

Creating vessel elements in vitro: Towards a comprehensive understanding of the molecular basis of xylem vessel element differentiation

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Abstract Xylem is an essential conductive tissue in vascular plants, and secondary cell wall polymers found in xylem vessel elements, such as cellulose, hemicellulose, and lignin, are promising sustainable bioresources. Thus, understanding the molecular mechanisms underlying xylem vessel element differentiation is an important step towards increasing woody biomass and crop yields. Establishing in vitro induction systems, in which vessel element differentiation is induced by phytohormonal stimuli or by overexpression of specific transcription factors, has been vital to this research. In this review, we present an overview of these in vitro induction systems, and describe two recently developed in vitro induction systems, VISUAL (Vascular cell Induction culture System Using Arabidopsis Leaves) and the KDB system. Furthermore, we discuss the potentials and limitations of each of these new in vitro induction systems for advancing our understanding of the molecular mechanisms driving xylem vessel element differentiation.

Key words: in vitro induction system, KDB system, VISUAL, VND, xylem vessel elements.

Introduction

Xylem is one of the conductive tissues found in vascular plants and is essential for transporting water and nutrients from roots to shoots. The water-conducting activity of the xylem relies on pipe-like structures consisting of cells called vessel elements. Xylem vessel elements have thick secondary cell walls (SCWs), which are composed of cellulose, hemicellulose, and lignin (Turner et al. 2007). These thick SCWs are the major source of woody biomass and represent a valuable source of renewable energy (Yang et al. 2013). Understanding the molecular basis of vessel element formation is an important step towards improving water conduction in plants to increase crop yields, and also towards controlling the quantity and quality of woody biomass.

Because of their physiological importance, xylem vessel elements have been the focus of many molecular plant biology studies. However, as xylem is embedded in the plant body, it is difficult to access xylem vessel elements for observation or molecular studies. In addition, since xylem tissues contain several kinds of

cells other than vessel elements, i.e., xylem fibers and parenchyma cells, isolating vessel elements from native xylem tissues poses a challenge. To overcome these issues, several in vitro induction systems have been developed, in which vessel element differentiation can be induced by phytohormonal stimuli (Fukuda and Komamine 1980; Kondo et al. 2014, 2015, 2016; Kubo et al. 2005; Oda et al. 2005; Pesquet et al. 2010; Tan et al. 2018) or by overexpression of specific transcription factors (Oda et al. 2010; Yamaguchi et al. 2008, 2010). Using these systems, homogenous cells at the same stage of differentiation can be collected and analyzed, facilitating the identification of novel factors involved in specific stages of cell differentiation. Indeed, in vitro induction systems have already revealed many regulatory aspects of xylem vessel element differentiation, including novel key regulators of xylem vessel cell differentiation and the stage-specific regulation of the transcriptome, proteome, and metabolome (Demura et al. 2002; Endo et al. 2015; Fukuda 1997, 2004; Goué et al. 2013; Ito et al. 2006; Kawabe et al. 2018; Kondo et al. 2014, 2015, 2016; Kubo et al. 2005; Li et al. 2016; Motose et al. 2004;

Abbreviations: APL, ALTERED PHLOEM DEVELOPMENT; PCD, programmed cell death; SUC2, SUCROSE-PROTON SYMPORTER 2; SCW, secondary cell wall; VISUAL, vascular cell induction culture system using Arabidopsis leaves; VND, VASCULAR-RELATED NAC-DOMAIN.

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Noguchi et al. 2018; Oda and Fukuda 2012, 2013; Oda et al. 2010; Ohtani et al. 2016, 2018; Pesquet et al. 2010; Schuetz et al. 2014; Takenaka et al. 2018; Tan et al. 2018; Watanabe et al. 2015, 2018).

In this review, we summarize the established in vitro induction systems of xylem vessel elements and the considerable findings obtained using these systems. With an emphasis on recently established in vitro induction systems, VISUAL (Vascular cell Induction culture System Using Arabidopsis Leaves) (Kondo et al. 2014, 2015, 2016) and the KDB system (Tan et al. 2018), we discuss future directions in research examining vessel element differentiation using these systems.

Development of in vitro induction systems for xylem vessel element differentiation

The first effective in vitro induction system of xylem vessel elements was developed by Fukuda and Komamine (1980) (Table 1), who established a simple and stable in vitro vessel element differentiation system using a *Zinnia elegans* (*Zinnia*) mesophyll cell culture. The induction stimuli in their system were two phytohormones, auxin and cytokinin, and ca. 50% of cultured cells differentiated

into xylem vessel elements 3 days after the treatment (Fukuda and Komamine 1980). The easy preparation of *Zinnia* cell samples and synchronized cell differentiation made this system ideal for characterizing the temporal cytological changes that occur during differentiation, and led to the isolation of many genes involved in xylem vessel cell differentiation (Fukuda 2004; Turner et al. 2007). A comprehensive microarray analysis using this system revealed step-wise changes in gene expression associated with distinct stages of xylem vessel element differentiation (Demura and Fukuda 1993, 1994; Demura et al. 2002), demonstrating that *Zinnia* mesophyll cells undergo a procambium cell stage followed by a xylem precursor cell stage as they differentiate into vessel elements. In addition, this gene expression analysis identified many candidate genes hypothesized to function in xylem vessel element differentiation (Demura et al. 2002).

When the whole genome sequence of *Arabidopsis thaliana* (*Arabidopsis*) became available in 2000 (Arabidopsis Genome Initiative 2000), the advantage of using *Arabidopsis* for molecular biology studies became apparent. Several groups reported new in vitro induction systems for vessel element formation using *Arabidopsis* cell suspension cultures manipulated

Table 1. In vitro xylem vessel element differentiation systems developed in herbaceous angiosperm species.

Species	Materials	Transgenes	Inducers	Light requirement ^b	Final frequency of ectopic xylem vessel element differentiation (%)	Days required to reach the final frequency of ectopic xylem vessel element differentiation (d)	References
<i>Zinnia elegans</i>	Isolated mesophyll cell	No	Auxin and cytokinin	Dark	50	3	Fukuda and Komamine 1980
<i>Arabidopsis thaliana</i>	Cultured cells	No	Auxin, boric acid and brassinosteroid	—	50	3	Kubo et al. 2005
<i>Arabidopsis thaliana</i>	Cultured cells (AC-GT13) ^a	35S GFP(S65T) TUA NOS	Removal of auxin followed by application of brassinosteroid	—	30	4	Oda et al. 2005
<i>Arabidopsis thaliana</i>	Cultured cells	No	Auxin, cytokinin and brassinosteroid	—	40	3	Pesquet et al. 2010
<i>Arabidopsis thaliana</i>	Cultured cells containing inducible VND6 construct	P _{G10-90} VND6 LexA VP16 hER T _{E9} NOS	Estrogen and brassinosteroid	—	80	2	Oda et al. 2010; Oda and Fukuda 2012
<i>Arabidopsis thaliana</i>	Cultured cells/plants containing inducible VND7 construct (T87)	35S VND7 VP16 GR NOS	Glucocorticoid (dexamethasone, DEX)	—	10	7	Yamaguchi et al. 2010
<i>Nicotiana tabacum</i>	Cultured cells containing inducible VND7 construct (BY-2)	35S VND7 VP16 GR NOS	Glucocorticoid (dexamethasone, DEX)	—	70	3	Yamaguchi et al. 2010
<i>Arabidopsis thaliana</i>	Leaves and cotyledons	No	Bikinin, auxin, and cytokinin	Light	ND	3	Kondo et al. 2014, 2015, 2016
<i>Arabidopsis thaliana</i>	Cotyledons	No	Brassinosteroid, auxin, and cytokinin	Light	ND	5	Tan et al. 2018

^aAC-GT13 is a cell suspension transformed with a vector containing a GFP(S65T)-tubulin alpha (TUA) fusion gene (Kumagai et al. 2001) that allows visualization of cortical microtubule rearrangement in living xylem cells. ^bLight or dark conditions are required to induce cell differentiation where indicated. —No indication about requirement of light or dark conditions. 35S and P_{G10-90}, strong constitutive promoters; LexA, DNA-binding domain of the bacterial repressor; VP16, activation domain of herpes virus protein; hER, the carboxyl region of the human estrogen receptor; GR, hormone-binding domain of rat glucocorticoid receptor; T_{E9}, *rbcs E9* poly(A) addition sequence; NOS, terminator of nopaline synthase; GFP, green fluorescent protein; ND, not determined.

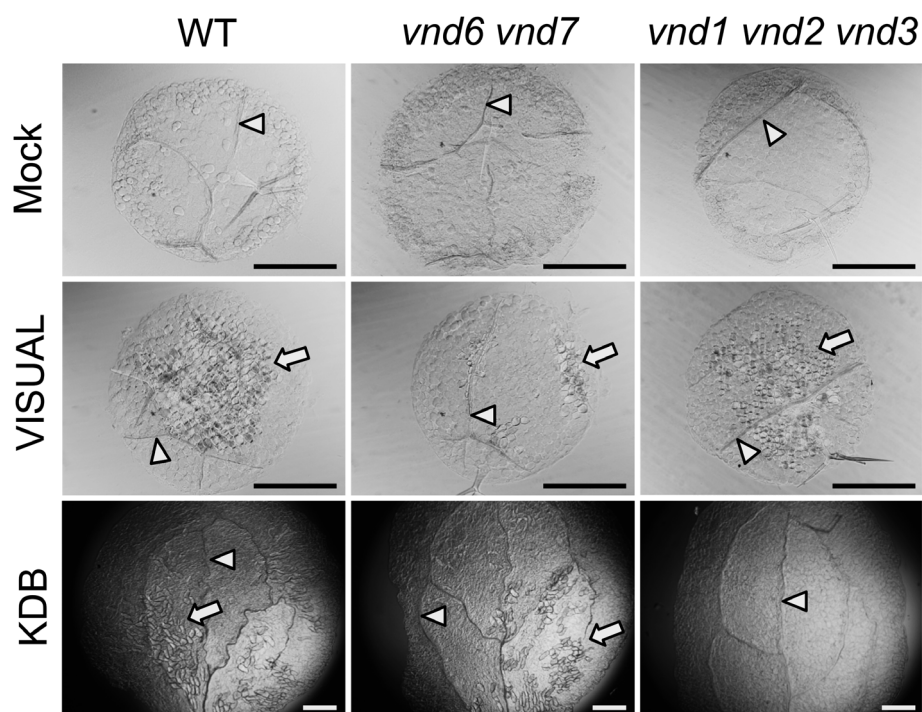


Figure 1. VISUAL and the KDB system have different dependencies on VND family genes. Ectopic xylem vessel element differentiation was induced in cotyledon leaf disks of wild type (WT), *vnd6 vnd7*, and *vnd1 vnd2 vnd3* using VISUAL (Kondo *et al.* 2014, 2015, 2016) or the KDB system (Tan *et al.* 2018). Mock-treated leaf disks were shown as the control (upper panels). White arrowheads and arrows indicate native xylem vessels and ectopic xylem vessel elements, respectively. Scale bars, 500 μ m.

with key phytohormones, i.e., auxin, cytokinin, and brassinosteroids. Kubo *et al.* (2005) established an *Arabidopsis* cell suspension system in which brassinosteroid and boric acid induce xylem vessel element differentiation (Table 1). In this system, approximately 50% of cells differentiate into xylem vessel elements within 7 days of treatment. Oda *et al.* (2005) induced ectopic xylem vessel elements in *Arabidopsis* cell suspensions by removing auxin and applying brassinosteroid to produce a differentiation rate of ca. 30% after 4 days of culture (Oda *et al.* 2005). Additionally, Pesquet *et al.* (2010) reported that the addition of auxin, cytokinin, and brassinosteroid induces the differentiation of *Arabidopsis* cell cultures into xylem vessel elements at a rate of 40% after 3 days of culture (Table 1). These *in vitro* induction systems were key to identifying critical factors in vessel element differentiation, such as members of the VASCULAR-RELATED NAC-DOMAIN (VND) family of transcription factors that induce xylem vessel element differentiation (Kubo *et al.* 2005) and the microtubule-associated proteins regulating cortical microtubule alignment for SCW patterning (Oda *et al.* 2005; Pesquet *et al.* 2010).

Based on the findings by Kubo *et al.* (2005), transgenic *Arabidopsis* cell suspensions were created containing either an estrogen-inducible VND6 construct or a glucocorticoid-inducible VND7 construct (Oda *et al.* 2010; Oda and Fukuda 2012; Yamaguchi *et al.* 2008,

2010). Both systems have high rates of ectopic vessel element differentiation (ca. 80–90%) and have been used to determine the transcriptional networks downstream of VND6 and VND7 (Ohashi-Ito *et al.* 2010; Yamaguchi *et al.* 2011; Zhong *et al.* 2010). Moreover, the VND7-inducible system has revealed many novel cellular and molecular mechanisms involved in xylem vessel element differentiation (Endo *et al.* 2015; Goué *et al.* 2013; Kawabe *et al.* 2018; Li *et al.* 2016; Noguchi *et al.* 2018; Ohtani *et al.* 2016, 2018; Schuetz *et al.* 2014; Takenaka *et al.* 2018; Watanabe *et al.* 2015, 2018).

Two recently developed similar *in vitro* induction systems: VISUAL and the KDB system

Recently, two *in vitro* induction systems have been developed that can be used to examine xylem vessel element differentiation in diverse *Arabidopsis* mutant and reporter lines: (1) VISUAL (Vascular cell Induction culture System Using *Arabidopsis* Leaves) (Kondo *et al.* 2014, 2015, 2016) and (2) the KDB system (Tan *et al.* 2018) (Figure 1). In VISUAL, *Arabidopsis* leaf disks or excised leaves are cultured with auxin and cytokinin along with bikinin, a compound that strongly activates brassinosteroid signaling by inhibiting the BRASSINOSTEROID-INSENSITIVE 2 (BIN2) kinase, a negative regulator of brassinosteroid signaling in

Arabidopsis (Kondo et al. 2014). As a result, both xylem vessel elements and phloem sieve cells can be ectopically induced in this system (Kondo et al. 2016).

Similarly, in the KDB system, the excised *Arabidopsis* cotyledons are treated with cytokinin (Kinetin) and auxin (2,4-D), but brassinosteroid signaling is activated by adding a brassinosteroid (Brassinolide) (Tan et al. 2018). In contrast to the VISUAL system, the KDB system induces xylem vessel element differentiation only, confirmed by the lack of *SUCROSE-PROTON SYMPORTER 2* (*SUC2*) expression, a phloem marker gene encoding a sucrose transporter (Truernit and Sauer 1995), and of *ALTERED PHLOEM DEVELOPMENT* (*APL*) expression, a key regulator of phloem development (Bonke et al. 2003). Among the members of the VND family, VND1 through VND3 are the main contributors to the differentiation of ectopic xylem vessel elements in the excised *Arabidopsis* cotyledons induced by the KDB system (Tan et al. 2018). Since VISUAL can induce both xylem vessel elements and phloem sieve cells while the KDB system induces strictly vessel elements, a comparative study between these systems could provide further clues into the xylem-specific and phloem-specific molecular processes in vascular cell differentiation.

Future challenges for studies of xylem vessel element differentiation using in vitro induction systems

VISUAL and the KDB system are valuable tools for studying xylem vessel element differentiation; furthermore, the availability of *Arabidopsis* resources facilitates analyses of gene functions and of the molecular mechanisms underlying xylem vessel element differentiation. However, a drawback of these two in vitro systems is that cotyledon and leaf tissues contain several types of cells, and these systems lack the synchrony of cell differentiation and homogeneity of other in vitro cell suspension-based approaches, such as the systems developed by Fukuda and Komamine (1980) and Kubo et al. (2005). High-resolution analytical methods, such as single-cell/molecule-level resolution methods, are highly sensitive to such factors.

Figure 2 shows the current model of xylem vessel element differentiation, based on a combination of results from mutant analyses and in vitro induction systems (Ohashi-Ito and Fukuda 2010; Růžička et al. 2015; De Rybel et al. 2016). In vitro induction systems typically contain the following steps: 1) conversion of differentiated leaf cells to procambium-like cells, 2) cellular differentiation into xylem precursor cells, and 3) activation of VND family proteins to induce molecular events, such as SCW formation and programmed cell death (PCD), for xylem vessel element differentiation

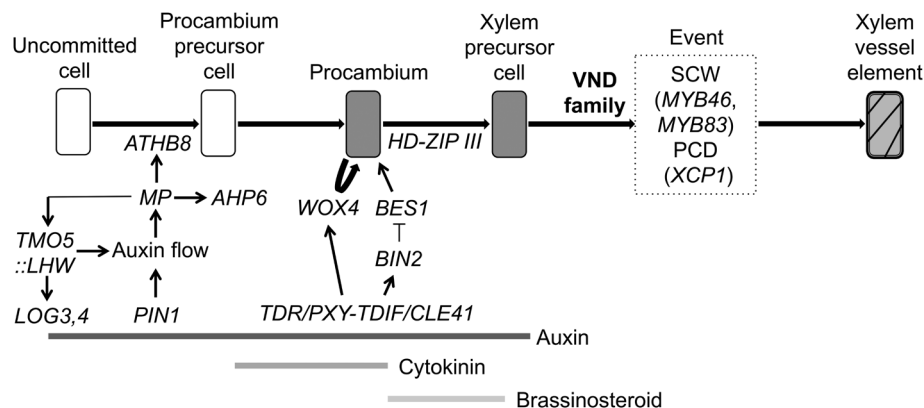


Figure 2. Proposed model of the process of xylem vessel element formation. The vascular cell formation is initiated with procambium cell development by the homeodomain-leucine zipper (HD-Zip) III gene *ATHB8*, which is regulated by *MONOPTEROS* (*MP*) encoding an auxin response transcription factor. For the *MP* expression, the regulation of auxin distribution by PIN-FORMED 1 (*PIN1*), an auxin efflux carrier protein, is important. *MP* also directly upregulates the expression of a basic helix-loop-helix (bHLH) type transcription factor gene, *TARGET OF MP 5* (*TMO5*), as well as a key cytokinin signaling inhibitor *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6* (*AHP6*). Heterodimeric complexes of *TMO5* and another bHLH protein, *LONESOME HIGHWAY* (*LHW*), promote cytokinin production by upregulating the expression of cytokinin biosynthesis genes, *LONELY GUY3* (*LOG3*) and *LOG4*, which regulate cell division activity. Two regulatory pathways mediated by a mobile secreted peptide, such as *TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR* (*TDIF*)/*CLE41*, which is perceived by the plasma membrane receptor *TDIF RECEPTOR* (*TDR*)/*PHLOEM INTERCALATED WITH XYLEM* (*PXY*), control procambium cell activity. The first pathway results in the proliferation of cambial cells through the activity of a transcription factor, *WUSCHEL HOMEODOMAIN RELATED 4* (*WOX4*), and the second pathway triggers the differentiation of cambial cells into xylem vessel elements through the kinase *BRASSINOSTEROID-INSENSITIVE 2* (*BIN2*), which inhibits the transcription factor *BRI1-EMS SUPPRESSOR 1* (*BES1*). After the establishment of xylem precursor cells, the VND family proteins upregulate the expression of an entire set of genes required for xylem vessel element differentiation, including those encoding SCW transcription factors (e.g., *MYB46* and *MYB83*) and PCD (e.g., *XCP1*).

(De Rybel *et al.* 2016; Nakano *et al.* 2015; Ohashi-Ito and Fukuda 2010a; Růžička *et al.* 2015). Interestingly, the results of the VISUAL and KDB systems suggest that vessel elements are differentiated through different molecular pathways in these systems; despite similarities in differentiation induction stimuli (auxin, cytokinin, and brassinosteroid), the main contributors to ectopic differentiation of xylem vessel elements in VISUAL are VND6 and VND7, with low requirement for VND1 through VND3 (Kondo *et al.* 2015; Figure 1), among the VND family proteins including 7 family genes in *Arabidopsis* (Kubo *et al.* 2005). By contrast, the KDB system is highly dependent on VND1 through VND3, but does not require VND6 or VND7 (Tan *et al.* 2018; Figure 1). These results suggest the existence of several possible routes towards xylem vessel element differentiation, especially before VND protein activation (Figure 2). Which parts of these molecular pathways overlap or differ between VISUAL and the KDB system? How flexible are the molecular routes underlying xylem vessel element differentiation, and what factors regulate this flexibility? Comparative analysis among multiple *in vitro* induction systems will provide important insights into the molecular underpinnings of xylem vessel element differentiation.

Concluding remarks and perspective

Over the past 40 years, research based on *in vitro* induction systems has elucidated many molecular processes involved in xylem vessel element differentiation. After substantial progress in xylem research that identified key regulators of each step in xylem vessel cell differentiation (Figure 2), new *in vitro* induction systems will expand the scope of research in the field of xylem vessel element differentiation. Emerging trends include applying high-resolution analysis to *in vitro* induction systems, focusing on the specific angle of molecular system for xylem vessel element differentiation (Kawabe *et al.* 2018; Kondo *et al.* 2016; Ohtani *et al.* 2018; Takenaka *et al.* 2018; Watanabe *et al.* 2015, 2018), and conducting *trans*-omics analyses using *in vitro* induction systems (Derbyshire *et al.* 2015; Li *et al.* 2016; Noguchi *et al.* 2018; Ohtani *et al.* 2016). Such studies will further improve our understanding of the molecular processes underlying xylem cell differentiation.

In addition to the approaches discussed above, comparative analysis of multiple types of *in vitro* induction systems will likely elucidate various molecular routes to vessel element differentiation in plant cells. Culture conditions in *in vitro* systems can be easily controlled, facilitating tests of the robustness of each molecular route. Information gleaned from these systems can be used to improve and modify xylem properties

in economically important trees for biomaterial and bioenergy applications.

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