

# IN VITRO CULTURE OF ORCHIDS: THE ROLES OF CLASS-1 KNOX GENE IN SHOOT DEVELOPMENT

## A REVIEW

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## ABSTRACT

*In vitro* culture of orchids has been developed for many purposes. Some native orchids and commercial orchid hybrids are propagated using seed germination or cut explants such as leaves, shoot tips, and roots to produce large numbers of orchid plantlets. This technique is widely used for the purpose in conservation of natural orchid species and industry of commercial orchid hybrids. However, the molecular genetic mechanism behind growth and development of these orchids during *in vitro* culture is still unclear, and needs to be elaborated. Recent advanced in transgenic technology in orchid is very helpful for studying the mechanism of action of key genes in various stages of orchid development during *in vitro* culture. In this review, an attempt to understand the role of class-1 KNOX gene and its relationship with other genes in the initiation of shoot apical meristem (SAM) for shoot development from orchid protocorm (a tubercle of developing orchid embryo) and PLBs (Protocorm Like Bodies) during *in vitro* culture will be discussed. It will answer the question about how the shoot formation can be controlled during growth and development of orchid cells in *in vitro* culture.

*Key words: In vitro, orchids, shoot development, KNOX, transgenic*

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## INTRODUCTION

Orchids are members of *Orchidaceae*, which is one of the largest families among flowering plants (Dressler 1993; Arditti 1992). Some species of orchids exhibit beautiful flowers, so they were developed for commercial purposes (Hew & Yong 1997). Wide range of flower colors, size and shapes, year-round availability, and long flowering life by several weeks to month are the prime attraction of this genus (Kuehnle 2007). However, many people trade in orchids for various purposes, for instance to be collected as a pot plant, or made wreaths for outdoor and indoor decoration in ceremonial events, that caused over collecting of the native orchid plants from their natural habitat in the forests. Therefore orchid species are under major threat worldwide due to over exploitation by collectors and enthusiasts. Existing regulations have set the orchid trade, which people are not allowed to sell orchid straight from the forest. For commercial use, one must do the multiplication first to produce mass quantities of plants, then the propagated plants/ plantlets can be sold in the form of orchid hybrids (Irawati 2002). However, recently, the orchid consumers change their habits; they prefer to buy native orchid species, instead of orchid hybrids. This will certainly threaten the existence of orchid species in nature. Therefore, conservation management for commercial purposes as well as the preservation of the existing plants should be made, i.e mass propagation of valuable orchids and flowering induction to generate much amount of flowers, for both cross pollination to get the next generation and producing a lot of cut flowers for flower arrangements for decoration. The efficient orchid transformation system (Yu et al. 2001; Belarmino & Mii 2000; Semiarti et al. 2007; 2010; 2014) will facilitate studies on gene function, that helpful to improve the quality and quantity of orchid plants to get excellent traits.

## PLANT TISSUE CULTURE (*IN VITRO* CULTURE)

Plant tissue culture or *in vitro* culture is a technique to grow cells, tissues, organs on artificial medium with aseptic condition and appropriate physical conditions in a culture flask (George & Sherrington 1984; Hussain et al. 2012). The basic concept is a plant cell has autonomous ability to conduct metabolism for their growth and life and totipotent in which the cell can regenerate into a whole plant (George et al. 2008). It means that plant can be dissected into smaller parts termed as explants and under the appropriate condition the explants can be developed into a whole plant. It is a promising method for applied plant science, including agriculture and plant biotechnology (Hew & Yong 2007). Theoretically, all plants cell possesses the genetic information and cellular machinery necessary to generate the whole organism. Using this technique, mass number of plants that are genetically identical to parental plant can be produced. Two concepts, plasticity and totipotency, are the central processes to understand the regeneration in plant cell culture (George & Sherrington 1984). Plant growth regulators (PGR's) play an essential role in determining the development pathway of plant cells and tissues in culture medium (Naing et al. 2011). The type and the concentration of PGR's used depend mainly on the species of the plant, the tissue or organ cultured and also the objective of the experiment. The high concentration of auxin generally favors root formation, whereas the high concentration of cytokinin promotes shoot regeneration. A balance of both auxin and cytokinin leads to the development of mass of undifferentiated cells known as callus.

Mass propagation of orchid is possible by producing millions of plantlets using tissue culture techniques. Orchids can also be rapidly propagated through tissue culture techniques by using shoot tips (Saiprasad et al. 2002), leaf (Chen et al. 2001), and stem nodes (Pathania et

al. 1998). Micropropagation of orchids is the most frequently used convenient technique for their exploitation as a major trade in developed countries (Goh & Tan 1982; Sagawa & Kunisaki 1982). Mass propagation of the orchid seedlings *in vitro* could be achieved with a suitable basal medium devoid of growth regulators as orchid seeds have sufficient growth hormones to germinate and develop into seedlings in nature. Moreover, epiphytic species can germinate both in the light and dark and seem to require light only for the induction or improvement of shoot and/or root formation (Arditti 1992).

Two kinds of growth are possible *in vitro*: organized growth and unorganized growth (George & Sherrington 1984). Organized growth occurs either when organized plant parts such as apical meristem of shoots and roots, leaf initials, young flower buds, and small fruits are transferred to culture. Unorganized growth, which is never found in nature, occurs fairly frequently when pieces of whole plants are cultured *in vitro*. In orchid, the seeds are usually very small in size, and generally not equipped with food reserves in the form of endosperm, in nature the seeds will germinate after symbiosis with mycorrhiza fungi (Veyret 1974; Arditti 1992). In order to get the entire plant of orchid from the seed it is necessary to germinate the orchid seeds in the *in vitro* culture system. Since the orchid seeds are lack of endosperm the *in vitro* system provides an artificial nutrition as a medium which is needed for the seeds to germinate and grow to become whole orchid plants (Kumar et al. 2002; Naing et al. 2011). Rare and threatened orchid species are propagated by seeds rather than by vegetative methods (Kumar et al. 2002). The advantages of immature seeds used for micropropagation in tissue culture are included no loss of seeds by sudden natural dehiscence, easy to surface sterilized, increased rate of seed germination, early start by immature seeds and immature seed culture helps in getting seedlings from wide crosses where embryos in mature seeds often get aborted (Parab & Krishnan 2012). Therefore, *in vitro* techniques will immensely aid conservation measures of orchid species.

Under aseptic conditions on artificial medium, orchid seeds will grow into protocorms (Arditti & Ernst 1993; Semiarti et al. 2007; Mercuriani et al. 2012). Protocorm is a tubercle cell mass which then can grow into seedlings and eventually into whole plants (Veyret 1974; Suryowinoto 1984). *In vitro* propagation involving a callus phase is considered rather difficult morphogenetic pathway in orchids (Arditti & Ernst 1993; Kumar et al. 2002; Naing et al. 2011). Thus plant regeneration from orchid culture usually occurs via protocorm. Meanwhile, if a piece of plant as explant cultured aseptically then a compact mass of cells like protocorm emerged, it terms as protocorm like bodies (PLBs) (Arditti and Ernst 1993; Kumar et al. 2002; Naing et al. 2011). Regeneration from seeds via protocorm-like bodies (PLBs) has become the preferential method for the production of orchids. Regeneration of plantlets in orchids through callus usually occurred through PLB

formation, which is an intermediary somatic embryo phase (Saiprasad et al. 2002; Naing et al. 2011). Parab and Krishnan (2012) suggested that callus differentiation into PLBs was found dependent on concentration of cytokinin and auxin used (Arditti & Ernst 1993; Kumar et al. 2002; Naing et al. 2011).

In some orchids, PLBs were converted into plantlets when cultured on medium containing lower concentrations of BAP. Kumar et al. (2002) obtained plantlets from PLBs of *Rhynchosstylis retusa* and *Cymbidium elegans* that cultured on Murashige and Skoog (MS) medium supplemented with 60 mM sucrose, 2.5  $\mu$ M BAP and 2.5  $\mu$ M IAA. On the other hand, PLBs can also be encapsulated in alginate gel beads to form synthetic seeds, which could be subsequently germinated on basal medium supplemented with 0.5  $\mu$ M BAP. Thus, *in vitro* culture can be used as a good tool to study the gene function in growth and developmental process of orchids (Arditti & Ernst 1993).

To grow plant cells and organs *in vitro* properly, the knowledge of the basic mechanisms of plant growth and development is required. Behaviors of plant cells or explants in tissue culture medium are unpredictable (George & Sherrington 1984). Visible manifestation of cell differentiation includes greening of callus, variation in the cell wall thickness, and biogenesis of certain cytoplasmic organelles (George et al. 2008). Differentiation in such tissues involves differences in the basic metabolic pathways. It has been assumed that differentiated plant cells retain their ability to revert to embryogenic condition and generate a complete new plant through somatic embryogenesis or organogenesis (Dey et al. 1998). Very little is known about the molecular mechanism of *in vitro* differentiation. Furthermore, callus cultures of certain plants require external supply auxin and cytokinin in order to maintain cell division. These conditions suggested that cell differentiation involves the activation of certain genes and repression of the others, which control different basic metabolic or anabolic pathways. Since the cultivation of plant material *in vitro* means manipulating the genome of plants, it is important to know the process of what happens with the genome when the cells or organs of plants grown on artificial medium, in a tube (*in vitro*), where the conditions may be very different to their natural condition *ex vitro*. We know that plant traits encoded by a group of genes in polygenic. During its life cycle, plant growth and development consists of three phases, namely, the embryonic phase, the phase of vegetative and reproductive phase (Howell 1998). Each phase was escorted by a group of genes that work together to form specific proteins that are organized to coordinate to form a protein complex that plays a role in producing organs of plants in this phase, and then sequentially will hold a working network with a group of genes in the next phase by inducing the next phase of the group of key genes. Gene products of the next phase will hold a negative feedback suppresses the activity of the gene

pool before. And so on, upregulation of genes will be switched on next gene, and downregulation of genes group will switch off the previous phase. Genes work in spatial and temporal (Howell 1998). Therefore, information about the specific function of the gene would be useful in manipulating plant cells under in vitro conditions. The expression of EMB-1 gene either spatial or temporal is detectable in zygote and somatic embryo as well (Dey et al. 1998). This suggests that normal embryogenesis process is independent of surrounding maternal tissue. Furthermore, RKD4 gene is a novel key regulator of the earliest stage of plant development. RKD4 functions are required after fertilization. Loss of function RKD4 showed embryo-specific developmental defect (Waki et al. 2011). Thus, RKD4 is preferentially expressed in early embryos. Dey et al. (1998) reported that the different approaches to explore the early events of differentiation are mutant analysis, differential screening of transcripts and ectopic expression of regeneration specific genes. Therefore, information about the specific function of the gene would be useful in manipulating plant cells under in vitro conditions, and in vitro culture can be used as a good tool to study the gene function in growth and developmental process of orchids (Arditti & Ernst 1993). Resistance of some orchids to several antibiotic is helpful in transgenic technology using *Agrobacterium tumefaciens*. Mercuriani et al. (2012) reported that *Phalaenopsis amabilis* resistance to Hygromycin phosphotransferase, in which 1 week application of 10 mg/l hygromycin caused the death of 50% protocorms (LD 50). This data indicate that the appropriate concentration of antibiotic can be used for selection of transformant plants after gene transferred in orchid system. Therefore, we can transfer genes using *Agrobacterium* to determine the function of some important gene in orchids.

### KNOX GENES AND ITS FUNCTION IN PLANT

Plant homeobox genes have been expected to function in important developmental process (Jackson et al. 1994; Hake et al. 1995). A maize homeobox gene codes for protein that localizes to the nuclei of cells of the SAM and in all axillary meristems, in terminal and lateral inflorescence meristems and in both male and female floral meristems (Kerstetter et al. 1994; Ritter et al. 2002). In plants, homeobox genes categorized into five class (Chan et al. 1998), one of which is the class1 of the *KNOTTED1-like homeobox (knox)* genes have been detected as transcriptional factors for the maintenance of the SAM and the development of aboveground organs (Yu et al. 2000; Ritter et al. 2002; Scofield et al. 2008). In maize, as well as in other plant species, a number of mutants defected in meristem fate or identity, that exhibit abnormal shoot and inflorescence development, for example teosinte branched1 (tb1) that exhibits excessive branching of shoot (Doebley et al. 1997), and barren stalk1 (ba1) mutant is defective in axillary meristem development (Ritter et al. 2008), tassel seed4 (ts4) that mutant of maize with highly branched

inflorescences (Irish 1997). In *Arabidopsis*, class1-KNOX genes *STM*, *BP/KNAT1*, *KNAT2* and *KNAT6* have been detected play important roles for the function of SAM and carpel development (Scofield et al. 2008), indicates that *KNOX* genes are involved in both vegetative and reproductive development of aboveground organs. Semiarti et al. (2001) showed that accumulation of *BP/KNAT1* transcripts were detected in the leaves of *asymmetric leaves2 (as2)* mutant of *Arabidopsis*, transcripts of the class1-KNOX gene family, which is involved in the formation and maintenance of a meristem state, ectopically accumulate in the mature leaves of *as1* and *as2* mutants. Explants of the *as1* and *as2* leaf mutants produced multishoots from the basal part of the leaves, suggesting that in the absence of AS2 function, the ectopic expression of *KNAT1* gene abnormally initiate numerous adventitious SAM in leaves and produced shoots. This evidence provided the role of *BP/KNAT1* gene on lateral meristem formation and the network that should be occurred between homeobox genes and leaves specific genes (such as *AS2* gene) to maintain the normal plant architecture. *AS1* and *AS2* genes play a role in repressing the expression of the *KNOX* genes in mature leaves, which might cause the maintenance of the determined cell state of leaf cells and is important for the formation of petioles and symmetric leaves (Byrne et al. 2000; Semiarti et al. 2001). *AS1* and *AS2* genes are also involved in the formation of adaxial-abaxial polarity and flat leaf laminas. *AS2* gene is also involved in early development of floral organs. Transcript of *AS2* were detected in inflorescence meristems, floral meristems and primordia of all floral organs of *A. thaliana*, but it decreased in the late stage of floral organ primordia (Keta et al. 2012). Although in *Arabidopsis*, understanding of the function of the class1-KNOX has improved. However, function of the class-1 *KNOX* genes in monocot has still not been so clear, because over expression data does not tell the real function of a gene (Machida 2015, Personal Communication). To understand the genetic regulation in shoot development in orchids, our group used the *Arabidopsis KNAT1* and *AS2* genes as molecular markers in transgenic system of orchids. Windiastri and Semiarti (2009) obtained transgenic *Phalaenopsis amabilis* orchids expressed *Arabidopsis AS2* transgene under the control of 35S CaMV promoter that exhibit dwarf shoots with abnormal leaf shapes, i.e rectangular, trumpet-like, oval, fused leaves, lobed-leaves compared to the lancet shape of leaves in wild type or non transgenic plants. The abnormal phenotype of leaves in transgenic orchids is similar to the phenotype of leaves in *as2* mutant of *Arabidopsis*, although the severe asymmetric leaves was not obtained in orchid. These results suggest that there might be homologous gene of *AS2* in orchid genomes.

In a model plant *Arabidopsis*, roles of leaves genes such as *ASYMMETRIC LEAVES1 (AS1)* and *ASYMMETRIC LEAVES2 (AS2)* in leaf development revealed that the *as1* and *as2* mutants of *A. thaliana* exhibit

1) pleiotropic abnormal phenotypes, including *asymmetric leaf 1/KNOX* gene family, which is involved in the formation and maintenance of a meristem lobes, malformed venation patterns, and downwardly curled leaves, 2) transcripts of the class state, ectopically accumulate in the mature leaves of *as1* and *as2* mutants (Semiarti et al. 2001), 3) AS1 is a nuclear protein that has a myb domain (Byrne et al. 2002), AS2 is a nuclear protein that belongs to the AS2/LOB protein family (Iwakawa et al. 2002), 4) AS1 and AS2 together with other genes (HAT1, HAT2, etc) are involved in the determination of adaxial-abaxial polarity (Terakura et al. 2006). *AS1* and *AS2* genes play a role in repressing the expression of the *KNOX* genes in mature leaves, which might cause the maintenance of the determined cell state of leaf cells and is important for the formation of petioles and symmetric leaves. *AS1* and *AS2* genes are also involved in the formation of adaxial-abaxial polarity and flat leaf laminas. *AS2* gene is also involved in early development of floral organs. Transcript of *AS2* were detected in inflorescence meristems, floral meristems and primordia of all floral organs of *A. thaliana*, but it decreased in the late stage of floral organ primordia (Keta et al. 2012). Based on the result of the work on 35S:STM-GR transgenic Arabidopsis *stm* plants, that STM activation rescued the *stm* mutant phenotype, and resulted in rapid 3-8-fold elevation of *AtIPT7* mRNA levels detected by RT-PCR, Yanai et al. (2005) revealed that in the central zone of the SAM, *KNOX1* proteins induce the expression of cytokinin biosynthesis gene *AtIPT7s* among other targets, causing the accumulation of cytokinin. Activation of the primary cytokinin response gene *ARR5* was also detected, that STM activation resulted in a 2-fold increase of the *ARR5*mRNA levels. Interestingly, that cytokinin can partially rescue the *stm* phenotype. Sakamoto et al. (2001) showed that *KNOX* homeobox protein directly suppresses the expression of a gibberellin biosynthetic gene in the SAM of tobacco. In agreement with Sakamoto et al. (2001), Hay et al. (2002; 2004) found that gibberellin and other phytohormones pathway mediate *KNOTTED1*-type homeobox function in plants with different body plants. Yanai et al. (2005) discovered that the cytokinin levels are reduced by the absence of *KNOX1*, in parallel *KNOX1* protein repress GA biosynthesis. In Arabidopsis, AS1-AS2 protein complex functions in regulation of proximal-distal leaf length by directly repressing 3 members of class 1 *KNOX* homeobox genes (*BP*, *KNAT2*, *KNAT6*) that are expressed in the meristem periphery below leaf primordial (Ikezaki et al. 2010). AS1-AS2 directly represses the abaxial gene, *ETTIN/AUXIN-RESPONSE-FACTOR3* (*ETT/ARF3*), and indirectly represses *ETT/ARF3* and *ARF4* through *tasiR-ARF*. AS1-AS2 acts as a key regulator for the establishment of adaxial-abaxial polarity through the repression of *ETT/ARF3* and *ARF4* (Iwasaki et al. 2013). Therefore, it can be proposed the working model of *KNOX1* proteins are as factors keep cytokinin levels high and GA levels low in SAM. It also can be summarized that

class1 *KNOX* genes are the central balancers of hormone levels to keep indeterminacy of SAM and the maintenance of a stable organization of the meristem, while continuously producing organs from its margins resulting in the growth and development of shoots and aboveground organs of plants. Logically, to induce callus or regenerate plants from explants in *in vitro* culture, we need to understand the genetic regulation that giving treatment phytohormones (cytokinin, auxin, GA and others) in the culture medium is likely to enable the homeobox genes and its receptor activation in explant cells. It means necessary to carefully decide the use of concentration of exogenous phytohormones and type of explants (shoot tips, leaves, stems or roots), due to the biosynthesis of endogenous phytohormones and some genes regulation will always maintained in plant cells.

### KNOX GENES IN ORCHIDS

In orchids, an orchid homeobox gene, *Dendrobium Orchid Homeobox1 (DOH1)* has been isolated from orchid hybrid *Dendrobium Madame Thong-In* (Yu et al. 2000). The gene contains the well-conserved homeodomain, the flanking ELK domain and the relatively conserved *KNOX* domain, its structurally similar to maize *KN1*, Arabidopsis *STM*, and Rice *OSH1*. *DOH1* was strongly expressed in stems (young and old stems) and vegetative Shoot apical meristem (*VSAM*), but moderately to weakly expressed in transitional SAMs (*TSAMs*) and floral buds, indicating that *DOH1* is required for maintenance of the basic plant architecture and floral transition in orchids. *DOH1* mRNA accumulates in meristem-rich tissues and its expression is greatly down regulated during floral transition. Overexpression of *DOH1* in orchid completely suppresses shoot organization and development. Transgenic orchid plants expressing both sense and antisense mRNA for *DOH1* exhibit abnormal shoot and leaf developments, suggesting that *DOH1* plays a key role in maintaining the basic plant architecture of orchid through control of the development of SAM and shoot structure. In addition, the reciprocal expression of *DOH1* and *DOMADSI* (Floral transition gene) during floral transition, indicates that downregulation of *DOH1* in the SAM is required for floral transition, indicates that *DOH1* is a possible upstream regulator of *DOMADSI* (Yu et al. 2000; Yu & Xu 2007).

Based on homology sequence to the *DOH1* cDNA, our group isolated 10 independent cDNAs of *DOH1* homologous from our natural moth orchid *Phalaenopsis amabilis*, designated as *Phalaenopsis Orchid Homeobox1 (POH1)* (Semiarti et al. 2008). We confirmed the genetic regulation in the development of protocorms and seedlings in *in vitro* culture and the *in vivo* floral transition using reverse transcriptase-PCR with *POH1* specific primers. Sulistianingsih (2012) found abnormal shoot and leaf phenotype of Gamma Co-60-irradiated *P. amabilis* mutants that defected in the C-terminal of *POH1* locus. Interestingly, one of the abnormal shape of leaves, that is

trumpet-like shape occurred in Gamma Co-60-irradiated *P. amabilis* mutants was very similar to that of 35S::KNAT1 transgenic *P. amabilis* (Fig. 1 and Fig. 2A).

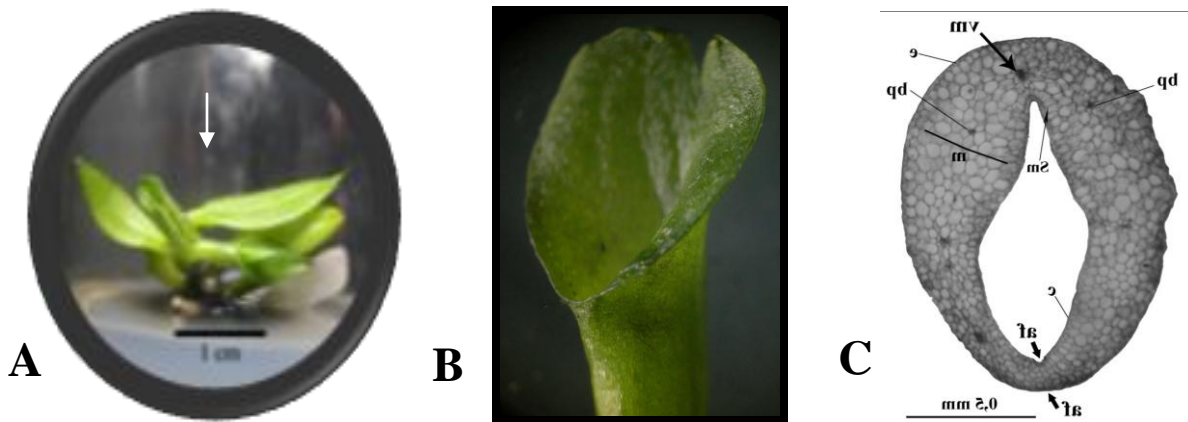


Figure 1. Abnormal leaf shape of Gamma Co-60-irradiated *P. amabilis* mutants. A. Mutant with trumpet-like shape as the most severe leaf phenotype; B. The distal part of leaf; C. Anatomical structure of the leaf. Arrow points to a trumpet-like leaf in *P. amabilis* mutant. Scale bars: 1 cm (A), 2 mm (B and C).

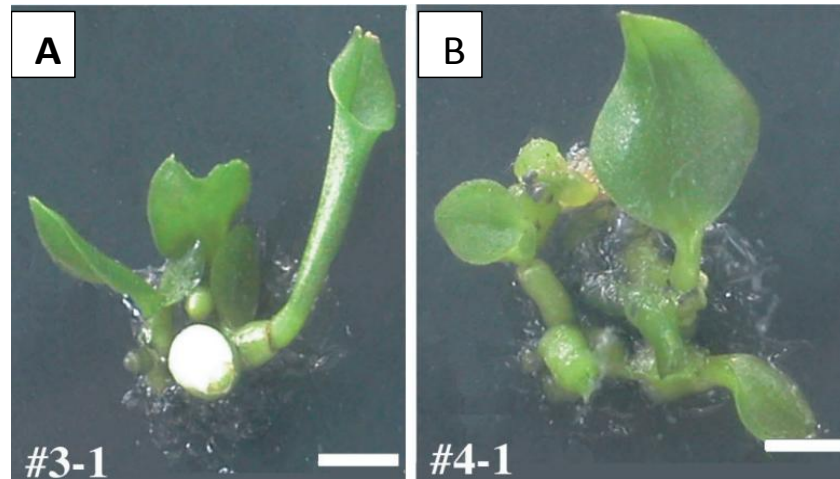


Figure 2. Abnormal leaf phenotypes of 35S::KNAT1 transgenic *P. amabilis* orchids. A. Trumpet-like shape of leaf in transgenic plant #3-1, B. Rectangular shape of leaf in transgenic plant #4-1. Arrow points to a trumpet-like leaf in transgenic *P. amabilis* #3-1. Scale bars: 1 cm (after Semiarti et al. 2007).

In orchids, generally plant regenerated from callus via PLBs, in a basal medium supplemented with phytohormon cytokinin and auxin in certain ratio (Ishii et al. 1998; Zhao et al. 2008), although it is not the case in *Dendrobium fimbriatum* orchid (Sharma et al. 2005), *Dendrobium chrysotoxum* that plants can be regenerated from PLBs in both without and with phytohormons in culture medium (Roy et al. 2007). This is in agreement with our data that we got PLBs grew from callus emerged from leaf segment explants of 35S::KNAT1 transgenic *P. amabilis* and *Coelogyne pandurata* cultured on phytohormon-free medium. Further, the plantlets developed from those PLBs (Semiarti et al. 2007; 2010). However, 35S::KNAT1 *Vanda tricolor* transgenic orchid did not exhibit multishoot, indicates that genotype roles may also involved in the shoot formation (Dwiyani et al. 2010).

Box et al. (2012) have isolated four class 1-*KNOX* genes from *Dactylorhiza fuchsii* (*DfKNI-4*) that show flower-specific function in that orchid, predominantly expressed in developing floral organs such as the spur-bearing labellum (*DfKN2*) and the inferior ovary (*DfKNI-4*). In agreement with the finding of Box et al. (2012) and Greco et al. (2012), on the function of *KNOX* gene in flower development, Rudall et al. (2013) proposed the *KNOX* functions in labellum development based on the observation on the small flowered terrestrial orchid *Herminium monorchis* as a model plant. This remarkable new genetic finding to speculate the function of *KNOX* in the development of the characteristic, and often elaborate, lobed morphology of the orchid labellum. Ma et al. (2015) reported a *KNOTTED1-LIKE HOMEODOMAIN PROTEIN1* (*KD1*) is highly expressed in both leaf and flower abscission zones. Reducing the abundance of transcripts of this gene in

tomato (*Solanum lycopersicum*) by both virus-induced gene silencing and stable transformation with a silencing construct driven by an abscission-specific promoter resulted in a striking retardation of pedicel and petiole abscission. Flower-specific *KNOX* phenotype in the orchid *Dactylorhiza*.

Although the roles of class1-*KNOX* genes in plants, especially in orchids growth and development remains unclear, we should agree to the assumption that class1-*KNOX* genes are the important genes that activate in very early stage of plant development. However, if it confirmed,

timing of *KNOX* expression could be crucial in establishing the diverse range of floral morphologies that at least partly accounts for the exceptional species richness exhibited by orchids (Rudall et al. 2013). The much-researched functional morphology of the orchid flower could therefore reflect extreme synorganization and the associated overlap in gene expression between organs (Box et al. 2012). Some genes which are known their functions and used in the study of the shoot formation, growth and development in plant are listed in Table. 1.

Table 1. Study of genes related to the shoot formation, growth and development

Gene	Plant	Gene function	Author
<i>KNOTTED1</i> -like homeobox ( <i>knox</i> )		transcriptional factors for the maintenance of the SAM and the development of aboveground organs	Yu et al. 2000; Ritter et al. 2002; Scofield et al. 2008
<i>teosinte branched1 (tb1)</i>	<i>Zea mays</i>	exhibits excessive branching of shoot	Doebley et al. 1997
<i>barren stalk1 (ba1)</i>	<i>Zea mays</i>	defective in axillary meristem development	Ritter et al. 2008
<i>tassel seed4 (ts4)</i>	<i>Zea mays</i>	highly branched inflorescences	Irish 1997
<b>STM, BP/KNAT1, KNAT2 and KNAT6</b>	<i>A.thaliana</i>	function of SAM and carpel development	Scofield et al. 2008
<i>asymmetric leaves2(as2)</i>	<i>A.thaliana</i>	formation and maintenance of a meristem state. Leaf mutant produced multishoots from the basal part of the leaves	Semiarti et al. 2001
<b>AS2</b>	<i>A.thaliana</i>	decreased in the late stage of floral organ primordia	Keta et al. 2012
Arabidopsis <b>KNAT1</b> and <b>AS2</b>	<i>P. amabilis</i>	dwarf shoots with abnormal leaf shapes, i.e rectangular, trumpet-like, oval, fused leaves, lobed leaves	Windiastris and Semiarti 2009
<b>AS1</b> and <b>AS2</b> together with other genes ( <b>HAT1, HAT2</b> , etc)	<i>A.thaliana</i>	involved in the determination of adaxial-abaxial polarity	Terakura et al. 2006
Dendrobium Orchid Homeobox1 ( <b>DOH1</b> )	<i>Dendrobium Madame Thong-In</i>	maintenance of the basic plant architecture and floral transition in orchids	Yu et al. 2000
Phalaenopsis Orchid Homeobox1 ( <b>POH1</b> )	<i>P. amabilis</i>	genetic regulation in the development of protocorm and seedling in <i>in vitro</i> culture and the <i>in vivo</i> floral transition	Semiarti et al. 2008
Phalaenopsis Orchid Homeobox1 ( <b>POH1</b> )	<i>P. amabilis</i>	Formation of shoot and leaf phenotype of Gamma Co-60-irradiated <i>P. amabilis</i>	Sulistianingsih 2012
<b>DfKN2</b>	<i>Dactylorhiza fuchsii</i>	developing floral organs such as the spur-bearing labellum	Box et al. 2012
<b>DfKN1-4</b>	<i>Dactylorhiza fuchsii</i>	inferior ovary	Box et al. 2012
<i>KNOTTED1</i> -like homeobox protein1 ( <b>KDI</b> )	orchid	leaf and flower abscission	Ma et al. 2015

#### A MODEL FOR THE ROLE OF CLASS1-KNOX GENES IN ORCHID PLANT TISSUE CULTURE

Based on our observations in 35S::KNAT1-transgenic Moth orchids *P. amabilis* and some Indonesian natural orchids, namely: Black orchids *Coelogyne pandurata*, *Dendrobium aphyllum*, *Spathoglottis plicata* and *Vanda*

*tricolor*, transgenic orchids show multishoots production from protocorm that finally could be regenerated to be intact multiplantlets, in agreement with the findings of Yu et al. (2001), its suggest that the roles of Class1-*KNOX* gene family in orchid *in vitro* tissue culture is the

transcription factor of genes that maintain the SAM identity and in turn induce shoot development (Fig. 3).

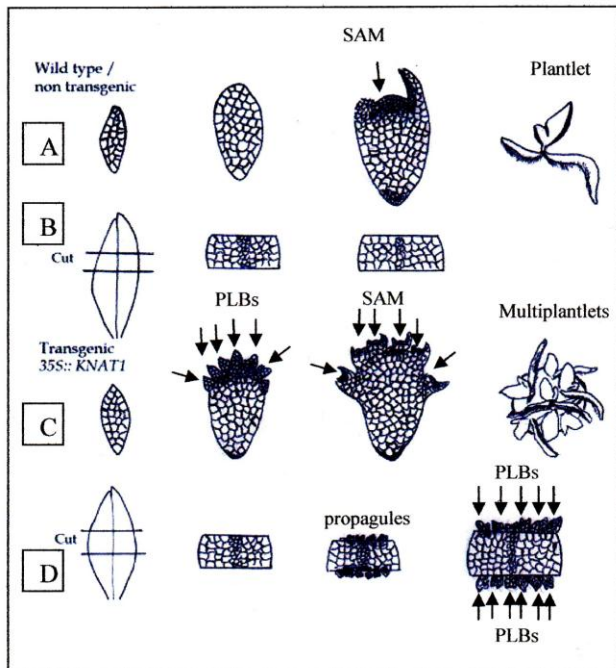


Figure 3. Schematic comparison of shoot development between Wild-type and 35S::KNAT1 transgenic of *P. amabilis* orchid plants. A and B are WT, C and D are 35S::KNAT1 transgenic plants cultured on basal media (phytohormone-free media). A and C. developmental stages from embryo to plantlet. B and D are developmental stages from leaf segments. WT-embryo grows into a single plant, on the other hand, from the transgenic embryo developed abnormal multishoots that finally produced multiplantlets from the SAM and distal/tip area. In B, WT-leaf segment explant produced no plant, but transgenic plants leaf segments produced propagules, that grew into PLB and it become many shoots.

Here, we propose a working model of the class1-*KNOX* gene in orchid tissue cultures as follows: 1) Class1-*KNOX* gene is required to start the growth of cells in the early stages of growth of SAM or dedifferentiated cells of explant by stimulating the cytokinin and auxin biosynthesis genes families to induce cell division and produce callus or propagules in the wounding part of the leaf segment explants, and 2) *KNOX* genes will maintain the physiological state of the callus as meristematic cells with indeterminate state to achieve the number of cells as the SAM-like cells, 3) initiate networking with groups of genes transition phase of indeterminate meristematic phase leading to a determinate stage for plant organ formation. If *KNOX* proteins excessively functions in various organs of plant or leaf segments, the misexpression of the class1-*KNOX* genes might be occurred that resulting in the emergence of adventitious meristems from the peripheral cells of explants and genetic networking occurs then closed system in *in vitro* conditions led them to grow as intact shoots and finally regenerate intact plants.

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