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Reprogramming of plant cells induced by 6b oncoproteins from the plant pathogen *Agrobacterium*

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Abstract Reprogramming of plant cells is an event characterized by dedifferentiation, reacquisition of totipotency, and enhanced cell proliferation, and is typically observed during formation of the callus, which is dependent on plant hormones. The callus-like cell mass, called a crown gall tumor, is induced at the sites of infection by *Agrobacterium* species through the expression of hormone-synthesizing genes encoded in the T-DNA region, which probably involves a similar reprogramming process. One of the T-DNA genes, *6b*, can also by itself induce reprogramming of differentiated cells to generate tumors and is therefore recognized as an oncogene acting in plant cells. The *6b* genes belong to a group of *Agrobacterium* T-DNA genes, which include *rolB*, *rolC*, and *orf13*. These genes encode proteins with weakly conserved sequences and may be derived from a common evolutionary origin. Most of these members can modify plant growth and morphogenesis in various ways, in most cases without affecting the levels of plant hormones. Recent studies have suggested that the molecular function of 6b might be to modify the patterns of transcription in the host nuclei, particularly by directly targeting the host transcription factors or by changing the epigenetic status of the host chromatin through intrinsic histone chaperone activity. In light of the recent findings on zygotic resetting of nucleosomal histone variants in *Arabidopsis thaliana*, one attractive idea is that acquisition of totipotency might be facilitated by global changes of epigenetic status, which might be induced by replacement of histone variants in the zygote after fertilization and in differentiated cells upon stimulation by plant hormones as well as by expression of the *6b* gene.

Keywords *Agrobacterium* · Crown gall tumor · Epigenetic regulation · Histone chaperone · Histone variants · Oncoprotein 6b · Reprogramming

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Introduction

Agrobacterium tumefaciens, also known by its updated name *Rhizobium radiobacter*, is a plant pathogen that causes tumors at the infected sites in its host plants. It is well known that these soil-borne bacteria have plasmids, called tumor-inducing (Ti) plasmids, that are not essential for their survival but are required for infection of and parasitic interaction with the host plant (for a review, see Pitzschke and Hirt 2010). In an infected plant, a specific region in the plasmids, called transfer DNA (T-DNA), is excised out, transferred into the host nuclei, and integrated into the chromosome (Chilton et al. 1977). The Ti plasmids contain *virulence* genes that function in the excision, transfer, and integration of T-DNA and are located outside of the T-DNA regions (for a review, see Zhu et al. 2000). The presence of T-DNA itself is not required for these processes but is essential for the generation of a tumor, called a crown gall, after the T-DNA integration into the plant genome (see Fig. 1a for the appearance of a crown gall).

Tumor formation is caused by enhanced division of undifferentiated cells at the sites of infection and is generally believed to involve reprogramming of differentiated plant cells. This abnormal morphogenesis is largely due

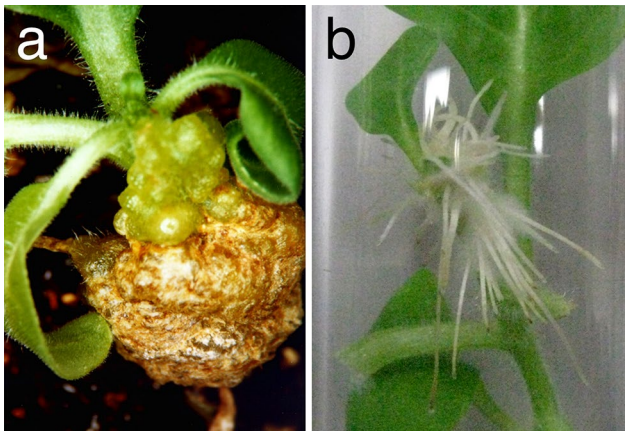

Fig. 1 Neoplastic disease caused by *Agrobacterium* infection. **a** Crown gall tumor developed on the *N. tabacum* stem infected by *A. tumefaciens*. **b** Hairy roots generated from the *N. tabacum* stem after infection by *A. rhizogenes*

to the production of two important plant hormones, auxin and cytokinin. The well-characterized genes for hormone production, *ipt/tmr* (encoding isopentenyl transferase), *iaaM/tms1* (tryptophan mono-oxygenase), and *iaaH/tms2* (indolacetamide hydrolase), are present in T-DNA and are responsible for tumor formation (Morris 1986).

When cultured in vitro, tumor cells can continue to divide in an undifferentiated state to form a cell mass without requiring an external supply of plant hormones. The similarly reprogrammed cell mass is known as the callus, which can be induced in explants when exposed to both auxin and cytokinin. Historically important experiments showed that plant organogenesis in tissue culture is strongly dependent on the auxin/cytokinin ratio. When the concentration of cytokinin is high, shoot meristems are ectopically induced, thus forming adventitious buds, whereas adventitious roots are induced if a higher concentration of auxin is supplied (Skoog and Miller 1957). Correspondingly, a mutation of the *ipt/tmr* gene in the T-DNA causes rooty tumors, whereas a mutation in *iaaM/tms1* results in shooty tumors (Morris 1986). Therefore, the crown gall tumor is generally regarded as equivalent to the callus developed *in planta* instead of in vitro.

In addition to the *ipt* and *iaa* genes, T-DNA contains a gene called *6b* that promotes tumorigenesis but is not essential for gall formation. This gene is found in all *A. tumefaciens* and *A. vitis* strains with gall-forming ability (Otten and De Ruffray 1994), and it has the ability to induce tumors by itself on the stems of *Nicotiana glauca* and *Kalanchoe tubiflora* without the assistance of other T-DNA genes (Hooykaas et al. 1988). When ectopically expressed in plants by a strong promoter, *6b* causes many different symptoms (Grémillon et al. 2004; Helfer et al. 2003; Kakiuchi et al. 2007), and most prominently induces a callus-like cell mass from explants without an external supply of auxin and cytokinin (Terakura et al. 2006; Wabiko and Minemura 1996). It is therefore believed that 6b, acting as an oncoprotein, induces an undifferentiated state in plant cells and stimulates cell division.

The 6b proteins show weak sequence similarity to some proteins encoded by the T-DNA of root-inducing plasmids (Ri plasmids) in another bacterial species of the *Agrobacterium* genus, *Agrobacterium rhizogenes* (updated as *Rhizobium rhizogenes*). *A. rhizogenes* ectopically induces abnormal root-like organs, called hairy roots, on the infection sites of host plants (Nilsson and Olsson 1997). The hairy roots have neoplastic characteristics, extraordinarily high growth rates, and a highly branching appearance (see Fig. 1b for the appearance of hairy roots). In addition, shoot regeneration is induced spontaneously when hairy roots are grown in vitro. The process of hairy root formation accompanies stimulation of cell division as well as reacquisition of cell identity and, therefore, can be regarded as the reprogramming of plant cells.

Similar to Ti plasmids, the T-DNA in Ri plasmids is transferred into host cells at the infection sites and is integrated into the genome (Huffman et al. 1984; White et al. 1983). In *A. rhizogenes*, T-DNAs in Ri plasmids commonly contain the root locus (*rol*) genes, *rolA*, *rolB*, and *rolC*, which are necessary and sufficient for hairy root induction. Even when individually expressed in *Nicotiana tabacum*, each *rol* gene can induce hairy root phenotypes at different efficiencies (Cardarelli et al. 1987; Spena et al. 1987). Among the products of these genes, rolB and rolC are diversely related to each other and to 6b in terms of their amino acid sequences. Based on their sequence similarities, *6b* in Ti plasmids and *rolB* and *rolC* in Ri plasmids are likely to have originated from a common ancestral gene and are collectively called the *plast* family (after their hypothetical function of conferring plasticity to plants) (Levesque et al. 1988). *ORF13* and *ORF14* in Ri plasmids and some other T-DNA genes are also regarded as members of the *plast* family because they show weak sequence similarity to *rolB*, *rolC*, and *6b* (Fig. 2). The evolutionary origin of this protein family is unclear because genes similar to those in this family have not been found outside of T-DNA in *Agrobacterium* species, except for a few *Nicotiana* species that have acquired T-DNA by horizontal gene transfer (Aoki 2004). Recent study reports another exception, *Laccaria bicolor*, a mycorrhizal fungus possessing genes encoding *plast*-like proteins, which, however, are only distantly related to any members of *plast* family, and hence would require further careful evaluation to be decisively classified into *plast* family (Mohajjel-Shoja et al. 2011). Until recently, the cellular and molecular functions of the *plast* genes have been controversial, although various hypothetical explanations have been offered for the effects of *plast* genes.

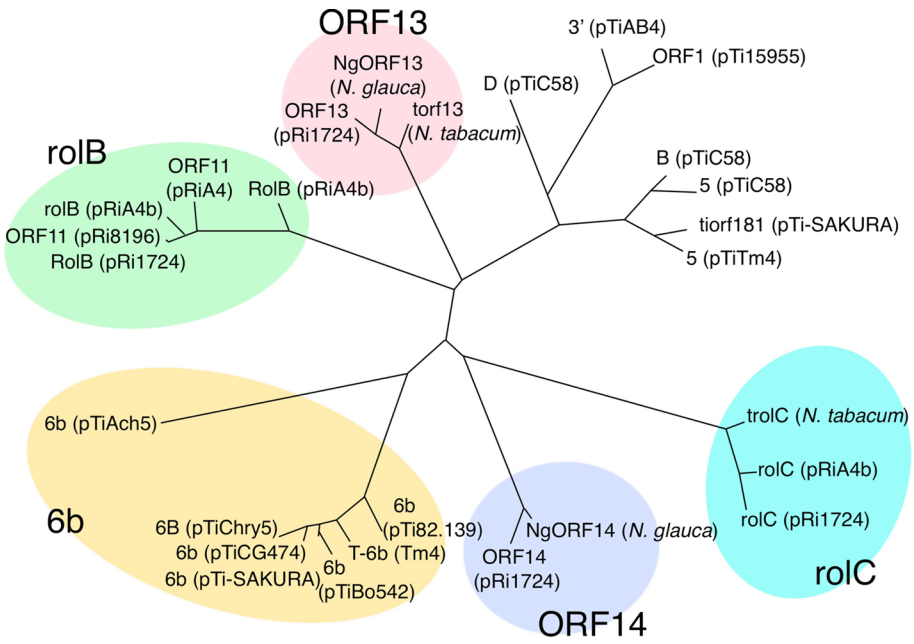

Fig. 2 Phylogenetic relationships of *plast* family proteins. The *plast* family mainly comprises five subfamilies that correspond to 6b in Ti plasmids, and rolB, rolC, ORF13, and ORF14 in Ri plasmids from *Agrobacterium* species. A few *Nicotiana* species also contain members of this family because of horizontal gene transfer from *Agrobacterium*. Proteins from *Agrobacterium* are shown together with the names of the plasmids that encode the proteins. When proteins are from *Nicotiana* species, the scientific names are provided in parentheses. The tree was constructed using the entire amino acid sequence of each protein using the web tool Phylogeny.fr (Dereeper et al. 2008; http://www.phylogeny.fr/)

Although the morphogenic activities of *A. tumefaciens* and *A. rhizogenes* differ substantially (i.e., tumors versus hairy roots), the *plast* genes share some common morphological effects when expressed individually in plants. Mohajjel-Shoja et al. (2011) showed that morphological changes induced by *rolC* were similar to those induced by *6b*. By contrast, only 6b proteins have C-terminal extensions that are rich in acidic amino acids, which may be related to the functions unique to 6b (Kitakura et al. 2002, 2008; Terakura et al. 2007). Therefore, when debating the functions of the *plast* genes, one should consider both the commonality among the *plast* family proteins and the specificity of individual subfamilies. The aim of this review is to summarize pioneering and recent studies on *6b* oncogenes and other *plast* genes, and to try to provide a unified view of the molecular function of *6b* for the reprogramming of plant cells by considering the interconnections between those studies.

Hormone-related functions proposed by pioneering studies

The *6b* gene has intrinsic activity for inducing small tumors in specific plant species when introduced through *Agrobacterium* infection (Hooykaas et al. 1988). Because the *ipt* and *iaa* genes are both critical for tumor formation upon *Agrobacterium* infection, the actions of 6b were extensively investigated in connection with the genes related to hormone production. Tinland et al. (1989) showed the synergistic interaction between *6b* and hormone synthesis genes (*iaa* and *ipt*) in tumor formation. Infection of either *ipt* or *iaa* alone could not induce tumors but, when combined with *6b*, each could increase the tumorigenic ability in the stem of tobacco (*Nicotiana rustica* and *N. tabacum*). Similar enhancing effects of *ipt* and *iaa* genes on 6b action were also observed by Canaday et al. (1992).

As one possible interpretation of these synergistic interactions, it was suggested that 6b may have auxin-like activity because the *iaa* gene interacts similarly with *ipt* in inducing tumor formation. However, the introduction of 6b does not necessarily mimic the auxin phenotype in various experimental systems. For example, 6b did not induce roots on *Kalanchoe* stems when infected, as did the *iaa* genes by increasing auxin content (Canaday et al. 1992; Hooykaas et al. 1988). Ectopic expression of 6b causes various developmental alterations such as enations (secondary leaf blades arising along the veins of the abaxial leaf side), curly or tubular leaves, and altered morphology of flowers (see Fig. 3 for examples of morphological changes). However, these phenotypic changes were not observed when *iaa* genes (*iaaM* and *iaaH*) were expressed in *N. tabacum* (Grémillon et al. 2004; Sitbon et al. 1992).

In auxin-dependent rolABC-based rooting assays in *N. rustica*, 6b could not replace the exogenous auxin for

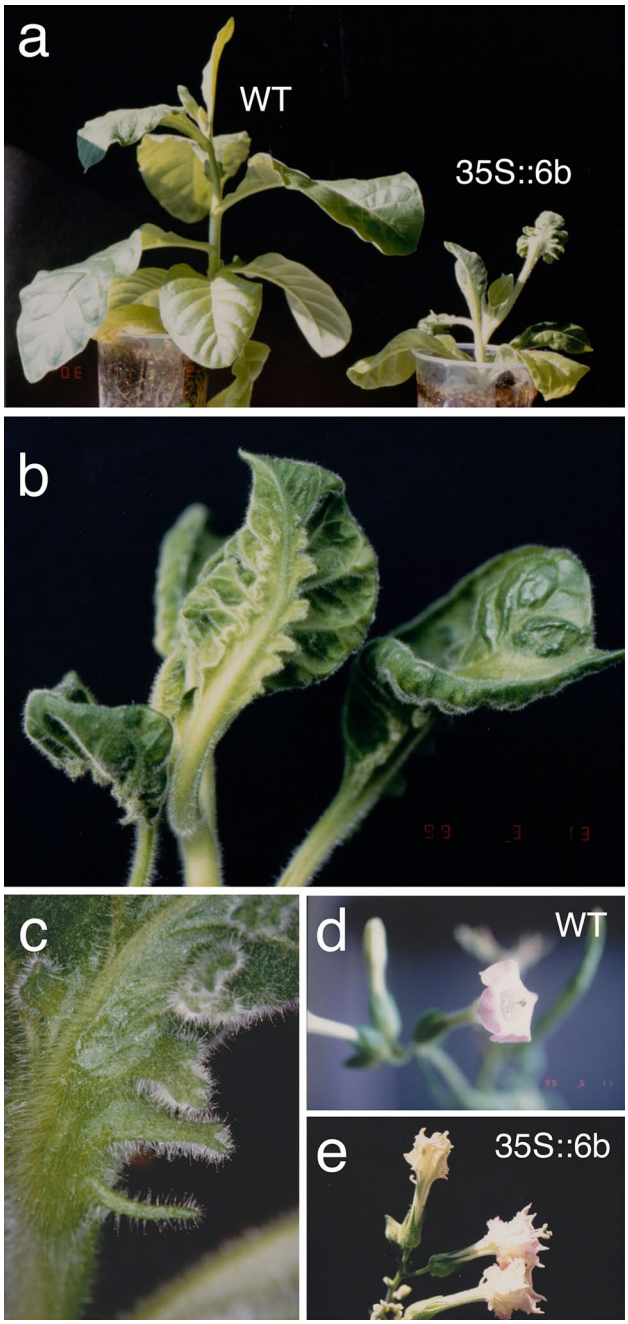

Fig. 3 Abnormal morphogenesis of 6b-expressing plants. **a** Comparison of plant architecture between wild-type and 35S::6b tobacco (*N. tabacum*). The 35S::6b tobacco plants typically show the dwarfed phenotype. **b** Abnormal leaf development in 35S::6b tobacco plants. The 6b-expressing plants show curly or tubular leaves with abnormally long petioles. **c** Enation-like syndrome occurring on the 35S::6b tobacco leaves. The strong phenotype induced by 6b expression is associated with secondary leaf blades arising along the veins of the abaxial leaf side. **d**, **e** Comparison of flower morphology between the wild-type (**d**) and 35S::6b (**e**) tobacco plants

growth inhibition of rolABC-transformed roots (Tinland et al. 1990). Instead, considering the observation that 6b enabled the growth of rolABC-transformed roots at a high auxin concentration, which normally inhibits root growth, these authors proposed that the role of 6b is to reduce the negative effects caused by high concentrations of auxin. Finally, in transgenic *N. tabacum* in which 6b was conditionally expressed, 6b showed an auxin-like effect that promoted the expansion of leaf discs. However, 6b-expressing leaf discs showed neither an increase of IAA content nor induction of IAA-responsive genes (Clément et al. 2006). Expansion of the leaf discs might have occurred because of the increase in osmolyte concentration induced by 6b. Another study investigated the roles of 6b in polar auxin transport and concluded that basipetal auxin transport in the hypocotyl was inhibited by 50–80 % when 6b was expressed in *N. tabacum* (Kakiuchi et al. 2006). It was proposed that this inhibition is due to the accumulation of phenolic compounds whose metabolism is affected by 6b (Gális et al. 2004).

The cytokinin-related roles of 6b have also been proposed by several other authors (Spanier et al. 1989; Wabiko and Minemura 1996). Spanier et al. (1989) observed that inductive effects of cytokinin on shoot regeneration were reduced by the introduction of 6b into *N. tabacum* leaf discs. The results led the authors to hypothesize that the action of the 6b product might be to reduce the level of cytokinin itself or to lower the sensitivity toward this hormone. Inconsistently, it was shown that 6b alone could induce shooty calli in *N. tabacum* leaves and that leaves from the regenerated plants showed a strong ability to generate shoots spontaneously in the absence of exogenously supplied cytokinin (Wabiko and Minemura 1996). However, cytokinin levels in 6b-expressing shooty calli did not differ greatly from those in the normal shoots, clearly indicating that cytokinin synthesis is not the main role of 6b.

Despite a lot efforts made for elucidating the relationship of 6b to auxin and cytokinin, these studies have not always produced consistent results, making it difficult to determine the involvement of 6b in hormone-related processes. It should be noted that most of the above-mentioned studies of 6b were performed before the recent progress in hormone studies, mainly during the past decade, which uncovered the molecular mechanisms of hormone perception, signaling, and transport (Shan et al. 2012). These studies have also provided the various molecular tools used to analyze and manipulate hormone signaling. Future approaches based on these new procedures and knowledge should lead to breakthroughs in the understanding of whether and how 6b might be linked to the molecular components of hormone signaling.

Possible functions in transcriptional regulation

Reprogramming generally requires marked changes in cell status, such as cell identity and division activity, and is

therefore likely to involve altered expression of numerous genes. Several lines of evidence in different plant species support the idea that the role of *6b* and other *plast* genes may be to modify the transcription program in host nuclei to facilitate reprogramming of plant cells.

Expression of 6b in *N. tabacum* under the strong cauliflower mosaic virus (CaMV) 35S promoter ectopically upregulated class I *KNOX* homeobox genes in mature leaves (Terakura et al. 2006). Corresponding to these effects of 6b on shoot regeneration, class I *KNOX* genes are generally expressed in undifferentiated cells in the meristem and can induce ectopic meristems in leaves (Tamaoki et al. 1997). Therefore, one possible explanation is that 6b may affect the expression of class I *KNOX* genes, which may help induce the conversion from differentiated cells into meristematic cells. Class I *KNOX* genes were ectopically expressed in mature leaves of tomato (*Lycopersicon esculentum*) expressing ORF13 (Stieger et al. 2004), suggesting that the inductive effect on class I *KNOX* genes may be common for other members of the *plast* family and may be conserved in different plant species.

Cell cycle-related genes may also be common targets affected by *plast* gene products. Strong expression of 6b upregulates cell cycle-related genes in mature leaves that are normally inactive (Terakura et al. 2006). These include genes for cyclins (*CYCB1* and *CYCD3;1*) and a Myb transcription factor (*NtmybA2*) that activates *CYCB1* expression, and a gene required for cytokinesis (*NACK1*). Similar upregulation of cell cycle-related genes was also reported in tomato plants that express ORF13 (Stieger et al. 2004). Correspondingly, the effects of 6b expression on cell division are apparent in transgenic *N. tabacum* plants, which have leaves with additional layers of small cells and enation-like protrusions. Similarly, upregulation of cell cycle-related genes correlates well with increased cell proliferation in ORF13-expressing tomato plants, which was suggested by an increase in the number of histone H4-expressing cells in shoot apical meristems and accelerated formation of leaf primordia (Stieger et al. 2004).

Other *plast* gene products, rolB and rolC, have also been studied for their effects on gene expression. Cecchetti et al. (2007) compared gene expression in protoplasts isolated form wild-type and *rolB*-transformed *N. tabacum* leaves, and identified the cDNA named *ROX1* as having a putative function of repressing stamen growth partly by inhibiting cell division. The authors suggested that ROX1 may act as a downstream effecter of rolB and may alter flower morphology. Another series of reports focused on the effects of *rol* genes on the activation of antioxidant genes (Bulgakov et al. 2012; Shkryl et al. 2010). Expression of either *rolB* or *rolC*, driven by the CaMV35S promoter, reduced the cellular production of reactive oxygen species (ROS) and concomitantly upregulated the genes encoding ROS-detoxifying enzymes, such as cytosolic ascorbate peroxidase, catalase, and superoxide dismutase. ROS may play dynamic roles in developmental programs (Tsukagoshi et al. 2010), and a reduced ROS level correlates with specific developmental abnormalities such as reduced apical dominance, abnormal flower development, parthenocarpy, and curled leaflets (Sagi et al. 2004), which, in part, resemble those observed in plants expressing *rol* genes (Nilsson and Olsson 1997).

More global gene expression analysis was performed in *Arabidopsis thaliana* (henceforth Arabidopsis) plants expressing 6b fused to the glucocorticoid receptor. Nuclear localization of the fusion protein and its biological effects, such as induction of callus-like cell mass, are both dependent on the presence of dexamethasone (DEX). This analysis showed that nuclear importation of the 6b protein is essential for its biological activities, which suggests a role of 6b in nuclear events such as gene expression (Terakura et al. 2006). In the microarray analysis, application of DEX resulted in marked downregulation of many auxin-responsive genes such as AUX/IAA, which acts as a negative regulator of auxin-inducible transcription mediated by auxin response factors (Terakura et al. 2007). The ACC synthase genes, which are involved in ethylene biosynthesis in response to auxin, were also identified as downregulated genes. The modified expression of auxin-responsive genes might be related to the roles of 6b in auxin-related processes, as proposed by earlier studies of *plast* genes (see above). Downregulation was observed as early as 6 h after induction of 6b, suggesting that 6b may participate in transcriptional regulation by acting on the immediate downstream targets such as AUX/IAA. At present, there is no published information about whether induction of 6b immediately affects other potential targets, such as class I *KNOX* genes and cell cycle-related genes. A follow-up report is needed to provide additional information on the genome-wide responses caused by 6b and the physical interactions between 6b and the potential target genes in vivo.

In accordance with the effects of 6b on gene expression, 6b interacts with nuclear proteins that may function in transcriptional regulation (see below, Kitakura et al. 2002, 2008). It should be noted that rolB–GFP fusion protein can also localize to the nuclei of *N. tabacum* cells (Moriuchi et al. 2004). It may be interesting to determine whether the observed gene expression changes reflect the direct effects of 6b and other *plast* gene products on target genes or the indirect consequence of phenotypic alterations.

Proteins interacting with 6b

To understand the molecular functions of 6b, several attempts have been made to identify the proteins that

interact with 6b and other *plast* gene products. Corresponding to their effects on gene expression, some reports have suggested mechanisms through which 6b may have direct effects on host proteins and thereby control transcriptional and posttranscriptional programs in host nuclei. Another study also suggested that 6b may play unexpected roles by affecting the epigenetic status of host chromatin, as described below.

NtSIP1, a trihelix family transcription factor

A series of studies using the yeast two-hybrid system have identified proteins that bind to 6b from *N. tabacum* (Kitakura et al. 2002, 2008; Terakura et al. 2007). One of the identified proteins, NtSIP1 (for *Nicotiana tabacum* 6b–interacting protein 1), is a putative transcription factor of the trihelix family. The proteins of this family contain a plant-specific DNA-binding domain with a trihelical structure that is conserved among different plant species (Kaplan-Levy et al. 2012). Consistent with its potential roles in transcriptional regulation, NtSIP1 protein, with its putative nuclear-localization signals, is present in the nuclei of *N. tabacum* cells and assists in 6b protein localization to nuclei. The 6b protein does not have a conserved DNA-binding domain but instead has the ability to activate transcription of a reporter gene when transiently expressed as a fusion with the GAL4 DNA-binding domain in *N. tabacum* cells (Kitakura et al. 2002). The C-terminal acidic region of 6b is required for binding to NtSIP1 in yeasts, transcriptional activation in *N. tabacum* cells, and induction of shoot-bearing calli in the absence of plant hormones. Therefore, it was suggested that 6b acts as a transcriptional coactivator and regulates its target genes with the aid of the potential DNA-binding ability of NtSIP1 to exert its effects on reprogramming.

NtSIP2, a nucleolar protein similar to transposase

Another 6b-interacting protein, NtSIP2, was found to be weakly homologous to the Ptta/En/Spm family of plant transposases (Kitakura et al. 2008). Although the biological significance of the observed similarity in the protein sequence is unclear, NtSIP2 localizes to nuclei, particularly to nucleoli, and can interact with both 6b and NtSIP1 in a yeast two-hybrid assay. Therefore, one may speculate that NtSIP2 may affect the properties of the predicted NtSIP1–6b complex in nucleoli. From this viewpoint, it is noteworthy that the 6b protein is present in both the subnucleolar area and nucleoplasm. In the Arabidopsis genome, two genes, AT1G40087 and AT3G30200, are annotated as Ptta/En/Spm family plant transposases and show weak similarity to NtSIP2. However, at present, there is no information about the biological activity of these Arabidopsis genes. For further studies of this protein, it would be interesting to test whether the homologous proteins in Arabidopsis also interact with 6b and whether they play significant roles in mediating 6b-induced morphological alterations and hormone-independent callus formation through their loss-of-function mutations.

AGO1 and SERRATE, key components of the miRNA pathway

Interactions between 6b and NtSIP1, a trihelix family protein, suggest a direct role of 6b in controlling gene expression at the transcriptional level. Wang et al. (2011) proposed that the microRNA (miRNA) pathway, which provides the machinery for posttranscriptional regulation, may be a target of 6b. Using in vitro pull-down and in vivo coimmunoprecipitation assays, they showed that 6b interacts with AGO1 and SERRATE (SE), which are key components of miRNA-mediated posttranscriptional regulation (Mallory and Bouché 2008). It was also shown that expression of 6b under the strong CaMV35S promoter causes defects in the miRNA pathway in Arabidopsis, leading to decreased levels of miRNAs such as miR162, miR164, and miR319. Correspondingly, 6b-expressing plants show elevated levels of the known targets of these miRNAs, including *DCL1*, *CUC1*, and *NAC1* (Wang et al. 2011). Strongly serrated leaves, one of the phenotypes of 6b-expressing plants, were phenocopied by loss-of-function mutations of *AGO1* and *SE*, suggesting that the effects of 6b on plant morphology are partially mediated by the miRNA pathway that is impaired by 6b. It should be also noted that 6b-induced changes in the levels of miRNAs and their target transcripts did not necessarily overlap with those in the *se* mutant, suggesting the need for further careful comparison of the changes in gene expression. The authors also showed that 6b could interact in vitro with host ADP-ribosylation factor and ADP-ribosylate SE, although 6b-induced ADP ribosylation was not confirmed in vivo (Wang et al. 2011).

Histone H3

The search for 6b-interacting proteins using yeast two-hybrid screening has also identified histone H3, one of the core histones comprising the histone octamer (Terakura et al. 2007). This finding and further analysis led to the unexpected conclusion that 6b has a histone chaperone activity that generally assists nucleosome assembly and disassembly by binding directly to core histones. The histone octamer comprises two molecules each of histones H2A, H2B, H3, and H4, and forms the core particle of the nucleosome, the basic repeating unit of chromatin, together with 147 base pairs of DNA wrapped around it. Because

histones are highly basic proteins that interact strongly with negatively charged DNA, histone chaperones are required to shield their positive charge to facilitate the correct transfer to DNA by avoiding unwanted interactions with DNA. The core histones comprise an N-terminal tail and a C-terminal histone fold. The N-terminal tail is known as the target of various posttranslational modifications, whereas the histone fold at its C-terminus is involved in interactions with other core histones in the formation of nucleosomes. *In vitro* interaction assays have shown that 6b binds specifically to the C-terminal histone fold of histone H3 but not to the N-terminal tail or to other core histones.

The interaction between 6b and histone H3 was confirmed in vivo by bimolecular fluorescence complementation assays in *N. benthamiana* and by coimmunoprecipitation in *N. tabacum* cells (Terakura et al. 2007). Consistently, 6b protein is copurified in the chromatin fraction from *N. tabacum* cells, probably because of the salt-sensitive weak electrostatic interaction with chromatin. Supercoiling assays and digestion by micrococcal nuclease have shown that 6b can mediate assembly of the nucleosomes on DNA molecules in vitro (Terakura et al. 2007). The C-terminal acidic region of 6b is required for the induction of hormone-independent callus formation, binding to histone H3, and histone chaperone activity, suggesting that the effects of 6b on plant cell division are dependent on the histone chaperone activity of 6b. This finding accords with the observed alteration in the gene expression profile in 6b-expressing plants, because histone chaperone activity can influence gene expression at the genome-wide scale by modulating local and global chromatin status (Avvakumov et al. 2011).

Possible functional diversification of 6b and other *plast* gene products

Considering the similarity in amino acid sequences and biological activities, one may expect different members of the *plast* family to share some common molecular functions. However, such commonalities in molecular functions have not been reported for any members of the *plast* family. Earlier studies had proposed that rol proteins may have enzymatic activities, such as glucosidase (Estruch et al. 1991a, b) and tyrosine phosphatase (Filippini et al. 1996). However, this has not been confirmed and has since been called into question (Nilsson and Olsson 1997). Similar to the case of 6b, some reports identified proteins that interact with the products of *ORF13* and *rolB*, which differ from those that interact with 6b (Moriuchi et al. 2004; Stieger et al. 2004). Therefore, the identified protein–protein interactions may be specific to the corresponding subfamily of the *plast* gene family. At present, it is not clear whether members of the *plast* family share a common biological pathway that is targeted by these proteins.

ORF13 interacts with retinoblastoma-related protein (RBR)

Overexpression of ORF13 causes marked alterations in plant development and morphogenesis, including formation of a callus in carrot (*Daucus carota*) discs (Fründt et al. 1998). The ORF13 product was reported to interact with RBR protein in tomato and to modify the expression of genes related to cell proliferation (Stieger et al. 2004). It is well known that RBR plays central roles in cell proliferation and differentiation through inhibitory interaction with the E2F/DP heterodimer, which activates transcription of various cell cycle-related genes (Gutzat et al. 2012). Therefore, inhibition of host RBR seems to be an elaborate strategy for pathogens to induce local cell proliferation at the site of infection. The Rb protein is a target of mammalian DNA tumor virus oncoproteins, such as simian virus 40 large-T antigen and adenovirus E1A (DeCaprio et al. 1998; Whyte et al. 1998). Some geminiviruses, known as viral plant pathogens, produce the viral replication protein RepA, which interacts with and possibly inhibits the host RBR and induces a cellular environment that is amenable to viral replication (Gutierrez 2000). Therefore, inhibitory interaction with RBR is an attractive explanation for the effects of *plast* genes on the induction of plant cell division. The LXCXE motif, which is responsible for ORF13–RBR interaction, is absent in proteins encoded by other members of the *plast* family (Helfer et al. 2002), which suggests that this interaction is specific to the ORF13 subfamily.

RolB interacts with 14-3-3 proteins

Yeast two-hybrid screening of a *N. tabacum* cDNA library identified a 14-3-3 protein, Nt14-3-3ωII, which interacts with rolB protein (Moriuchi et al. 2004). The 14-3-3 proteins belong to a conserved protein family found in all eukaryotes that can generally physically interact with cellular proteins with various functions (Denison et al. 2011). Although rolB protein does not have common recognition motifs for binding to 14-3-3 proteins, its binding to Nt14-3-3ωII in vivo was confirmed in *N. tabacum* cells expressing tagged rolB protein. Mutational analysis suggested that binding to the 14-3-3 protein is required for both its nuclear localization in *N. tabacum* cells and full activity for generating hairy roots from *N. tabacum* leaf segments (Moriuchi et al. 2004). However, the amino acid sequence required for the interaction is specific to rolB and is not conserved in other *plast* gene products, which makes it unlikely that the

14-3-3 protein is the common molecular target affected by other members of the *plast* family.

Mode of action of 6b for reprogramming

6b may directly target transcription factors

The discovery of NtSIP1, a trihelix transcription factor, as the interacting partner of 6b led to the proposed model of 6b action in which 6b binds directly to transcription factors and controls their activity, thereby influencing the gene expression program. A similar mode of action is known for the bacterial phytopathogens, *Xanthomonas* species, which express the effecter protein called TAL (transcription activator-like). After delivery into host cells, TAL binds to specific promoter sequences and by itself can directly activate gene expression related to bacterial infection and disease symptoms (Boch and Bonas 2010). XopD, which is expressed in *X. campestris*, is another example of a pathogenic effecter with a similar mode of action to that of 6b in the control of host transcription. It has been shown recently that XopD can target one of the Myb transcription factors, MYB30, in Arabidopsis (Canonne et al. 2011). MYB30 activates the production of very long-chain fatty acids (VLCFAs) and thereby induces defense and cell death-related responses (Raffaele et al. 2008). When transferred to the host nuclei, XopD interacts with MYB30 and inhibits the activation of VLCFA-related genes to suppress the plant defense response (Canonne et al. 2011). Therefore, binding to and hijacking host transcription factors may be one major strategy of pathogens that is effective in influencing host cells and facilitating the establishment of pathogenic interactions.

This line of debate about 6b function requires more knowledge about the role of NtSIP1 in plant growth and development, apart from its interaction with 6b. The trihelix protein family contains 30 members in Arabidopsis, which are classified structurally into five subgroups. One subgroup, called the SIP1 clade, contains NtSIP1 and 12 Arabidopsis proteins (Kaplan-Levy et al. 2012). In recent studies, two closely related SIP1 group genes, 6b-interacting protein 1-like 1 and 2 (ASIL1 and ASIL2), were shown to repress the expression of late embryo development genes in seedlings, thereby establishing the phase transition from embryogenesis to vegetative growth (Gao et al. 2009). ASIL1 specifically recognizes a GT box-like promoter motif that is frequently observed in various plant genes. Another member of the SIP1 group that is more distantly related to NtSIP1, FRIGIDA-INTERACTING PROTEIN2 (FIP2), was identified as a protein that interacts with FRIGIDA (FRI), which positively regulates the expression of FLC, a key repressor of flowering in Arabidopsis (Geraldo et al. 2009). These reports on the trihelix proteins in the SIP1 clade do not identify an immediate link between their biological functions and 6b-induced morphological alteration and reprogramming. This may be because only a few trihelix proteins have been studied in some detail, and further systematic investigations are awaited. It will be interesting to test genetically whether some members of the trihelix family mediate the effects of 6b on plant morphogenesis in Arabidopsis. The genome-wide identification of target genes regulated directly by NtSIP1 would also help increase the understanding of the physiological functions of NtSIP1 as well as its potential roles in mediating 6b actions toward reprogramming.

Histone chaperones as critical players in reprogramming

The formation of nucleosomes and higher-order structures contributes to the packaging of long DNA into chromatin and to making the DNA molecule inaccessible to various cellular proteins, including transcription factors. The status of chromosome condensation is not uniform along the entire chromosome, which, instead, comprises euchromatin with a decondensed and open structure and highly condensed heterochromatin (Misteli 2007). It is well known that the local condensation/decondensation status of chromatin is a critical determinant of transcriptional activity and that this is largely dependent on covalent histone modifications, such as acetylation, methylation, and ubiquitination. In addition to histone modification, histone chaperones can affect the epigenetic status of chromatin mainly by replacing canonical histones with histone variants, which differ in their amino acid sequences from the canonical histones and are known for all core histones except for the H4 family (Skene and Henikoff 2013).

In contrast to the canonical histones, which are expressed specifically in the S phase of the cell cycle and are deposited into nucleosomes in a replication-dependent manner, histone variants are incorporated into nucleosomes in a replication-independent manner. Histone chaperones are responsible for the replication-independent deposition of histone variants and can influence epigenetic chromatin status by two main mechanisms: (1) deposition of newly synthesized histone variants that can erase the covalent modification of nucleosomal histones, and (2) specific histone variants with distinct properties that differently influence the local epigenetic status of chromatin when incorporated into nucleosomes.

Variant histones in the H2A and H3 classes in Arabidopsis have been studied thoroughly, and the genome-wide correlations between their enrichment and gene expression levels have been investigated. A variant of H2A, H2A.Z, is enriched at the transcriptional start sites of actively transcribed genes, and enrichment of this variant in gene bodies correlates with gene responsiveness (Coleman-Derr and

Table 1 Chaperones for specific histone variants and their homologs in *Arabidopsis*

Histone variants	Chaperone complex	Human protein	*Arabidopsis* homologs		
			Gene	Protein name	Reference
H3.1	CAF1 complex	p150	AT1G65470	FAS1	For review see, Ramirez-Parra and Gutierrez (2007)
		p60	AT5G64630	FAS2	
		p48/RbAp48	AT5G58230	MSI1	
H3.3		HIRA	AT3G44530	HIRA	Nie et al. (2014)
H3.3		ASF1A, ASF1B	AT1G66740	ASF1A	Zhu et al. (2011)
			AT5G38110	ASF1B	
H3.3	DAXX-ATRX	ATRX	AT1G08600	ATRX	Wollmann et al. (2012)
		DAXX	NF		
H3.3		DEK	AT3G48710	AtDEKA	Otero et al. (2014)
			AT5G63550	AtDEKB	
			AT4G26630	AtDEKC	
			AT5G55660	AtDEKD	
H2A.Z	SWR1/SRCAP		SWR1-like complex genes (see reference)		For review see, March-Díaz and Reyes (2009)
H2A.Z		NAP1	AT4G26110	AtNAP1; 1	Liu et al. (2009)
			AT2G19480	AtNAP1; 2	
			AT5G56950	AtNAP1; 3	
			AT3G13782	AtNAP1; 4	
			AT1G74560	NRP1	Zhu et al. (2006)
			AT1G18800	NRP2	
H2A.X	FACT	SPT16	AT4G10710	SPT16	Duroux et al. (2004)
		SSRP1	AT3G28730	SSRP1	

NF not found

Zilberman 2012). H2A.W, another type of H2A variant, marks heterochromatin and maintains transposon silencing (Yelagandula et al. 2014). A variant type of histone H3, H3.3, tends to localize to actively transcribed genes, especially at the 3′ end of such genes (Stroud et al. 2012).

Typically, each histone variant has a corresponding dedicated chaperone that acts in its deposition into chromatin. Arabidopsis has homologs that correspond to some, but not all, such chaperones (for review see Zhu et al. 2012), such as the chromatin assembly factor 1 (CAF1) complex (Ramirez-Parra and Gutierrez 2007), histone regulatory homolog A (HIRA) (Nie et al. 2014), SWR1 chromatin remodeling complex (March-Díaz and Reyes 2009), nucleosome assembly protein 1 (NAP1) (Liu et al. 2009), NAP1-related protein (NRP) (Zhu et al. 2006), and facilitate chromatin transcription (FACT) (Duroux et al. 2004) (Table 1). It should be noted that 6b binds to both the canonical histone H3 and H3.3 variants in a yeast two-hybrid assay and therefore may potentially possess the ability to deposit both types of H3 histones (Terakura et al. 2007).

Reprogramming is generally regarded as the process of reacquiring a pluripotent or totipotent state in differentiated cells that are committed to a particular lineage. Reprogramming of animal cells is typically induced by the expression of the "Yamanaka factors" (Oct4, Sox2, Klf4, and c-Myc) or by transfer of a nucleus into an enucleated egg or oocyte. Studies of isolated nuclei transferred into *Xenopus* oocytes have shown that reprogramming accompanies the deposition of oocyte-derived H3.3 into chromatin and that the H3.3 incorporation at the *Oct4* locus is required for the reprogramming of the isolated nuclei (Jullien et al. 2012). Global replacement of histone variants was also reported for H2A variants in mouse zygotes (Nashun et al. 2010) and for H2A and H3 variants in nuclei transferred to enucleated mouse oocytes (Nashun et al. 2011). More recent study investigated the roles of other histone variants, TH2A and TH2B, which are highly expressed in oocytes and contribute to activation of the paternal genome after fertilization (Shinagawa et al. 2014). Expression of TH2A and TH2B increases the generation of pluripotent cells induced by the Yamanaka factors and can induce reprogramming even without Sox2 and c-Myc (Shinagawa et al. 2014). Taken together, these reports suggest that the dynamic replacement of histone variants that normally occurs in the zygote is critical for reprogramming of somatic cells.

The possibility that histone variants are involved in reprogramming of plant cells has also been suggested. Recent studies in Arabidopsis have emphasized the importance of H3.3 in reprogramming. Wollmann et al. (2012) examined the dynamics of H3.3 enrichment during leaf development, when actively proliferating cells in the undifferentiated state undergo developmental transition into terminal differentiation and cessation of cell division. The authors concluded that the developmental gene activation and repression are linked to an increase and decrease in H3.3 variants, respectively. The authors also suggested that the resetting of covalent histone modification by replacement with H3.3 is associated with global gene expression changes during the transition from the undifferentiated to terminally differentiated state (Wollmann et al. 2012). Another recent study, investigating early events after fertilization, showed rapid replacement of H3.3 variants in zygotes (Ingouff et al. 2010). Both maternally and paternally inherited H3.3 variants were rapidly removed from zygotic nuclei because of an active process that was independent of DNA replication. This finding suggests that the replacement of H3 variants may reset the covalent modification of H3, which may then affect the global epigenetic status and contribute to acquisition of zygotic totipotency (Ingouff et al. 2010).

In animals, replacement of H3.3 requires HIRA, which forms a complex with calcineurin-binding protein (CABIN), ubinuclein 1 (UBN1) and UBN2, and another histone chaperone, ASF1 (Amin et al. 2012; Balaji et al. 2009; Tagami et al. 2004). Arabidopsis contains homologs for a full set of these components, which may similarly form a protein complex, as suggested by the protein–protein interactions and colocalization observed in vivo (Nie et al. 2014). Similar to the animal counterparts, HIRA also binds to H3.3 in Arabidopsis (Nie et al. 2014). In *Drosophila*, HIRA and yemanuclein, a UBN1 homolog, are essential for replication-independent incorporation of H3.3 into chromatin. The HIRA complex is essential for replication-independent incorporation of H3.3 into the male pronucleus after fertilization in *Drosophila* (Loppin et al. 2005). However, in Arabidopsis, HIRA is not required for fertilization and early embryogenesis, suggesting that H3.3 replacement in the zygote may depend on other H3.3 chaperones. Instead, HIRA may play a role in dedifferentiation of protoplasts isolated from Arabidopsis roots (Nie et al. 2014). Loss of HIRA by mutation resulted in significant reduction of the number of genes that are down- or upregulated during dedifferentiation, which suggests that HIRA-dependent H3.3 incorporation may affect epigenetic status and contribute to transcriptional reprogramming during dedifferentiation of plant cells.

Concluding remarks and perspectives

Various mechanisms responsible for the actions of *plast* genes have been proposed based on different experimental approaches. In part, this may reflect the divergence in the members of the *plast* family, which may have effects specific to the corresponding subfamily. 6b has a unique protein structure—the C-terminal acidic regions—which is essential for its interaction with NtSIP1 and NtSIP2, and for its histone chaperone activity (Kitakura et al. 2002, 2008; Terakura et al. 2007). Because the presence of this region is required for its biological activities, 6b and other *plast* gene products may target different molecules in plant cells, thereby affecting distinct biological pathways. Considering the pleiotropic effects of individual members, each member of the *plast* family can also be considered to have multiple molecular functions in promoting parasitic interactions. Loading multiple functions into one effecter protein may be the common strategy of phytopathogens for achieving a successful interaction with host cells (Métraux et al. 2009), especially to increase the morphogenic effects of T-DNA that encodes only a small number of proteins in *Agrobacterium* species.

In both animals and plants, reprogramming is closely associated with deposition and replacement of histone variants (Skene and Henikoff 2013). In both cases, a mechanistic relationship has been suggested between naturally occurring reprogramming in zygotes and induced reprogramming of somatic cells. Recent studies have suggested that H3.3 variants are particularly important for reprogramming in plants (Ingouff et al. 2010; Nie et al. 2014; Wollmann et al. 2012). Together with the finding that 6b with histone chaperone activity binds to both H3.1 and H3.3, it is tempting to speculate that replacement of H3.3 may affect global gene expression changes, leading to the acquisition of totipotency, in zygotes after fertilization and in somatic cells upon external stimulation. Local changes of epigenetic status may also be achieved by recruitment of the histone chaperones to the target loci via the interaction with specific DNA-binding proteins, as observed for the interaction between 6b and NtSIP1 (Kitakura et al. 2002). Following this line of argument, it would be of great interest to study the effects of 6b on the distribution of H3 variants across the Arabidopsis chromosomes.

The mechanisms responsible for the formation of calli have long been one of the central enigmas in plant physiology. Although still fragmentary, accumulating evidence suggests that changing the epigenetic status across the genome is associated with this reprogramming process (Perianez-Rodriguez et al. 2014). This association is illustrated most clearly by the spontaneous callus formation upon mutations in specific genes involved in epigenetic

regulation, such as histone modifiers and chromatin remodeling factors (Ikeuchi et al. 2013). The identification of 6b as a histone chaperone provides additional evidence for the involvement of epigenetic regulation in reprogramming and sheds light on the dynamics of histone variants as an additional layer of epigenetic regulation that may be important for reprogramming during developmental transitions, callus formation, and formation of crown gall tumor induced by *Agrobacterium* oncoproteins.

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