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Mode of Action of Plant Hormones and Plant Growth Regulators During Induction of Somatic Embryogenesis: Molecular Aspects

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Abstract Plant hormones play critical roles in the establishment of somatic embryogenesis. During this process, somatic plant cells reverse their state of differentiation, acquire pluripotentiality and set up a new developmental program. The identification of the regulatory mechanisms that govern the key events of somatic embryogenesis requires molecular and genetic investigations. One critical issue is how plant hormones and growth regulators act to mediate somatic embryogenesis. Do they function as simple stimuli or participate directly, as central signals, in the reprogramming of the somatic cells towards an embryogenic fate? The latter scenario is now well supported by a number of studies that provide evidence of close interconnections between plant hormones and the molecular pathways that control somatic embryogenesis, including chromatin remodeling, gene expression patterning, reactivation of cell cycle and division and regulation of protein turnover. In this chapter we describe recent advances in the understanding of molecular and genetic mechanisms underlying the early stages of somatic embryogenesis. The roles and mode of action of plant hormones are especially emphasized.

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
ABA	Abscisic acid
ABP1	Auxin binding protein 1
ARF	Auxin-response factors
aza-C	5-Azacytidine
BBM	*BABY BOOM*
BAP	Benzylaminopurine
CDK	Cyclin-dependent kinase
DD-RT PCR	Differential display reverse transcription polymerase chain reaction
ER	Endoplasmic reticulum
GA	Gibberellin
IAA	Indole-3-acetic acid
LEC	*LEAFY COTYLEDON*
NAA	Naphthalene acetic acid
PGR	Plant growth regulator

PH	Plant hormone
PKL	*PICKLE*
SE	Somatic embryogenesis
(*SERK*)	*SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE*
(*WUS*)	*WUSCHEL*

1 Introduction

Somatic embryogenesis (SE) has been observed to be induced by different factors (reviewed by Jiménez 2001; Fehér et al. 2003). Independently of the nature of the external stimulus, the establishment of SE necessarily involves profound changes at the molecular level, such as the coordinated expression of different sets of genes that drive the switch from the current vegetative growth pattern to an embryogenic development. Thus, the identification of the genes that trigger key phases of SE, i.e. cell dedifferentiation, cell cycle reentry and establishment of a new embryogenic fate, is highly desirable. Additionally, the elucidation of the signaling pathways by which plant cells remodel their gene expression program is central to understanding the regulation of the SE process.

As discussed in detail elsewhere (Jiménez and Thomas, this volume), plant growth regulators (PGRs) are among the external stimuli most often employed to induce SE and to regulate the further development of embryogenic tissues. There was some controversy as to whether PGRs/plant hormones (PHs) act only as stimuli or are more directly involved in the mechanisms that regulate gene expression (Gaspar et al. 2003). However, during the last few years, a large body of experimental data supports the view that PHs play a central role in the establishment of SE. The understanding of the underlying mechanisms of PH action requires investigation of hormone receptors, signal transduction pathways, and genetic programs that lead to the final cell response.

The first step in any event associated with a response to a hormone is a proper recognition by the target cells. This recognition normally involves receptors, which are proteins associated with the cell membranes or are located in the cytoplasm. Receptors have been identified and characterized for hormone groups such as ethylene (Chang et al. 1993; Schaller and Bleecker 1995) and cytokinins (Inoue et al. 2001; Ueguchi et al. 2001). These receptors activate a signal transduction pathway that either induces or inhibits cellular functions, or controls gene expression (reviewed by Kulaeva and Prokoptseva 2004).

In the case of auxins, although some auxin-binding proteins have been isolated, it is still uncertain whether they represent receptors for different auxin-mediated processes (Gaspar et al. 2003). To date, auxin binding pro-

tein 1 (ABP1) is the best-studied putative auxin receptor protein. ABP1 predominantly accumulates in the lumen of the endoplasmic reticulum (ER), an unusual location for a hormone receptor (Barbier-Brygoo et al. 1989; Inohara et al. 1989). However, there is evidence that ABP1 is active in auxin responsiveness at the surface of the plasma membrane although it carries the ER retention motif (Rück et al. 1993; Thiel et al. 1993; Leblanc et al. 1999; Steffens et al. 2001; Shimomura et al. 1989). ABP1 has been shown to mediate early auxin responses such as auxin-induced electrical responses (Rück et al. 1993; Thiel et al. 1993; Zimmermann et al. 1994; Bauly et al. 2000) and cell expansion (Jones et al. 1998; Chen et al. 2001a, b). However, its involvement in auxin-induced gene expression has not been proved yet. Although mechanisms responsible for auxin signal transduction from receptor to genome are still poorly known, significant progress has been achieved in auxin-regulated gene expression (reviewed by Hagen and Guilfoyle 2002).

The PGRs most widely used to induce and regulate in vitro SE are auxins and cytokinins. It has been observed that members from both hormone groups regulate the cell cycle and trigger cell divisions (Francis and Sorrell 2001), two very important factors that have been related to initiation of SE (Dudits et al. 1991, 1995; Yeung 1995). Recent data provide evidence that the elaboration and execution of developmental programs require a proper control of the cell cycle and division, indicating the regulators of the cell cycle machinery as key determinants of SE.

In addition to their influence on cell cycle progression, PGRs/PHs have been demonstrated to trigger substantial changes in chromatin structure and alteration of transcription that lead to the formation of either dedifferentiated callus tissues or somatic embryos (Dudits et al. 1995). Studies on the links between PH action and gene expression have resulted in the cloning of several genes responsive to auxins, cytokinins, or to both hormones. Although, the functions of a number of these genes remain unknown, others have obvious connections with the cell cycle or developmental processes including SE.

In this chapter we describe those findings related to cell division and changes in the pattern of gene expression during early stages of SE and we further highlight how hormonal signals are integrated into these processes.

2
Reactivation of Cell Cycle and Division

The reactivation of the cell cycle and division in differentiated cells is indispensable for the initiation of plant developmental processes, including SE (Dudits et al. 1995). The cell cycle is usually divided in four sequential phases: G1, S (DNA replication), G2 and M (cytokinesis). The basic control mechanisms that regulate progression through the cell cycle are remarkably well conserved during evolution and operate mainly at the G1–S and G2–M tran-

sitions (reviewed by Stals and Inzé 2001; De Veylder et al. 2003; Dewitte and Murray 2003). These two key check points depend on highly conserved serine/threonine kinases, named cyclin-dependent kinases (CDKs), and their associated regulatory subunits called cyclins.

The two mentioned groups of hormones, auxin and cytokinins, are generally sufficient to stimulate and sustain the in vitro proliferation of most plant cell types and have therefore been the best documented direct regulators of the cell cycle progression. The expression of genes related to the *cdc2* gene, which encodes the catalytic subunit of the key G1–S and G2–M regulator cdc2 protein kinase, is upregulated by auxin in alfalfa (Hirt et al. 1991), soybean (Miao et al. 1993) and tobacco pith explants (John et al. 1993). Although auxin enhances *cdc2* gene expression, cotreatment with cytokinin is absolutely required to induce a basic *cdc2* expression in tobacco pith explants (John et al. 1993), illustrating the synergic regulation exerted by both growth regulators on cell proliferation. The observation that most systems require only exogenously added auxin to resume cell division suggests that the rate of endogenous cytokinin synthesis is sufficient to sustain growth (del Pozo et al. 2005). In situ analysis of *cdc2* expression in plants such as *Arabidopsis* (Martinez et al. 1992; Hemerly et al. 1993) and soybean (Miao et al. 1993) revealed that *cdc2a* expression is not only associated with cell proliferation but also precedes it, suggesting that it reflects a state of competence to divide (Hemerly et al. 1993).

More recently, auxin has been shown to upregulate the expression of an alfalfa A2-type cyclin, whose promoter contains auxin-response-like elements (Roudier et al. 2003). In addition, auxin treatment of alfalfa plants affects the spatial expression pattern of this cyclin by shifting its expression from the phloem to the xylem poles, where lateral root formation is initiated in response to auxin. This auxin-regulated spatial cyclin expression illustrates another aspect of the complexity of hormonal regulation of the cell cycle in planta.

Cyclins D represent important connections between PHs and the cell cycle. Consistent with its regulatory function in the cell cycle progression, cyclin CycD3 is expressed in tissues having a high rate of cell divisions, including shoot meristems, young leaf primordia, axillary buds, procambium and vascular tissues of developing leaves (Riou-Khamlichi et al. 1999). The *CycD3* gene is highly responsive to cytokinin in both cell cultures and whole plants and is rapidly induced by cytokinin during the G1 phase of cells reentering the cell cycle. Constitutive expression of this cyclin in transgenic *Arabidopsis* plants leads to diverse disorders, e.g., extensive leaf curling and disorganized meristems, and, importantly, it renders callus growth independent of cytokinin application (Riou-Khamlichi et al. 1999). This demonstrates that cytokinins promote cell division by inducing the *CycD3* expression at the G1–S phase transition.

Cytokinins have also been reported to play a regulatory role at the mitotic control point of the G2–M transition. This is well illustrated by the observa-

tion that application of the cytokinin biosynthesis inhibitor lovastatin blocks cells of tobacco BY-2 in G2 (Laureys et al. 1998). This effect is nullified by the addition of an exogenous cytokinin, such as zeatin. This is in line with previous observations that cytokinins accumulate transiently during the G2–M phase (Redig et al. 1996) and that the removal of cytokinin from the culture medium leads to an arrest in G2 of tobacco suspension cell cultures, which accumulate inactive CDK complexes (Zhang et al. 1996). The kinase activity of the latter is restored by either addition of cytokinin or by tyrosine dephosphorylation, suggesting that the inactivation of CDK complexes under cytokinin deprivation is due to phosphorylation of regulatory residues of the CDK subunit.

Although auxin and cytokinins are generally considered as the main hormonal signals triggering cell cycle progression, others PHs with enhancing or inhibitory functions participate in the cell cycle control by modulating the transcriptional expression of different cell cycle genes. For example, gibberellin (GA) stimulates CDK and cyclin accumulation in a tissue-specific manner (Sauter 1997). Abscisic acid (ABA) induces a decrease in Cdc2a-like kinase activity by increasing the expression level of a CDK inhibitor gene, namely the *ICK1* gene, whose product interacts with Cdc2a and CycD3 (Wang et al. 1998). Although its mode of action is still unclear, jasmonic acid has been reported to block synchronized BY2 cells in both G1–S and G2–M transitions (Swiatek et al. 2002).

3 Reprogramming of the Gene Expression Pattern

The establishment of totipotency and the subsequent induction and development of somatic embryos require reprogramming the cultures. This is in part achieved by synthesis of new RNA molecules. Therefore, early inductive molecular events have been investigated by monitoring gene transcripts that are synthesized under the influence of external stimuli that trigger the embryogenic fate. Several examples of changes in the expression of genes related to initiation of SE have been reported. Here, we make reference only to those works in which the change in gene expression can be traced back to PGRs/PHs.

In an attempt to identify genes that switch on the SE program, researchers have employed systematic approaches aimed at comparing the population of transcripts expressed in embryogenic conditions with the population of the transcript expressed in nonembryogenic conditions. This was carried out using techniques such as differential complementary DNA library screening, differential display reverse transcription polymerase chain reaction (DD-RT PCR), cold plaque screening and more recently microarrays. The application of these techniques to the induction phase of SE has been complicated by the

difficulty to identify and isolate embryogenic cells in the initial steps when no morphological changes are visible. However, improvements in in vitro culture systems and methods of molecular analysis have allowed progress in the exploration of the early phases of SE.

Nagata et al. (1994) isolated three auxin-regulated genes, *parA*, *parB* and *parC*, that are transiently expressed during the regaining of meristematic activity of tobacco mesophyll protoplasts. The corresponding transcripts were detected as early as 20 min after the beginning of incubation of protoplasts with auxin. Importantly, they were no longer detected after 48 h of culture when protoplasts started to divide, suggesting that they are specifically involved in the reentry into the plant cell cycle.

Kitamiya et al. (2000) isolated two carrot genes that are differentially expressed in hypocotyl cells induced to form somatic embryos by treatment with 2,4-dicholorophenoxyacetic acid (2,4-D) for 2 h. One of these genes, namely the *D. carota heat-shock protein 1* (*Dchsp-1*) gene, is related to low molecular weight heat-shock proteins and was found to be expressed during embryo development. The other gene has homology to the auxin-regulated genes, including *par A* (Takahashi et al. 1989), and thus was named *D. carota auxin-regulated gene 1* (*Dcarg-1*). Interestingly, there is a parallel relationship between the expression of *Dcarg-1* and the formation of somatic embryos. In addition, in contrast to *Dchsp-1*, *Dcarg-1* was not responsive to stress treatment and was not expressed during development of somatic embryos, implying that its function was not required for this process to occur.

Using DD-RT PCR, Yasuda et al. (2001) attempted to identify genes that are preferentially expressed during the early stages of auxin-induced carrot SE. Three transcripts that accumulate immediately after somatic cells divide to form cell clusters, but that do not accumulate or barely accumulate in nonembryogenic cell suspension cultures, were characterized. Although these genes represent potential key regulators of SE, a clear function has still not been attributed to them.

An important issue is how PH action on the gene expression level pattern is mediated. In a general view, the hormonal signal activates a signaling cascade that recruits specific transcription factors. These induce the expression of target genes, which in turn trigger the final response. Numerous genes have been described containing *cis*-acting elements in their promoter region that confer hormone responsiveness. Over the past 20 years, sequences that are upregulated or downregulated by PHs have been described for auxins (Guilfoyle et al. 1998; Ulmasov et al. 1999), ABA (Marcotte et al. 1992), GAs (Gubler and Jacobsen 1992) and ethylene (Meller et al. 1993).

The auxin-modulated gene expression system is based, at least in part, on two interacting protein families. The multifamily protein auxin-response factors (ARFs) can activate or repress target genes by directly binding to specific DNA sequences, i.e., auxin response elements (Ulmasov et al. 1999). In contrast, the auxin/indole-3-acetic acid (IAA) proteins do not bind to DNA

directly but can inactivate ARF transcription factors by interacting with them through heterodimerization (Tiwari et al. 2001). Auxin exerts its regulation on gene expression through modulation of auxin/IAA protein turnover via a specialized branch of the ubiquitin–proteasome pathway (Worley et al. 2000; Gray et al. 2001; Dharmasiri and Estelle 2002). Such a PH-regulated proteolysis has been shown to be involved in many aspects of plant developmental processes including SE.

4
Chromatin Structure and DNA Methylation

A specific gene expression program is the result of the balance between the part of the genome that is transcribed, i.e., euchromatin, and the part that is repressed, i.e., heterochromatin. Many aspects of plant development, including embryonic and meristem development, flowering and seed formation, involve modifications of chromatin structure that affect the accessibility of target genes to regulatory factors that control their expression (reviewed by Li et al. 2002). Since maintaining the cellular differentiated state largely relies on chromatin-dependent gene silencing, the cellular dedifferentiation and the switch to a new embryogenic program necessarily involve important changes in chromatin structure.

Zhao et al. (2001) identified two distinct phases of chromatin decondensation during in vitro induced dedifferentiation of tobacco mesophyll cells. The first was independent of any hormonal treatment and was linked to the acquisition of pluripotentiality or dedifferentiation of cells. In contrast, the second phase of chromatin decondensation required auxin and cytokinin treatment and was linked to the reentry into the S phase.

Dynamic changes in chromatin structure are influenced by both posttranslational modifications of histone amino terminal tails and direct modifications of the DNA, such as methylation. The degree of DNA methylation has been reported to influence plant morphogenesis (reviewed by Li et al. 2002). The overexpression of an antisense DNA methyltransferase copy in transgenic tobacco plants provokes development disorders, including small leaves, short internodes and abnormal flower morphology (Nakano et al. 2000). The role of DNA methylation in early phases of SE has been recently addressed by Yamamoto et al. (2005) by investigating the effects of 5-azacytidine (aza-C), an inhibitor of DNA methylation, on the induction of direct carrot SE. Aza-C treatment totally inhibited the formation of embryogenic cell clumps from epidermal carrot cells. When applied during morphogenesis of embryos, aza-C downregulated the expression of *C-LEC1*, an important gene that participates in the embryonic program (Sect. 6). Additionally, in untreated cells, a DNA methyltransferase gene transcript transiently accumulated after auxin application but before the formation of embryogenic cell clumps, suggest-

ing a direct role for DNA methylation in the establishment of embryogenic competence in carrot somatic cells.

Other chemical substances, such as the antibiotic kanamycin, have been observed to considerably modify the level of DNA methylation during plant in vitro culture (Bardini et al. 2003). In this case, DNA methylation is considered as a potential source of somaclonal variation, a phenomenon (often undesirable) observed in plant cell and tissue cultures (Caplan et al. 1998).

5
Some Key Regulators of the Vegetative-to-Embryogenic Transition

As already stated, different strategies have been used to identify genes that are differentially expressed during SE (Thomas 1993; Lin et al. 1996; Schmidt et al. 1997). Although several genes have been cloned, their function or functions often remain obscure. However, improvements in plant transformation protocols and the availability of new mutants allowed the characterization of genes that regulate the vegetative-to-embryogenic transition. The ectopic expression of these genes either enhances SE in in vitro cultures or even provokes spontaneous embryo formation on intact plants. One new challenge is to identify possible existing links between the PRG/pH and the genes that possibly influence the vegetative-to-embryogenic transition during SE.

5.1
The *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE* Gene

Most of the molecular markers of SE identified to date are related to late stages of embryo development. However, one gene, encoding a leucine-rich-repeat receptor-like kinase, has been found to be specifically upregulated during the very precocious phases of the SE process. The *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE* (*SERK*) gene was originally cloned from a carrot cell suspension culture where it was found to mark cells that are competent to form somatic embryos, i.e., cells in transition between the somatic and the embryogenic states (Schmidt et al. 1997). Using in situ hybridization, *DcSERK* expression was shown to first appear in single cells of embryogenic cultures induced with 2,4-D for 7 days. *DcSERK* expression continues until the 100-cell stage of the globular somatic embryo and then ceases. Interestingly, a similar *SERK* expression pattern was observed during early zygotic embryogenesis, suggesting that the same SERK signaling pathway is activated during both SE and zygotic embryogenesis (Schmidt et al. 1997).

Several homologs of the carrot *DcSERK* have been identified in monocots, e.g., maize (Baudino et al. 2001) and *Dactylis glomerata* (Somleva et al. 2000), and dicots, e.g., *Medicago truncatula* (Nolan et al. 2003), *Arabidopsis thaliana* (Hecht et al. 2001) and sunflower (Thomas et al. 2004). Plant

genomes contain several *SERK* genes. As an example, the *Arabidopsis SERK* gene family comprises five members (Hecht et al. 2001). The expression of *AtSERK1*, the *Arabidopsis* gene most closely related to the carrot *DcSERK*, also marks embryogenic competent cells in culture. As in carrot, the level of *SERK* expression increases in response to the auxin treatment used to induce somatic embryos. Auxin-dependent *SERK* expression was also reported for the *M. truncatula MtSERK* gene, which is upregulated by the auxin naphthalene acetic acid (NAA) but not by the cytokinin benzylaminopurine (BAP; Nolan et al. 2003). However, addition of BAP to the culture medium potentiates NAA-induced *SERK* expression, possibly by stimulating endogenous auxin synthesis. In the direct SE system of sunflower, *SERK* transcripts specifically accumulate in the future morphogenic region of explants within the first few hours of culture. Although the only PGR supplied in the medium is a cytokinin, analysis of the endogenous PH content revealed that the internal IAA concentration transiently increases in explants during this early period (Thomas et al. 2002). A link between auxin and *SERK* expression is also suggested by the accumulation of *SERK* transcripts in plant tissues that contain high auxin levels, e.g., vascular tissue and leaf primordia (Hecht et al. 2001; Thomas et al. 2004). However, since *SERK* is not induced by auxin in all the cell explants or cell cultures, it is probably not an integral part of the auxin machinery or its expression requires other, still unknown, factors (Hecht et al. 2001).

Evidence that *AtSERK1* is not only a good marker of embryogenic competent cells in *Arabidopsis* but is also involved in the establishment of the embryogenic competence comes from ectopic overexpression of the *AtSERK1* gene in *Arabidopsis* (Hecht et al. 2001). Although during normal growth transgenic seedlings do not show any specific phenotype, their embryogenic capacity is considerably enhanced (approximately 4 times compared with the wild type) during in vitro culture. A similar increase in embryogenic competence is conferred by mutation in shoot apical meristem regulatory genes such as *AMP1*, *CLV1* and *CLV2* (Mordhorst et al. 1998). The higher *AtSERK1* expression level in *amp1* cultures, in comparison with that in wild-type cultures, suggests that one role of AMP1 could be to downregulate the expression of *AtSERK1* after germination (Hecht et al. 2001).

The identification of SERK-activating ligand(s) as well as the downstream targets of SERK is highly desirable to further characterize the function(s) of SERK in both zygotic embryogenesis and SE.

5.2 The *BABY BOOM* Gene

Another gene that potentially activates signal transduction pathways leading to the induction of embryo development from differentiated somatic cells is the *BABY BOOM* (*BBM*) gene (Boutilier et al. 2002). It was identified

by a screening approach aimed at identifying genes differentially expressed during early phases of *Brassica napus* microspore embryogenesis. The *B. napus* microspore culture system relies on the ability of the vegetative cell of an immature pollen grain to develop into an embryo in response to high-temperature (above 25 °C) culture conditions (Custers et al. 1994).

The *BBM* gene encodes a protein that belongs to the AP2/ERF family, a plant-specific class of transcription factors that regulate several developmental processes, such as floral organ identity determination and control of leaf epidermal cell identity (reviewed by Riechmann and Meyerowitz 1998). It is preferentially expressed during embryo and seed development (Boutilier et al. 2002).

Overexpression of the *BBM* gene under the control of a constitutive promoter leads to the spontaneous formation of somatic embryos and cotyledon-like structures on different tissues of intact plants (Boutilier et al. 2002). Additionally, in vitro cultured explants, coming from *BBM*-overexpressing transgenic plants, display an enhanced capacity to regenerate through shoot organogenesis. This suggests that *BBM* plays a broader role in cell division and differentiation rather than being a specific element of the SE pathway.

Importantly, in contrast to *SERK*, ectopic expression of *BBM* is able to promote SE in the absence of exogenously applied PGR. It has been proposed that *BBM* could act by stimulating an increase of PH and/or increasing the cellular hormonal sensitivity (Boutilier et al. 2002). In that sense, Klucher et al. (1996) speculated that AP2/ERF domain proteins, being unique to plants, might have coevolved with plant-specific pathways such as PH signal transduction. Alternatively, it is also conceivable that the *BBM* product acts in a PH signaling pathway downstream of the hormone perception as previously shown for some other AP2/ERF domain proteins (Finkelstein et al. 1998; Menke et al. 1999; Gu et al. 2000; Banno et al. 2001; van der Fits and Memelink 2001).

5.3 The *LEAFY COTYLEDON* Genes

Arabidopsis mutants that display abnormalities in embryo development represent powerful tools to investigate the molecular pathways underlying SE. The *LEAFY COTYLEDON1* (*LEC1*) and *LEAFY COTYLEDON2* (*LEC2*) genes were identified originally as loss-of-function mutants showing defects in both embryo identity and seed maturation processes (Meinke et al. 1994; West et al. 1994). *Lec* embryos present a reduction in desiccation tolerance and do not accumulate normal storage materials. In addition, *lec* mutants exhibit other anatomical characteristics, including the presence of trichomes on cotyledons, which in *Arabidopsis* wild-type plants are specific to true leaves (Meinke et al. 1994; West et al. 1994; Stone et al. 2001). The pleiotropic effects of *lec* mutations pinpoint the *LEC* genes as central regulators of embryo and seed development.

Identification and analysis of the *Arabidopsis LEC1* and *LEC2* genes confirmed their regulatory roles in embryogenesis and provided significant insight into their functions. Both *LEC* genes encode seed-expressed transcriptional activators. *LEC1* encodes a protein related to the heme-activated protein 3 subunit of the CCAAT box-binding factor, a eukaryotic transcription factor (Lotan et al. 1998). *LEC2* encodes a protein that contains the plant-specific B3 domain (Stone et al. 2001), which is found in several plant transcription factors including ABA INSENSITIVE3 (Luerssen et al. 1998) and VIVIPAROUS1 (McCarty et al. 1989 and 1991).

Ectopic overexpression of either *lec1* or *lec2* results in the spontaneous formation of somatic embryos directly on the leaf surface, suggesting that *lec* genes play a role in conferring embryogenic competence to cells (Lotan et al. 1998; Stone et al. 2001). It also confers embryonic characteristics to seedlings. The expression of embryo-specific genes, such as those encoding cruciferin A, 2S storage protein and oleosin, in adult transgenic seedlings, confirms the activation and maintenance of embryo-specific programs in vegetative tissues. Interestingly, the *35S::LEC1* phenotype is relatively weak, i.e., only a few plants show sporadic embryo development, whereas the *35S::LEC2* phenotype is stronger and comparable to that observed for *35S::BBM* plants. The fact that the *BBM* and *LEC* genes exhibit similar putative functions as transcription factors, are both preferentially expressed in seeds, and confer to plants a similar phenotype when ectopically expressed suggests that they function in the same molecular pathway. However, *BBM* transcripts are present in *lec1* mutant seeds (Boutilier et al. 2002), indicating that the expression of *BBM* is not dependent per se on the presence of the *LEC1* protein. Thus, *BBM* could either function upstream of *LEC1* or operate in an *LEC1*-separated but overlapping pathway.

Recently, Yazawa et al. (2004) isolated a carrot functional homolog of *Arabidopsis LEC1*, as demonstrated by complementation experiments. In the SE system of carrot, the highest expression of *C-LEC1* was detected in cell clusters of 38–63 μm in diameter that were being cultured for induction of somatic embryos. Strikingly, cell clusters of this size are also those that are the most efficient for somatic embryo production (Satoh et al. 1986).

5.4 The *PICKLE* Gene

Another interesting *Arabidopsis* mutant is the *pickle* (*pkl*) mutant described by Ogas et al. (1997). At the opposite side of the *lec* phenotypes, a null mutation in the *PKL* gene induces embryonic characteristics in the roots of *Arabidopsis* seedlings, including accumulation of lipids and seed storage proteins normally found in seeds (Ogas et al. 1999; Rider et al. 2004). When excised and cultured on a medium lacking PGR, roots of *pkl* seedlings spontaneously develop somatic embryos. Exogenous application of GA is sufficient

to suppress the mutant phenotype, whereas decreasing the level of GA in germinating seeds increases significantly the penetrance of the *pkl* root phenotype (Ogas et al. 1997). These observations suggest that *PKL* functions in a GA pathway that controls the switch of root cells from an embryonic to a vegetative fate. The *PKL* gene encodes a CHD3-chromatin remodeling factor, and thus is likely to function as a negative regulator of transcription of embryo-specific genes (Eshed et al. 1999; Ogas et al. 1999). This is supported by the observation that *LEC1* and *LEC2* expression levels are significantly higher in *pkl* than in wild-type seedlings. Thus, expression of embryonic traits in *pkl* seedlings is highly suspected to be a consequence of the failure to repress expression, in a GA-dependent manner, of the master regulators of embryogenic identity, such as the *LEC* genes, during germination (Rider et al. 2003). However, as noted by Henderson et al. (2004), data that demonstrate a direct link between *PKL* activity and GA are still missing and thus it could not be absolutely decided whether repression of *LEC1* is or is not a GA-dependent event. The observation that GA can act in the absence of *PKL* to repress expression of the *pkl* root phenotype (Ogas et al. 1997) demonstrates that there also exists a *PKL*-independent pathway by which GA represses expression of embryonic traits. This is consistent with the recent metabolic analysis that revealed that *pkl Arabidopsis* roots accumulate some but not all seed-specific metabolites (Rider et al. 2004).

5.5 The *WUSCHEL* Gene

Using a genetic approach to identify gain-of-function mutations that can promote embryogenic callus formation from *Arabidopsis* root explants, Zuo et al. (2002) identified a gene, *PAG6*, that was found to be identical to *WUSCHEL* (*WUS*), a gene previously characterized as a key regulator for specification of stem cell fate in floral and shoot meristems (Laux et al. 1996). *WUS* encodes a homeodomain protein and is expressed in a small group of cells, namely, the organizing center, below the shoot meristem central zone, which contains the stem cells (Mayer et al. 1998; Schoof et al. 2000).

Overexpression of *WUS* induces the formation of highly embryogenic callus in the presence of auxin (Zuo et al. 2002). In addition, ectopic overexpression of *WUS* in transgenic plants directly induces somatic embryos from various vegetative tissues independently of any external PGR treatment. Therefore, *WUS* appears to be able to trigger the vegetative-to-embryogenic transition, bypassing the auxin requirement or taking advantage of the endogenous auxin flux (Zuo et al. 2002).

Interestingly, *WUS* cannot reprogram the shoot apex towards SE when overexpressed under the control of meristem-specific promoters such as CLV1, ANT (Schoof et al. 2000), LFY, AP3 and AG (Lenhard et al. 2001;

Lohmann et al. 2001). This raises the possibility that some factors could favor one or the other *WUS* function (a shoot meristem or an embryo organizer). Gallois et al. (2004) addressed this possibility by studying the effects of ectopic expression of *WUS* in roots. In the absence of additional cues, *WUS* expression in the root induced shoot stem identity and leaf development indicating that *WUS* establishes stem cells with an intrinsic shoot identity. However, when *WUS* is coexpressed with *LEAFY*, which is a master regulator of floral development (Weigel et al. 1992), *WUS* induces the formation of floral tissues. Finally, when exogenous auxin is supplied, the expression of *WUS* leads to the development of somatic embryos. This elegant work demonstrates that although *WUS* expression specifies an intrinsic shoot activity (in the absence of additional cues) it also makes cells developmentally flexible and able to be directed to floral organ or embryo development, depending on additional cues.

6 Concluding Remarks

PGRs/PHs are largely used to elicit in vitro SE and are therefore suspected to play important roles in this process; however, the question of their exact function remains open. One difficulty in elucidating the role of PGRs/PHs in SE is that they are likely to be involved at different levels. Although they are very efficient stimuli, they also represent signaling molecules that are an integral part of the molecular pathways underlying SE. As exogenous stimuli, they can occasionally be replaced by other treatments, including stresses such as osmotic or heat shock (Jiménez and Thomas, this volume). In contrast, it becomes obvious that endogenous PHs play essential roles in directing crucial SE-related events, including reentry into the cell cycle and dedifferentiation and redifferentiation of somatic cells. Recent developments in the elucidation of modes of action of PHs have shown that they trigger profound modifications in cellular gene expression patterns both by influencing chromatin structure and DNA methylation and by a finer and more specific transcriptional regulation of target genes.

Recent data suggest that the cellular embryonic competence is "actively" repressed in postembryonic plant tissues by proteins such as AMP1 or PICKLE. Derepression, e.g., by null mutation in repressor genes, opens the way to SE. However, somatic embryo induction is only activated when local tissue/cellular conditions, such as a proper hormonal balance, are appropriate. This would explain why all cells of *pickle* or *amp1* mutants do not uniformly enter an embryonic developmental program. The observation that different mutations induce similar embryonic phenotypes in postembryonic plants reflects the complexity of SE and the possible existence of overlapping pathways triggering this developmental process.

In recent years functional genomics allowed identification of several potential candidate genes that may be responsible for the establishment of the SE program. Although the participation of these genes in the induction of SE in wild-type plants has not been proved yet, they represent very exciting tracks to pursue in the exploration of molecular pathways underlying SE.

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