These results indicate that during vernalization and transition to generative stage different sets of genes are activated and de-activated in different organs which points to rather complex and dynamic processes obscuring identification of crucial control points (Filek *et al.* 2006a) (Fig. 2).

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