

# Changes in the Morphology and Cation Content of a *Bambusa edulis* Xylem Mutant, *vse*, Derived from Somaclonal Variation

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**ABSTRACT.** A xylem mutant (*vse*) was isolated from a *Bambusa edulis* (Odashima) Keng plantlet following vegetative micropropagation and subculture for 7 consecutive years and induced to proliferate in medium supplemented with 0.1 mg·L<sup>-1</sup> (0.5 μM) thidiazuron (TDZ) and to develop roots in medium supplemented with 5 mg·L<sup>-1</sup> (26.9 μM) α-naphthaleneacetic acid (NAA). Subsequent investigations comparing the growth habits of mutant plantlets with those of the wild type indicated that the growth of the former was retarded in a greenhouse. Several morphological abnormalities were observed in the *vse* mutant: it had thinner stems with fewer trichomes on the surface; the xylem vessels were smaller in diameter and contained crystal-like structures in the pith; the leaves were shorter and narrower with a sharp leaf blade angle; the roots were thinner and contained fewer xylem cells. The cation concentrations of both the mutant and wild type were similar in the *in vitro* analysis, except for those of iron and potassium, which were lower in mutant leaves *in vivo*. In 2-month-old mutant plants, iron chlorosis was observed on young leaves and a potassium deficiency was observed on older leaves. After 1 year of growth in the greenhouse, all of the wild-type plants had survived, but only 27% (16/60) of the mutant *vse* plants were alive.

*Bambusa edulis* is a semitropical evergreen species known locally in Taiwan as “gray feet green bamboo.” It is a medium-sized clumping bamboo that is cultivated extensively for the quality of its edible young shoots, which are considered to be a culinary delicacy in Taiwan, as well as for its wood products. It is also commonly used as an ornamental, both in the garden, where it can reach a height of 19.8 m, and in pots, with a more restricted height of 3 m. Although various propagation techniques are available for Bambusoideae in general and *B. edulis* in particular, including plant division, vegetative propagation, and seed propagation, these methods are severely limited when utilized for large- or mass-scale propagation. It has been proposed that only micropropagation—propagation by tissue culture—will meet the estimated demand for the mass propagation of 500,000 bamboo plants per year (Gielis et al., 2001).

In *B. edulis*, protocols for micropropagation (Lin and Chang, 1998) and somatic embryogenesis (Lin et al., 2003, 2004) have been successfully developed and used to maintain multiple shoots *in vitro* for 7 years. Rooted plantlets derived from the shoots maintained in these *in vitro* tissue culture systems were recently transferred to the greenhouse. We found that the vegetative tissue-derived somatic embryos (Lin et al., 2004) were retarded with respect to their growth characteristics and displayed chlorosis of the young leaves, indicating a nutrient imbalance. We denoted this aberrant plant type as the *vse* mutant. Since xylem is the pathway through which the plant transports its water and nutrients and given the economic potential of successful micropropagation techniques for bamboo, it is important to understand the relation between nutrient transport and vascular structure of the wild type and somaclonal variation in *B. edulis*. Consequently, the aim of the investigation reported here was to compare the wild type and the micropropagated *vse* mutant in terms of morphology, anatomy, and nutrient analysis in an attempt to clarify the relation between xylem structure and cation content in both types of bamboo. In addition, we examined the specific morphological changes that have occurred in the mutant relative to the wild type.

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**MEDIUM PREPARATION.** The basal medium comprised MS salts (Murashige and Skoog, 1962), 100 mg·L<sup>-1</sup> myoinositol (Sigma, St. Louis), 0.5 mg·L<sup>-1</sup> nicotinic acid (Sigma), 0.5 mg·L<sup>-1</sup> pyridoxine-HCl (Sigma), 1 mg·L<sup>-1</sup> thiamine-HCl (Sigma), 2 mg·L<sup>-1</sup> glycine (Sigma), 2 g·L<sup>-1</sup> Gelrite (Kelco, San Diego), and 30 g·L<sup>-1</sup> sucrose (Taiwan Sugar Co., Taipei, Taiwan). Growth regulators were added prior to the adjustment of the medium to pH 5.7. The media were autoclaved at 121 °C for 15 min.

**PLANT MATERIALS.** *Bambusa edulis* multiple shoots were used as explants and incubated on MS medium supplemented with 0.1 mg·L<sup>-1</sup> (0.5 μM) TDZ (Sigma) (Lin et al., 2004) to induce in vitro shoot proliferation. Each explant (wild type and *vse*) contained approximately five shoots. Root development was induced in the developing shoots following a 1-month culture on MS medium supplemented with 5 mg·L<sup>-1</sup> (26.9 μM) NAA (Sigma). The explants were maintained at 26 °C under a 16/8 h (light/dark) photoperiod with light supplied by fluorescent tubes (FL-30D/29, 40 W; China Electric, Taipei, Taiwan) at an intensity of 54 mol·m<sup>-2</sup>·s<sup>-1</sup>. The rooted shoots were transferred to a greenhouse without hardening.

**HISTOLOGICAL STUDIES.** Plant tissues were first fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 4 h at room temperature, then post-fixed in 2% (v/v) OsO<sub>4</sub> for 2 h, washed in phosphate buffer, dehydrated through an acetone series, infiltrated with Spurr's resin, and polymerized at 70 °C (Spurr, 1969). For light microscopy, the embedded tissues were cut into 0.7-μm-thick sections on a Reichert Ultracut S ultrathin microtome (Reichert, Vienna, Austria), stained with 0.1% toluidine blue, and viewed under a light microscope. The photographs of the sections were printed out and used for determining the number and diameters of the cells of the roots and shoots. The longest cross-section that did not cross the xylem was considered to be the diameter. The number of cells falling on this line was taken to be the cell number.

For viewing in the scanning electron microscope (model DSMS 950; Carl Zeiss, Oberkochen, Germany), the tissues were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 4 h at room temperature, dehydrated through an ethanol series (Dawns, 1971), dried in a critical point dryer (HCP-2; Hitachi, Tokyo), and coated with gold in an ion coater (IB-2; Giko Engineering, Tokyo).

**MEASUREMENT OF CATION CONTENT.** Leaves were collected from the wild-type and *vse* mutant plants cultured under different growth conditions (in vitro or different lengths of time in the greenhouse). The samples were first oven-dried at 65 °C for 3 d. The dried sample (0.1 g) was weighed into a crucible and placed in a muffle furnace at 200 °C for 2 h, followed by 400 °C for 1 h, and finally 550 °C for 1 h. The ashed samples were dissolved in 5 mL 2 N HCl and filtered through Whatman #42 filter paper. To measure the potassium and magnesium content of the ashed samples, 0.1 mL of filtered solution was diluted with 4.9 mL of deionized water and potassium and magnesium content determined by means of a Varian 20 Techtron atomic absorption spectrophotometer (Techtron, Melbourne, Australia). For calcium content measurement, 0.1 mL of the filtered solution, 3.9 mL deionized water, and 1 mL 5% lanthanum oxide were mixed for atomic absorption analysis (Martin-Prevel et al., 1987).

**STATISTICAL ANALYSIS.** The analysis of variance was conducted using Costat (CoHort Software, Monterey, Calif.). Duncan's multiple range test was used for mean separation when significance treatment effects existed (Duncan, 1955).

**MORPHOLOGY OF THE *vse* MUTANT PLANT.** The leaves of *vse* plantlets derived from multiple shoots maintained in continuous subculture for 7 years on MS medium supplemented with 0.1 mg·L<sup>-1</sup> TDZ were slightly smaller than those of the wild type at the 21-d stage (Fig. 1A), but there was no significant difference in shoot proliferation (Fig. 1B). Following the transfer of both the wild-type and *vse* rooted plantlets to the greenhouse, differences in growth characteristics became more visible (Fig. 1C–F). We measured stem diameter (Fig. 2A), leaf length (Fig. 2B), and leaf width (Fig. 2C) during in vitro culture and at the 3- and 6-month intervals, respectively. Our measurements showed that the difference in leaf size between the wild-type and *vse* mutant was

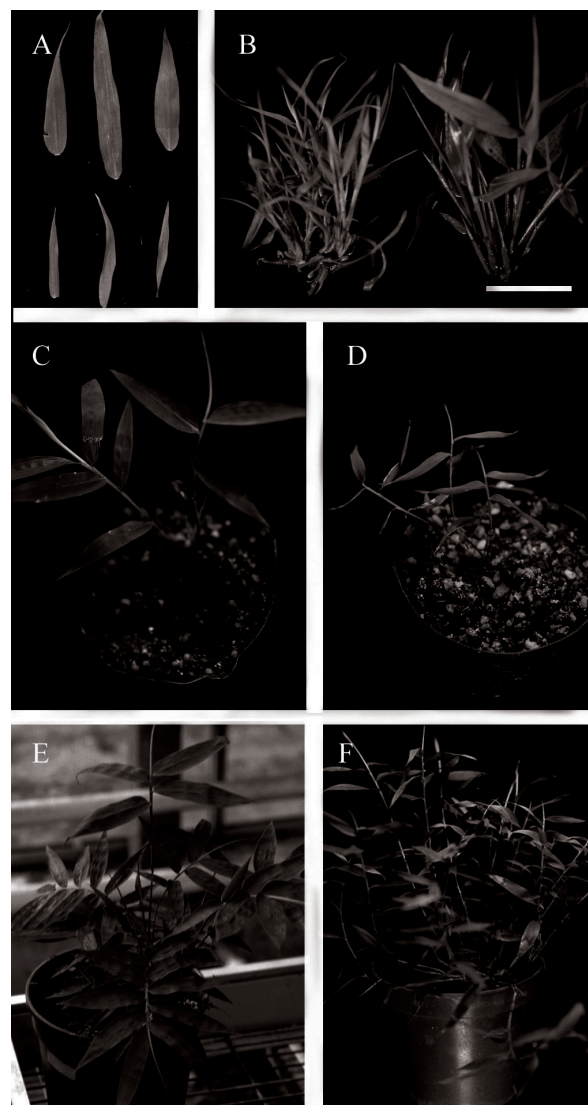


Fig. 1. Comparison of the *Bambusa edulis* *vse* mutant and wild-type phenotypes. (A) Leaves from a 21-d-old wild-type (upper) and *vse* (lower) mutant plant grown on MS medium (Murashige and Skoog, 1962) supplemented with 0.1 mg·L<sup>-1</sup> thidiazuron (bar = 1.5 cm). (B) Multiple shoots from a 21-d-old wild-type (right) and *vse* (left) mutant plant grown on MS medium supplemented with 0.1 mg·L<sup>-1</sup> thidiazuron (bar = 1.5 cm). (C) Three-month-old wild-type plantlet grown in the greenhouse (bar = 3 cm). (D) Three-month-old *vse* plantlet grown in the greenhouse (bar = 3 cm). (E) Six-month-old wild-type plantlet grown in the greenhouse (bar = 12 cm). (F) Six-month-old *vse* plantlet grown in the greenhouse (bar = 3 cm).



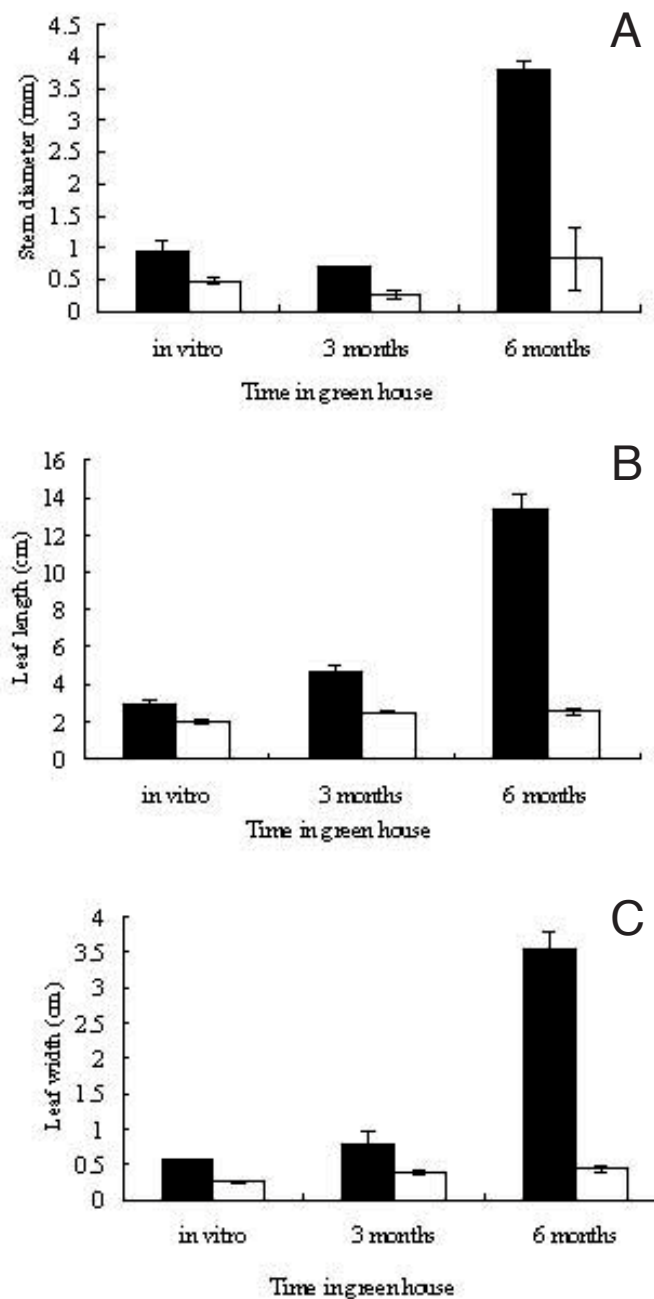


Fig. 2. Growth parameters of the *Bambusa edulis* wild-type and *vse* plants. (A) Stem diameter, (B) leaf length, and (C) leaf width of plants grown on Murashige and Skoog (1962) medium supplemented with 0.1 mg·L<sup>-1</sup> (0.5 μM) thidiazuron (in vitro), and in the greenhouse for 3 and 6 months. All results show the means ±SE (n = 5). Solid bars = wild type, open bars = *vse* mutant.

proportional to the duration of the growth period in the greenhouse, with the leaves of the wild type becoming significantly longer and wider than those of the *vse* mutant over time (Fig. 2B–C). The stem diameter of the wild type was significantly larger than that of the *vse* mutant by the 3- and 6-month stage (Fig. 2A, Table 1). There was a 100% survival rate of the wild-type plants (16/16) after 1 year, whereas only 27% (16/60) of the *vse* plants were alive after the same length of time.

**STEM STRUCTURE.** The structure of the stems of wild-type and *vse* mutant plantlets after 3 months of growth in the greenhouse was examined and compared (Fig. 3, Table 1). Horizontal sec-

Table 1. Comparison of the stem diameter, cell number in the stem section, diameter of the metaxylem, number of pith cells, and pith cell size in *Bambusa edulis* stems from 3-month-old wild-type and *vse* mutant plants.

	Stem diam (μm)	Stem cell (no.)	Metaxylem diam (μm)	Pith cell (no.)	Pith cell size (μm)
Wild type	814 a <sup>2</sup>	32.00 a	18.09 a	14.0 a	55.57 a
<i>Vse</i>	412 b	21.67 b	7.46 b	9.0 b	33.41 b

<sup>2</sup>Means followed by the same letter are not significantly different according to the least significant difference at *P* < 0.05 (Duncan, 1955). Data are the means of three independent stems (n = 3).

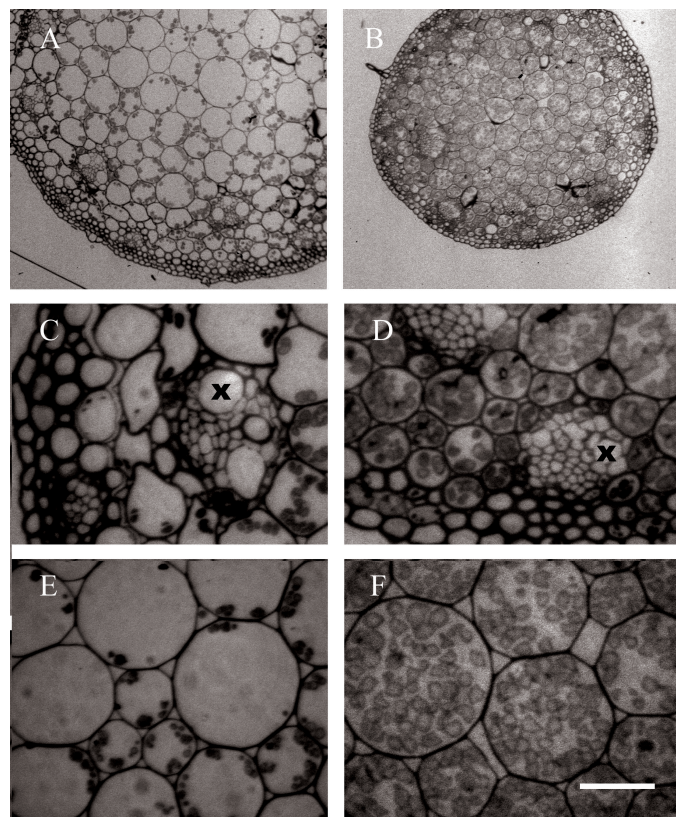


Fig. 3. Anatomy of the first internode from 3-month-old *Bambusa edulis* wild-type (left) and *vse* mutant (right) plantlets. (A) and (B): Transverse section of the first internode from the wild type and *vse* plantlet, respectively (100x magnification, bar = 50 μm). (C) and (D): Vascular structure of the wild-type and *vse* plantlet, respectively; x = xylem (200x magnification, bar = 25 μm). (E) and (F): Pith of the wild type and *vse* plantlet, respectively (200x magnification, bar = 25 μm).

tions of the stem revealed that the *vse* mutant had a reduced mean total number of cells, a reduced mean number of pith cells, and smaller xylem and pith cells relative to the wild-type plantlets (Table 1, Fig. 3A–D). In addition, the pith cells of the *vse* mutant contained large quantities of crystal-like structures (Figs. 3E–F; 4 A and C); these were conspicuously absent, or present at much lower levels, in their wild-type counterparts. In comparison with the *vse* mutant, the wild type had more trichomes on the surface of the stems (Fig. 4 B and D).

**LEAF STRUCTURE.** Following a 3-month culture in the greenhouse, the leaves of the *vse* mutant were thinner than those of the wild type (Fig. 5A–B). The leaves of the wild type were greener (Fig. 5C–D) due to the presence of more chloroplasts (Fig. 5A).

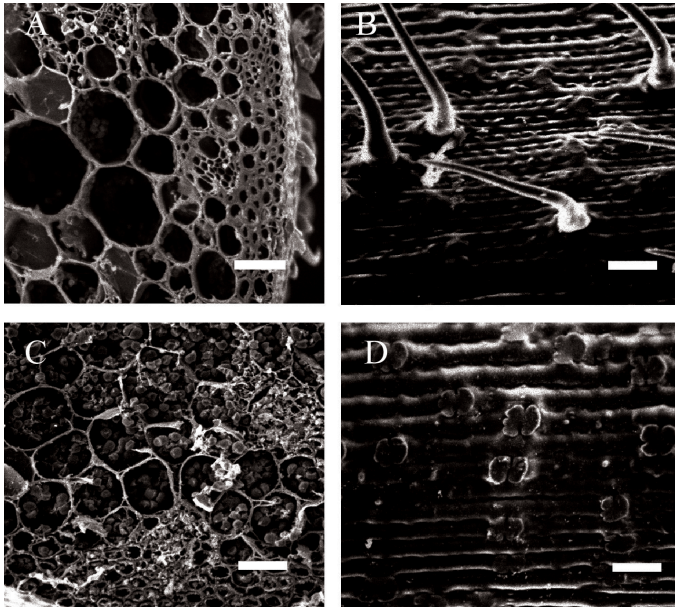


Fig. 4. Scanning electron microscope images of stem morphology of the *Bambusa edulis* wild-type and *vse* plantlets. (A) and (C): First internode transverse section of a 3-month-old wild-type (upper) and *vse* plant (lower). (400 $\times$  magnification, bar = 40  $\mu$ m). (B) and (D): Epidermis cell development in the wild type (upper) and *vse* mutant (lower) (175 $\times$  magnification, bar = 90  $\mu$ m).

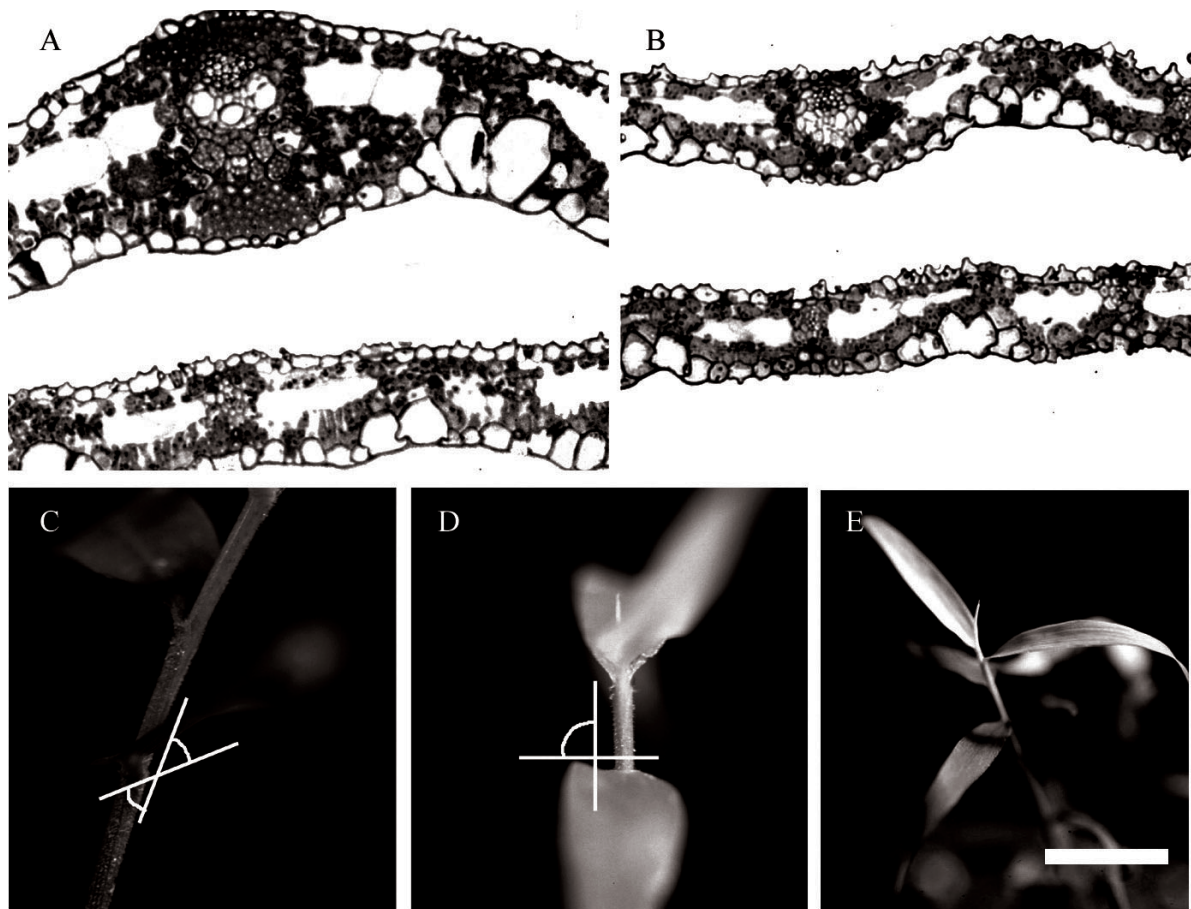


Fig. 5. Anatomy and morphology of a leaf from a 3-month-old *Bambusa edulis* wild-type and *vse* plantlet. (A) and (B): Transverse section of the leaves from the wild type and *vse* mutant, respectively (100 $\times$  magnification). (C) and (D): The angle of the leaf blade of the wild type and *vse*, respectively (bar = 4 mm). (E): Iron-deficient symptoms on the young leaves of *vse* (bar = 8 mm).

Chlorosis appeared in some of the young *vse* leaves (Fig. 5E). The angle of the wild-type leaf blade ( $44.6 \pm 5.5^\circ$ ,  $n = 17$ ) was more vertical than that of the *vse* mutant leaf blade ( $86.6 \pm 1.6^\circ$ ,  $n = 21$ ) (Fig. 5C–D), but the wild-type plantlet had fewer leaflets (*vse*: wild type =  $7.0 \pm 0.0$ :  $5.7 \pm 0.4$ ,  $n = 3$ ).

The morphology of the mesophyll cells changed in the *vse* mutant plantlets relative to the wild-type plantlets after 3 months of growth in the greenhouse (Fig. 6A–B). The number of chloroplasts and starch grains in the chloroplasts (Fig. 6C–D) decreased significantly in the mutant relative to the wild type (Table 2).

**ROOT STRUCTURE.** As a result of the reduced cell size and number, the diameter of the roots of the *vse* mutant plantlets cultured in vitro was significantly smaller than that of the wild-type roots (Table 3). Contrary to the situation observed in the stem, the diameters of the root xylem in both plantlet types were the same; however, the number of xylem cells was significantly reduced in the *vse* mutant (Fig. 7A–B; Table 3).

**CATION CONTENT.** The cation contents of the leaf samples removed from in vitro cultures of the *vse* mutant and wild type were almost identical when they were incubated in MS medium supplemented with  $0.1 \text{ mg} \cdot \text{L}^{-1}$  TDZ (data not presented). Following the transfer of the plantlets to the greenhouse, differences between the *vse* mutant and the wild type became apparent. The potassium content of the *vse* mutant was reduced relative to that of the wild type at 3 months (Table 4) as was the iron content at 2 months (Table 5). The levels of other cations, such as calcium, magnesium,



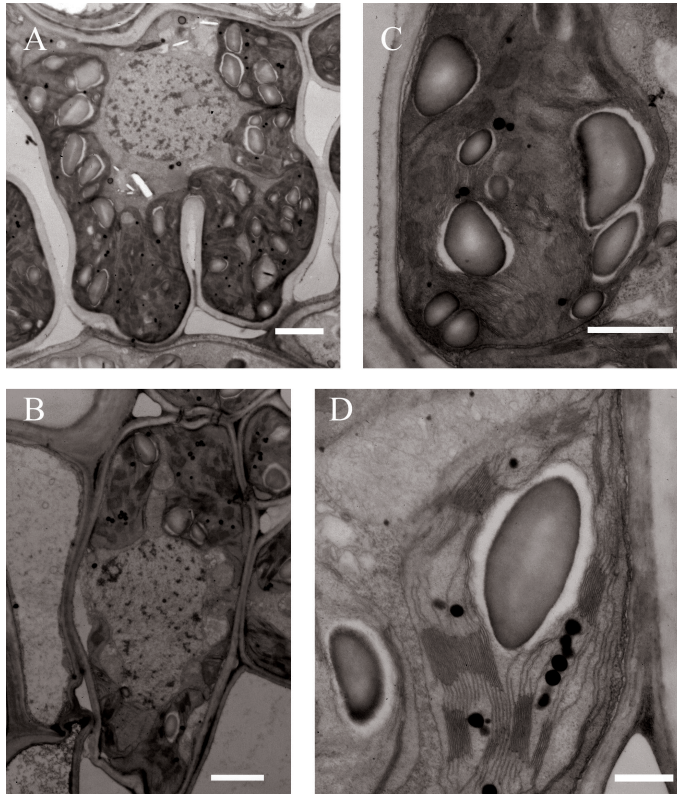


Fig. 6. Transverse electronic microscopy images of a leaf from a 3-month-old *Bambusa edulis* wild-type and *vse* plantlet. (A) and (B): Mesophyll of the leaves from the wild type (bar = 2  $\mu\text{m}$ ) and *vse* mutant (bar = 2  $\mu\text{m}$ ), respectively (100 $\times$  magnification). (C) and (D): The chloroplasts of the wild type (bar = 1  $\mu\text{m}$ ) and *vse* mutant (bar = 500 nm), respectively.

Table 2. Comparison of the mean number of chloroplasts and mean number of starch grains in *Bambusa edulis* leaves from 3-month-old wild-type and *vse* mutant plants.

	Chloroplast (no./cell)	Starch grain (no./chloroplast)
Wild type	11.0 a <sup>z</sup>	5.45 a
<i>vse</i>	7.2 b	1.90 b

<sup>z</sup>Means followed by the same letter are not significantly different according to the least significant difference at  $P < 0.05$  (Duncan, 1955). Data are the means of 10 mesophyll cells and 20 chloroplasts.

and zinc, appeared to be the same. The chlorosis observed in the upper young leaves of the *vse* mutant was probably due to an iron deficiency. In order to check for possible mineral imbalances, we determined potassium and iron concentrations in leaves at different positions on the plant. The potassium content of the first four leaves (upper) was higher than that of the last four (lower). Conversely, the concentration of iron in the lower four leaves was higher than that in the upper leaves (Table 6).

## Discussion

We have isolated a bamboo xylem mutant (*vse*) from multiple shoots subjected to continuous subculture for 7 years. Long-term subculture for the purpose of plant propagation is always full of risks due to a phenomenon known as somaclonal variation. In tobacco (*Nicotiana tabacum* L.) tissue culture, abnormalities are common in plants originating from long-term callus subculture, but they have not been found in those derived from short-term subculture (Syono and Furuya, 1972). Similarly, chrysanthemum [*Dendranthema morifolium* (Ramat.) Tzvel] plants derived from a 9-year-long callus culture were highly variable (Sutter and Langhans, 1981). In a variety of bamboo species, somaclonal variation has resulted in alterations in the color or shape of the stem. The bulbous internodes of *Bambusa ventricosa* McClure were lost following long-term tissue culture (Huang and Huang, 1995).

Somaclonal variation is most likely the cause of the genetic mutation that has resulted in the *vse* mutant. Although the mecha-

Table 3. Comparison of the root diameter, number of cells in the root section, xylem diameter, number of xylem cells and cortex cells, and size of the cortex cells in *Bambusa edulis* root during in vitro culture of the wild type and *vse* mutant. The medium is Murashige and Skoog (1962) basal medium supplemented with 5 mg·L<sup>-1</sup>  $\alpha$ -naphthaleneacetic acid.

	Root cells (no.)	Root diam ( $\mu\text{m}$ )	Xylem cells (no.)	Xylem diam ( $\mu\text{m}$ )	Cortex cells (no.)	Cortex cell size ( $\mu\text{m}$ )
Wild type	18.0 a <sup>z</sup>	570 a	6.0 a	27.8 a	5.6 a	31.2 a
<i>Vse</i>	13.3 b	297 b	3.0 b	23.8 a	5.3 a	17.6 b

<sup>z</sup>Means followed by the same letter are not significantly different according to the least significant difference at  $P < 0.05$  (Duncan, 1955). Data are from three independent stems (n = 3).

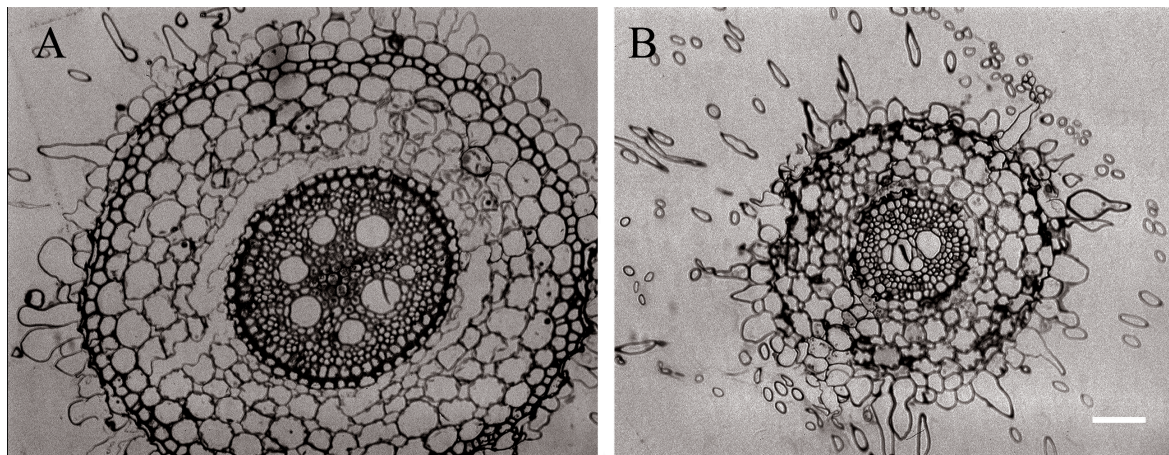


Fig. 7. Anatomy of the root from a 3-month-old *Bambusa edulis* wild type and *vse* plantlet. (A) and (B): Transverse section of a root from the wild type and *vse* mutant, respectively (100 $\times$  magnification, bar = 40  $\mu\text{m}$ ).

Table 4. Potassium concentration in *Bambusa edulis* wild-type and *vse* leaves under the different growth conditions.

	Potassium concn (%)		
	In vitro	3 months	6 months
Wild type	3.85 a <sup>2</sup>	2.08 a	1.76 a
<i>vse</i>	3.65 a	1.57 b	0.99 b

<sup>2</sup>Means followed by the same letter are not significantly different according to the least significant difference at  $P < 0.05$  (Duncan, 1955). Data are from three independent shoots ( $n = 3$ ).

Table 5. Calcium, magnesium, iron, and zinc concentrations in *Bambusa edulis* leaves of 2-month-old wild-type and *vse* plantlets.

	Calcium (%)	Magnesium (%)	Iron ( $\mu\text{g}\cdot\text{g}^{-1}$ )	Zinc ( $\mu\text{g}\cdot\text{g}^{-1}$ )
Wild type	0.38 a <sup>2</sup>	0.15 a	306.52 a	15.28 a
<i>vse</i>	0.43 a	0.13 a	131.40 b	11.07 a

<sup>2</sup>Means followed by the same letter are not significantly different according to the least significant difference at  $P < 0.05$  (Duncan, 1955). Data are from three independent stems ( $n = 3$ ).

Table 6. Potassium and iron concentrations in *Bambusa edulis* *vse* leaves at different developmental stages.

Nutrient concn	Potassium (%)	Iron ( $\mu\text{g}\cdot\text{g}^{-1}$ )
Upper (1st–4th)	2.47 a <sup>2</sup>	7.18 b
Lower (5th–8th)	0.99 b	49.44 a

<sup>2</sup>Means followed by the same letter are not significantly different according to the least significant difference at  $P < 0.05$  (Duncan, 1955). Data are from three independent stems ( $n = 3$ ).

nisms of somaclonal variations are not well known, it is accepted that chromosomal variation, mutagenesis, transposable element insertion, and DNA methylation are involved (see review by Jain, 2001). Explants with a large number of chromosomes and high-ploidy levels generally produce somaclonal variations resulting in aberrant plants more easily than those with a low number of chromosomes and low-ploidy levels (Creissen and Karp, 1985). *Bambusa edulis* contains 104 chromosomes (or 96 according to Chen et al., 2003), which implies a certain degree of chromosomal instability. However, there is a definite lack of information on the cytogenetics of *B. edulis* and, consequently, we cannot draw a definite conclusion regarding the chromosomal origin of the variation at this time.

In general, *vse* plants grew normally and did not exhibit any readily apparent morphological abnormalities under in vitro conditions other than that their leaves were slightly smaller than those of the wild type (Fig. 1). However, following the transfer of the plantlets to the greenhouse, the difference in growth habits between the *vse* mutant and the wild type increased over time, with the former showing reduced growth relative to the wild type (Fig. 2). A similar growth pattern was observed in an *Arabidopsis thaliana* (L.) Heynh xylem mutant. The *A. thaliana* *irx3* (irregular xylem3) mutant has a severe deficiency in secondary cell-wall cellulose deposition that leads to the collapse of xylem cells (Turner and Somerville, 1997). As in the bamboo *vse* mutant, the xylem elements of the *irx* mutant initially appeared to expand correctly, with most of the cells having a normal appearance, even in the young mature cell. The collapsed xylem phenotype of the *irx* mutant only became apparent in the mature stem, which is actively involved in the transport of water and nutrients (Turner and Somerville, 1997). Since tissue culture medium is generally rich in water and nutrients, explants need not develop a wider and

mature xylem for transport, thereby providing an explanation of why the xylems between the *vse* mutant and the wild type were not significantly different in vitro.

Any change in the diameter of a xylem vessel has a profound effect on the potential conductivity of the vessel. Cruz et al. (1992) pointed out that wider xylem vessels should contribute more to the overall hydraulic conductance of the xylem tissue than narrower vessels. Therefore, any increase in the diameter of the xylem vessel and/or number of xylem cells could promote stress resistance in crops. Singh and Sale (2000) observed that a wider diameter of the xylem vessels was correlated with high phosphorus levels in the plant, which in turn could increase white clover tolerance to drought. This observation could explain the fact that the *vse* mutant wilted quickly and had a low survival rate in the greenhouse.

Since xylem is the conductance pathway for water and nutrients throughout the plant, the thinner xylem vessels of the *vse* mutant could result in a nutrient deficiency following the transfer of the mutant plants to the greenhouse. We measured the concentrations of various cations in the *vse* mutants and wild-type plants grown in the greenhouse and found that the levels of potassium and iron were significantly reduced in the former compared to the latter (Table 1). Potassium is an osmotic regulator and can balance the negative charges within plant cells (Taiz and Zeiger, 2002), while iron is a component of many enzymes and light energy-transferring compounds involved in photosynthesis (Taiz and Zeiger, 2002). Since iron is an immobile nutrient, the deficiency appears first in the young leaves. The most common symptom of iron deficiency begins with an interveinal chlorosis of the youngest leaves, then evolves into an overall chlorosis, and ultimately ends in a totally bleached leaf (Taiz and Zeiger, 2002). Based on our results in bamboo, potassium is a mobile element but iron is an immobile one (Table 3).

Iron and potassium deficiencies were coincident. Previous investigations have also shown a relationship between these two cations. For example, Hart (1934) observed that maize and sugarcane plants showed an iron deficiency when they were deprived of potassium, while Hewitt and Bolle-Jones (1953) and Bolle-Jones (1955) observed that young chlorotic leaves were also potassium-deficient. These data indicate that a nutrient imbalance could result in deficiencies of other, seemingly unrelated nutrients. For example, a high application of phosphorus could hamper the mobility of iron (Alam, 1983). This relationship between potassium and iron balance in the *vse* mutant will be examined in a future investigation. However, it is possible that the cation contents of the *vse* mutant were affected by morphological alterations in the xylem cells as well as by impedance in element mobility due to the smaller diameter of the xylem vessels.

This paper is the first to report that the diameter of the xylem vessels and cation contents of the plant tissue are related in the monocot crop bamboo; specifically, that a change in the former growth characteristic can result in an alteration in the cation balance of bamboo plants. We are currently using a proteomic approach to investigate the genes involved in xylem development and mineral content in the bamboo, *B. edulis*.

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