

Dramatic changes in leaf development of the native *Capsicum chinense* from the Seychelles at temperatures below 24°C

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Abstract When a pepper cultivar (*Capsicum chinense* cv. Seychelles-2, Sy-2) native to the Seychelles was grown in Japan, all seedlings showed seasonal developmental abnormalities such as development of abnormally shaped leaves. Other pepper cultivars grew well in all seasons while the growth of cv. Sy-2 was stunted. In this study, we first examined the effects of various changes in temperature and photoperiod on the cv. Sy-2 phenotype. The results showed that temperatures lower than 24°C led to the formation of abnormal leaves. Second, morphological and anatomical analyses of cotyledons and true leaves developed at 28 and 20°C were conducted. The narrower and thicker cotyledons developed at 20°C had fewer palisade cells in the leaf-length direction, and more cells in the leaf-thickness direction. True leaves developed at 20°C were irregularly shaped, thicker and had smaller leaf area. In addition, true leaves developed at 20°C had fewer palisade cells in the leaf-length and leaf-width directions and had more cells in the leaf-thickness direction. Furthermore,

abnormal periclinal cell divisions in the mesophyll and/or epidermal cell layers were observed during leaf blade development at 20°C. These results suggest that the observed changes in cell proliferation and abnormal periclinal cell divisions were related, at least in part, to abnormal leaf development of cv. Sy-2 at temperatures below 24°C.

Keywords *Capsicum chinense* · Cell division · Leaf morphology · Pepper · Seychelles · Temperature sensitivity

Introduction

The Seychelles consists of about 100 islands between 4–10°S and 55–56°E in the Western Indian Ocean, stretching from 400 to 1,600 km from the coast of East Africa. The granitic islands are of Pre-Cambrian age (Miller and Mudie 1961) and, because of their long isolation, they are classified as ‘Oceanic islands’ although of continental origin (Baker and Miller 1963). The flora of the granitic islands of the Seychelles comprises many endemic species (Swabey 1970; Fleischmann et al. 2005).

Capsicum, a member of the *Solanaceae*, originates from, and was first domesticated in, South and Central America (Singh 2007). *Capsicum chinense* Jacq., which is popular in tropical regions, is thought to have been domesticated in the lowlands east of the Andes (Pickersgill 1969). *Capsicum* was introduced to Europe at the end of the fifteenth century, after the first voyage of Christopher Columbus, and its use spread rapidly over the Old World continents as a new source of fresh vegetable and spice.

In 1985, we collected seed samples from cultivated *Capsicum* of the Seychelles. When a pepper cultivar

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(*C. chinense* cv. Seychelles-2, Sy-2) native to the Seychelles was grown at Kyoto (35°N, 135°E) in Japan, newly developing leaves of all seedlings grown in spring and autumn showed abnormal morphology. In contrast, newly developing leaves in summer were normal. Because of the seasonal change of this developmental abnormality, day-length or temperature were predicted to affect the phenotype of cv. Sy-2. The Seychelles have a mild climate, with average temperatures ranging from 24 to 31°C during the year (Swabey 1970; Republic of Seychelles 2007). In the Seychelles, cv. Sy-2 is grown normally as a commercial pepper. Based on these observations, the phenotype of cv. Sy-2 seemed to be temperature sensitive. Peppers (*Capsicum annuum* L.) have an optimum growth temperature ranging from 21 to 29°C (Knott and Deanon 1967; Nonnecke 1989), and chilling injury is caused below 15°C (Harrington and Kihara 1960; Wang 1990). Other peppers, e.g., cv. Takanotsume (*C. annuum*) and cv. Fushimiamanaga (*C. annuum*) from Japan, cv. No.3341 (*C. chinense*) from Bolivia, cv. Habanero (*C. chinense*) and cv. Tabasco (*Capsicum frutescens* L.) from Mexico, and cv. No.3188 (*Capsicum baccatum* L.) from Peru, cultivated in our fields exhibited healthy growth in all seasons, while growth of cv. Sy-2 was stunted.

Temperature is one of the most important environmental factors affecting plant growth and development, as reflected by numerous research reports (Wallenstein and Albert 1963; Johnson 1967; Kane and Albert 1982; Deschamp and Cooke 1983; Sato et al. 2008). Furthermore, mutations in specific genes of *Antirrhinum majus* L. (Waites and Hudson 1995), *Nicotiana tabacum* L. (Samuelsen et al. 1997), and *Arabidopsis thaliana* (L.) Heynh. (Tsukaya et al. 1995; Pickett et al. 1996; Ozawa et al. 1998; Qi et al. 2004; Ichimura et al. 2006) have been reported to show clear phenotypes in a high/low temperature-sensitive manner.

The present study focuses on leaf morphogenesis in cv. Sy-2 as the most significant developmental abnormality. To investigate the environmental factors controlling abnormal leaf development of cv. Sy-2, the effects of various changes in temperature and photoperiod were examined. Also, morphological and anatomical analyses were conducted to document precisely how environmental factors affect the abnormal leaf development of cv. Sy-2.

Materials and methods

Plant materials and growth conditions

In 1985, we collected seed samples of cultivated *Capsicum* from the Seychelles. After observing the seasonal developmental abnormality in cv. Sy-2, we generated an inbred

line of cv. Sy-2 by self-fertilizing more than ten times. Seeds of cv. Sy-2 (*C. chinense*) from the Seychelles, cv. Takanotsume (*C. annuum*) and cv. Fushimiamanaga (*C. annuum*) from Japan, cv. No.3341 (*C. chinense*) from Bolivia, cv. Habanero (*C. chinense*) and cv. Tabasco (*C. frutescens*) from Mexico were sterilized with sodium hypochlorite solution containing 1% available chlorine and sown in a plastic container containing horticultural soil mix (Kureha, Tokyo, Japan). Plants were grown at various temperatures (20, 22, 24, 26 or 28°C), air humidity (50–80%), and photoperiods (8, 12 or 16 h) with a light intensity of 190 $\mu\text{mol m}^{-2} \text{s}^{-1}$ using cool white fluorescent lights. After germination, each plant was transplanted into a plastic pot containing 0.15 L horticultural soil mix. The temperature fluctuation was recorded by a thermo recorder (Tandd, TR-72S, Nagano, Japan). Also, F₁ and F₂ population were prepared by crossing cv. Sy-2 with cv. No.3341 to determine the inheritance pattern of the developmental abnormality.

Histology and anatomy

Leaf length and width were defined as the longest and widest parts of the leaf blade. We used the terms leaf-length direction, leaf-width direction, and leaf-thickness direction to describe directions of, and within, the leaf blade according to Tsuge et al. (1996). Leaves developed at 28 and 20°C were fixed in FAA (ethanol:water:formalin:acetic acid, 12:6:1:1 v/v) solution. For transverse sections, tissue samples were cut at the widest point of the leaf length. For longitudinal sections, tissue samples were cut along the midrib of the leaf. Samples were dehydrated in a graded ethanol series. Ethanol in the dehydrated samples was exchanged for Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany) by immersing the samples in liquid resin for 15 h. Samples were solidified in Technovit 7100 resin according to the manufacturer's protocol. Embedded samples were cut into 5 μm sections using a fully motorized rotary microtome (Leica, RM2155, Wetzlar, Germany). Sections were stained with a 0.05% toluidine blue solution for 10 min. The dyed sections were observed and photographed with a digital microscope (Keyence, VHX-100, Osaka, Japan). The average cell area was obtained from the measurement of at least 40 palisade cells in longitudinal and transverse sections. From these data, the total numbers of palisade cells in the leaf-length and leaf-width directions were estimated.

Statistical analysis

Data were expressed as means and standard error of the mean. Means were compared by a *t* test ($P < 0.01$).

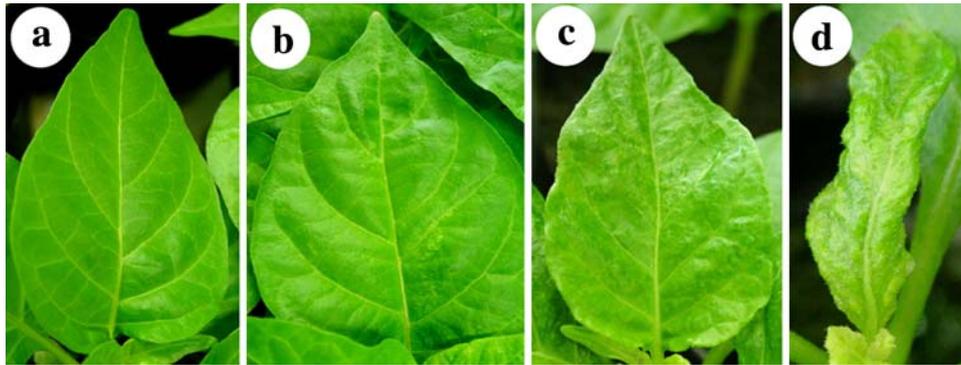


Fig. 1 Normal and abnormal morphologies observed at the fifth true leaves of *Capsicum chinense* cv. Seychelles-2 (cv. Sy-2). Abnormal leaf level (ALL) was quantified as follows: ALL 1, completely normal leaf (a); ALL 2, a few pale-green parts were observed in the leaf blade

(b); ALL 3, rough surface and pale-green parts observed over the whole leaf blade (c); ALL 4, abnormal shape (ex. asymmetric, wrinkled), rough surface and pale-green parts observed over the whole leaf blade (d)

Table 1 Relationship between growth temperature and abnormal leaf development of *Capsicum chinense* cv. Seychelles-2 (cv. Sy-2)

Growth temperature (°C)	Actual temperature range (°C)	Plants with abnormal leaves (%) ^a
28	28.0–29.0	0
26	25.5–26.3	0
24	23.5–25.5	0
22	21.8–23.6	100
20	19.0–20.7	100

^a (Plants with abnormal leaves/total number of plants) × 100. ALL3 and ALL4 (see Fig. 1) were judged to be abnormal leaves; 20 plants examined per treatment

Table 2 Relationship between day-length and abnormal leaf development of cv. Sy-2

Growth temperature (°C)	Actual temperature range (°C)	Day length (h)	Plants with abnormal leaves (%) ^a
28	28.0–29.0	8	0
		12	0
		16	0
22	21.8–23.6	8	100
		12	100
		16	100

^a (Plants with abnormal leaves/total number of plants) × 100; 20 plants examined per treatment

Results

Abnormal leaf development of cv. Sy-2 below 24°C

To identify environmental factors controlling abnormal leaf development in cv. Sy-2, the effects of various changes in temperature and photoperiod were examined. First, we examined the effect of temperature on abnormal leaf development by growing cv. Sy-2 at 28, 26, 24, 22, and 20°C. The abnormal leaf level (ALL) was quantified as follows: ALL 1, completely normal leaf (Fig. 1a); ALL 2, a few pale-green parts were observed in the leaf blade (Fig. 1b); ALL 3, rough surface and pale-green parts observed over the whole leaf blade (Fig. 1c); ALL 4, abnormal shape (ex. asymmetric, wrinkled), rough surface and pale-green parts observed over the whole leaf blade (Fig. 1d). We defined leaves showing abnormal shape and/or rough surface as ‘abnormal leaves’ (Fig. 1c,d). Abnormal leaves developed at 20 and 22°C, whereas normal leaves developed at 24, 26, and 28°C (Table 1). The actual temperatures of the 22 and 24°C treatments were 21.8–

23.6°C and 23.5–25.5°C, respectively. Thus, abnormal leaf development of cv. Sy-2 occurs only below 24°C. At 15°C, development of leaves was extremely slow or completely stopped (data not shown). Second, we examined the effect of photoperiod to abnormal leaf development. Regardless of photoperiod, cv. Sy-2 developed normal leaves at 28°C, whereas abnormal leaves developed at 22°C (Table 2). Leaves of cv. Takanotsume (*C. annuum*), cv. Fushimi-amanaga (*C. annuum*), cv. No.3341 (*C. chinense*), cv. Habanero and cv. Tabasco (*C. frutescens*) all developed normally even at temperatures below 24°C (data not shown). Thus, it was shown that temperatures lower than 24°C led to the formation of leaves with abnormal morphology. Furthermore, the inheritance pattern of the cv. Sy-2 phenotype was analyzed using F₁ and F₂ populations derived from a cross between cv. Sy-2 and cv. No.3341. The temperature-sensitive abnormal leaf phenotype segregated 0:1 in the F₁ population ($n = 15$) and 1:3 in the F₂ population ($n = 200$, P value = 0.76); thus, this phenotype seems to be controlled by a single recessive gene.

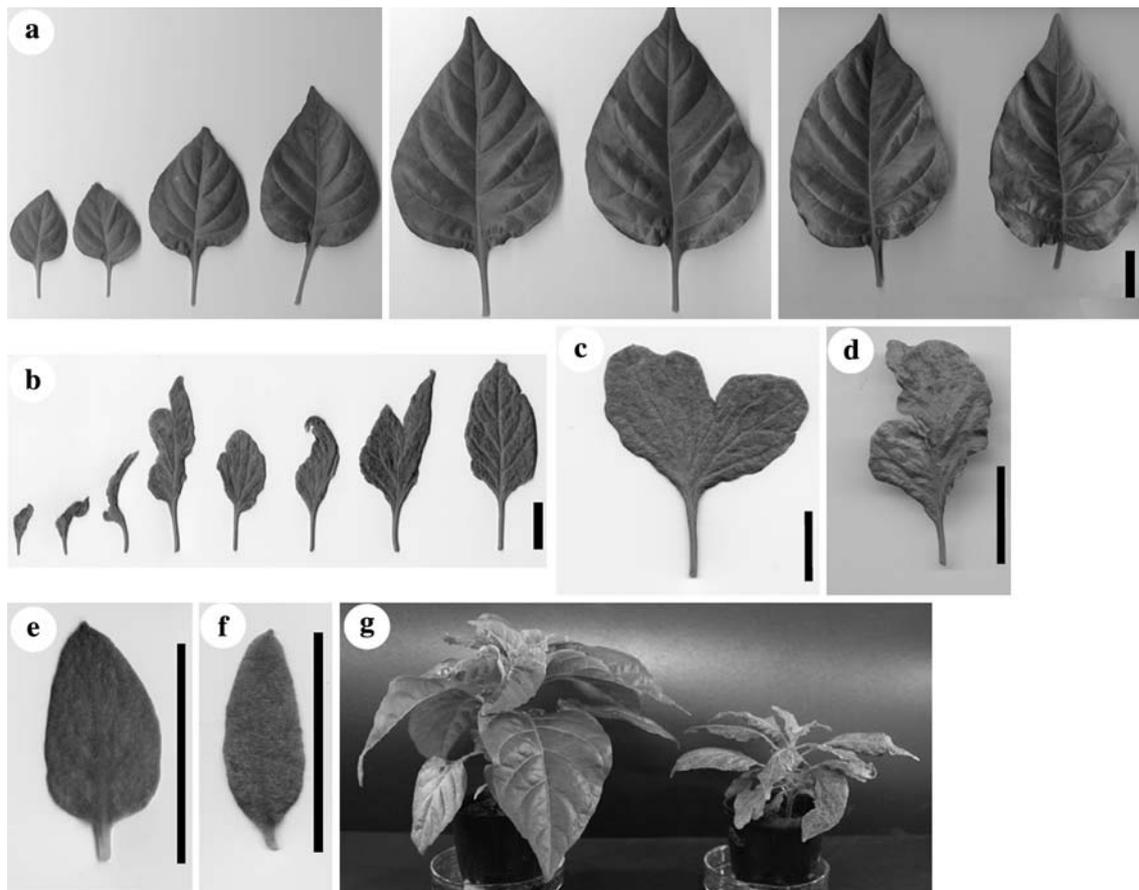


Fig. 2 cv. Sy-2 grown at 28 and 20°C. True leaves developed at a 28°C and b 20°C (first to eighth true leaves from left to right). Formation of c ectopic lamina and d wrinkled and lobed lamina at

20°C. Cotyledons developed at e 28°C and f 20°C. g Gross morphology of cv. Sy-2 grown at 28°C (left) and 20°C (right). Bars 2 cm

Morphological and anatomical analysis of cotyledons and true leaves of cv. Sy-2 developed at permissive and restrictive temperature

Morphological changes of cv. Sy-2 were analyzed in more detail by comparing plants grown at the permissive temperature of 28°C and the restrictive temperature of 20°C. At 28°C, cv. Sy-2 was morphologically normal (Fig. 2a, g). In contrast, cv. Sy-2 showed dwarfism and developed asymmetric true leaves at 20°C (Fig. 2b, g). In some cases, ectopic lamina (Fig. 2c) or wrinkled and/or lobed lamina (Fig. 2d) were formed. Cotyledons developed at 20°C were narrow compared with those developed at 28°C (Fig. 2e, f; Table 3).

As the morphological changes of cotyledons were somewhat more simple than those of true leaves, cotyledons were initially chosen for the anatomical study. Thicker cotyledons developed at 20°C than at 28°C (Fig. 3, Table 3). Fully expanded cotyledons at 20°C had fewer palisade cells in the leaf-width direction (77%), and more cells in the leaf-thickness direction (110%) (Table 3);

however, there was no significant difference in palisade cell size (Table 3).

The fifth true leaves had the normal size and shape of the true leaves (Figs. 2a, 4). Therefore, the fifth true leaves were chosen for the anatomical analyses. True leaves developed at 20°C were short in leaf-length direction, narrow in leaf-width direction compared with those developed at 28°C (Table 3), and also asymmetric (Fig. 2a, b). Thickness of fully expanded true leaves at 20°C severely increased compared with those at 28°C (Fig. 3, Table 3). Leaf thickness was constant through leaf blade at 28°C (Fig. 5a). In contrast, at 20°C, leaf thickness varied greatly at sites through curved leaf blade (Fig. 5b). Fully expanded true leaves at 20°C had fewer palisade cells in leaf-length direction (49%) and in leaf-width direction (43%) and had more cells in leaf-thickness direction (146%) (Table 3). Palisade cells were smaller than 28°C in leaf-thickness direction (73%) (Table 3). However, there were no significant differences in palisade cell size in leaf-length and width direction (Table 3).

Table 3 Morphological and anatomical analysis of the cotyledon and the fifth true leaf of cv. Sy-2 developed at 28 and 20°C. Values are means ± SE

	Cotyledon		True leaf	
	28°C	20°C	28°C	20°C
Leaf-length (mm)	17.2 ± 0.2	16.8 ± 0.3	125.6 ± 0.7	53.1 ± 2.5*
Leaf-width (mm)	9.3 ± 0.2	6.1 ± 0.1*	79.8 ± 1.4	28.7 ± 0.7*
Leaf-thickness (µm) ^a	277.8 ± 15.8	374.5 ± 13.3*	218.5 ± 4.0	369.7 ± 38.2*
Number of cells lined in				
Leaf-length direction ^b	341.6 ± 18.3	373.7 ± 8.7	3,786.9 ± 39.8	1,862.2 ± 117.8*
Leaf-width direction ^b	186.0 ± 11.5	142.8 ± 1.5*	2,513.2 ± 80.0	1,069.4 ± 85.6*
Leaf-thickness direction ^c	5.8 ± 0.1	6.4 ± 0.1*	5.6 ± 0.1	8.2 ± 0.5*
Cell size (µm) in				
Leaf-length direction ^b	53.0 ± 2.9	45.3 ± 1.6	33.2 ± 0.3	28.8 ± 1.7
Leaf-width direction ^b	49.7 ± 1.3	45.6 ± 1.8	31.9 ± 1.4	27.3 ± 1.7
Leaf-thickness direction ^b	61.2 ± 4.3	67.9 ± 1.8	70.5 ± 1.0	51.4 ± 2.6*

* $P < 0.01$ vs leaves developed at 28°C

^a At least 40 points measured in individual sections

^b Palisade cells were observed (see text)

^c Epidermal cells on the adaxial and abaxial sides of leaf were included. At least five leaves from five individual plants were examined for each treatment

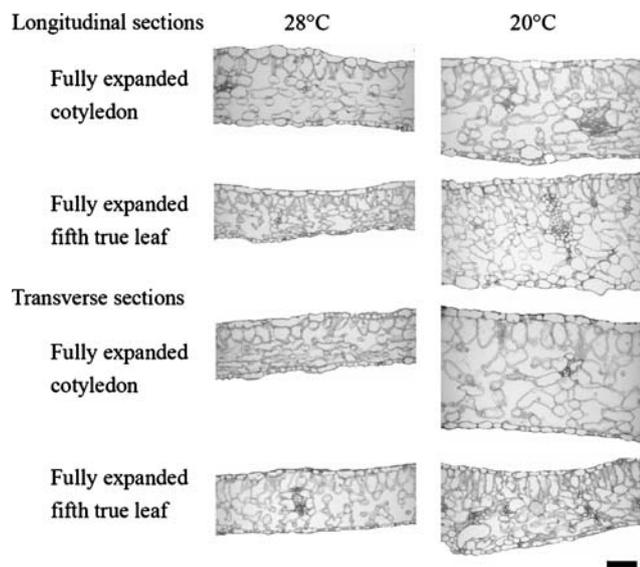


Fig. 3 Sections of fully expanded cotyledons and fifth true leaves of cv. Sy-2 at 28 and 20°C. The adaxial surfaces of the leaves are uppermost. Bars 100 µm

Transverse sections of leaves developed to leaf length (LL) 5, 10, 15 mm at 28 and 20°C were examined. During blade expansion, abnormal periclinal divisions in the mesophyll and/or epidermal cell layers were observed at 20°C (indicated by white arrowheads in Fig. 6f–h). Periclinal divisions typically involved clusters of cells. However, abnormal periclinal divisions were not observed at 28°C (Fig. 6b–d). Furthermore, transverse sections of leaf

primordia developed at 28 and 20°C were observed. At 20°C abnormal periclinal divisions were observed in the blade initiation sites of leaf primordium including epidermal cells (indicated by white arrowheads in Fig. 6e). Such abnormal periclinal divisions were not observed at 28°C (Fig. 6a).

Discussion

Abnormal leaf development of cv. Sy-2 below 24°C

The present study shows that the abnormal leaf development of cv. Sy-2 was caused by a small temperature decrease around 24°C (Table 1). Many studies have reported that day-length or differences in day and night temperatures affects plant growth and development (Went 1944; Dorland and Went 1947; Erwin et al. 1989; Myster and Moe 1995; Thingnaes et al. 2003); however, day-length did not affect the abnormal leaf development of cv. Sy-2 (Table 2). The optimum growth temperature for commonly cultivated peppers (*C. annuum*) is 21–29°C (Nonnecke 1989). Abnormal leaf development of cv. Sy-2 occurred below 24°C, well within the optimum growth temperature for peppers. Chilling injury to peppers occurs at temperature below 15°C (Harrington and Kihara 1960; Wang 1990). When the temperature falls below 15°C, growth of peppers is usually retarded, blossoms drop, and fruit-set ceases (Knott and Deanon 1967). However, the

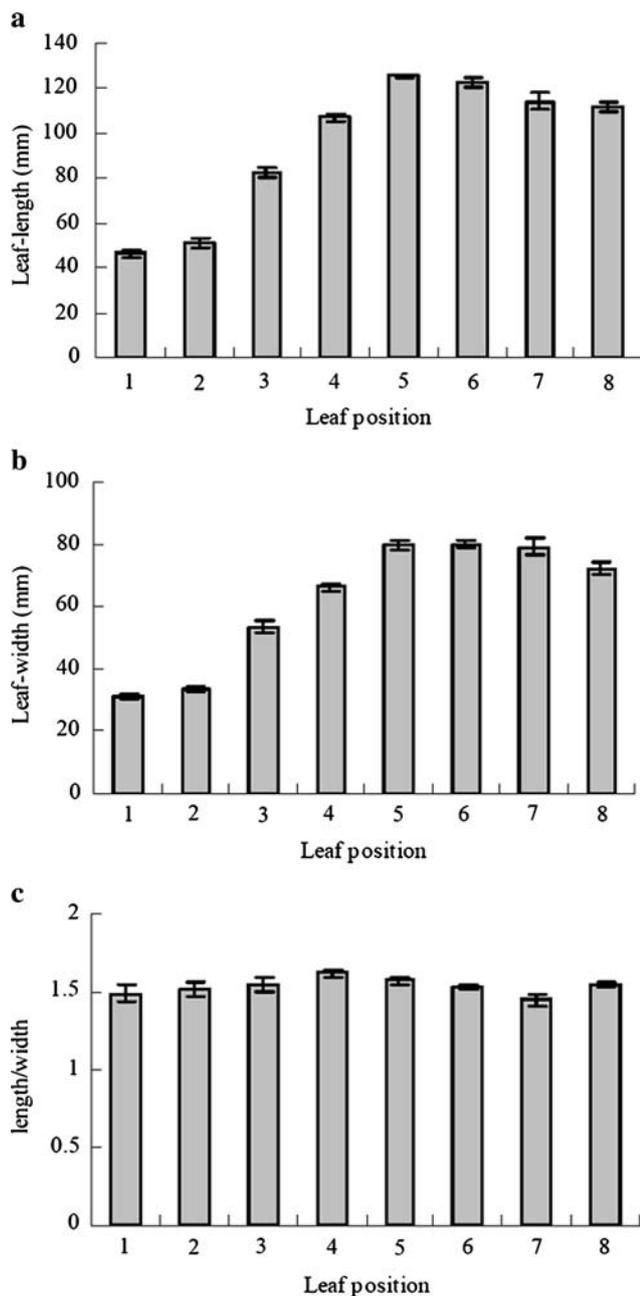


Fig. 4 Characterization of the true leaves of cv. Sy-2 developed at 28°C according to leaf position (x-axis). **a** Length, **b** width, **c** ratio of length to width of leaf blade. Error bars Standard error for results of five plants

developmental abnormalities of cv. Sy-2 below 24°C differed from the characteristics typical of chilling injury. In most case, no true leaves of cv. Sy-2 developed at 15°C (data not shown). Thus, abnormal leaf development of cv. Sy-2 is distinct from chilling injury.

The growth and development of laboratory-selected mutants has been reported to show a clear phenotype in a low-temperature-sensitive manner below temperatures of ~24°C. The *phantastica* mutants of *Antirrhinum majus*

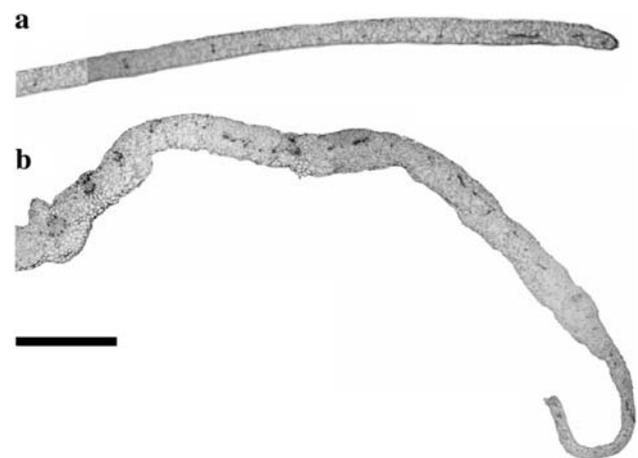
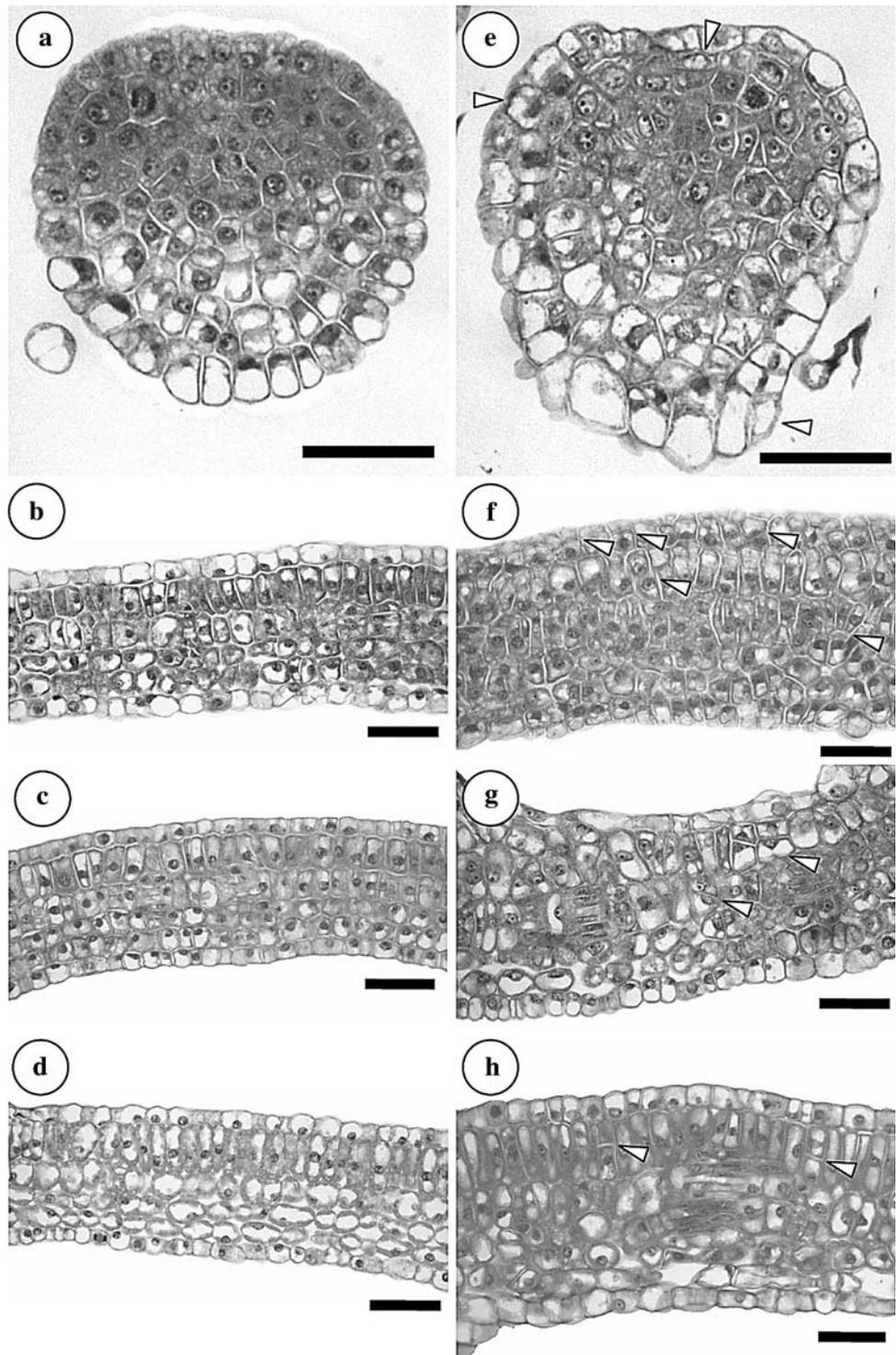


Fig. 5 Transverse sections of fully developed fifth true leaves of cv. Sy-2 at 28 and 20°C. Leaves developed at **a** 28°C and **b** 20°C. Bar 1 mm

develop leaves that lack dorsal cell types at 20°C, though leaves at 25°C are similar to those of wild-type (Waites and Hudson 1995). The *temperature dependent shooty* mutant of *Nicotiana tabacum* develops thick, narrow leaves at 21°C, although application of high temperature (30°C) reverses the mutant phenotype (Samuelson et al. 1997). The *acaulis2* mutant of *Arabidopsis thaliana* shows reduced length of inflorescence at 22°C, whereas it is almost normal at 28°C (Tsukaya et al. 1995). The *mekk1* mutant shows severe dwarfism at 22°C, whereas it is greatly suppressed at 28°C (Ichimura et al. 2006).

The temperature sensitivity of mutants can be caused by the structural instability of an expressed protein or because the underlying genetic pathways are inherently sensitive to temperature. The present study showed that the temperature-sensitive phenotype of cv. Sy-2 seemed to be controlled by a single recessive gene. Whether the temperature-sensitive mutation of cv. Sy-2 occurred in the Seychelles or not cannot be explained by the present study. However, as the minimum temperature seldom drops below 24°C throughout the year in the Seychelles (Swabey 1970; Republic of Seychelles 2007), it can be assumed that the temperature-sensitive phenotype of cv. Sy-2 will not be a problem for pepper production. Several studies have discussed the maintenance of cryptic mutations and their manifestation due to environmental changes such as a moderate change in temperature, and have provided evidence that HSP90 can serve as a buffer for cryptic mutations in *Drosophila melanogaster* and *A. thaliana* (Rutherford and Lindquist 1998; Queitsch et al. 2002; Sangster et al. 2008). Further study is needed to clarify whether the moderate climate of equatorial oceanic regions functions to maintain the temperature-sensitive mutations of other plants.

Fig. 6 Sections of fifth true leaves of cv. Sy-2 at 28 and 20°C. Transverse sections of leaf primordia developed at **a** 28°C and **e** 20°C. Sections of leaves **b** leaf length (LL) 5 mm, **c** LL 10 mm and **d** LL 15 mm developed at 28°C. Sections of leaves **f** LL 5 mm, **g** LL 10 mm and **h** LL 15 mm developed at 20°C. *White arrowheads* Abnormal periclinal divisions. *Bars* 50 µm



Morphological and anatomical analysis of cotyledons and true leaves of cv. Sy-2 developed at permissive and restrictive temperatures

Temperature-dependent developmental changes of cv. Sy-2 leaves at the restrictive temperature of 20°C were analyzed morphologically and anatomically. Cotyledons developed

at 20°C were narrower and thicker compared with those developed at 28°C (Fig. 2e, f; Table 3). As there was no significant difference in the palisade cell size, the smaller cell number in the leaf-width direction and the greater cell numbers in the leaf-thickness direction might be related to the narrow and thick-shaped cotyledon at 20°C (Fig. 2e, f; Table 3). A similar leaf phenotype is observed in the

angustifolia3 (an3) mutant, whose plate meristem activity is decreased (Horiguchi et al. 2005). However, compensation by increase in cell size was not observed in the cv. Sy-2-like *an3* mutant.

True leaves developed at 20°C had a smaller leaf area than those developed at 28°C (Fig. 2a, b; Table 3). As there was no significant difference in palisade cell size, the decrease in cell number in both the leaf-length and leaf-width directions might be related to the decreased leaf area at 20°C (Table 3). At 28°C, six cell layers in the leaf blades seemed to be maintained by a strict pattern of anticlinal division of each cell relative to the surface (Fig. 5a; Table 3). In contrast, the leaf thickness of true leaves increased and varied greatly at sites throughout the curved leaf blade at 20°C (Fig. 5b; Table 3). Moreover, the number of cells in the leaf-thickness direction increased in the thicker true leaves of cv. Sy-2 at 20°C (Table 3). The abnormal periclinal divisions in mesophyll cell layers during leaf blade expansion at 20°C might contribute to the formation of thicker leaves (Fig. 6f–h). Also, the variation in thickness throughout the leaf blade might be related to the rough surface of the leaf blade. Abnormal periclinal divisions were also observed in the epidermal cell layers (Fig. 6e, f). Donnelly et al. (1999) proposed that the basic mechanism of cell divisions in epidermal and mesophyll layers is presumably common, while regulatory pathways differ. At 20°C, cv. Sy-2 might have defects in both regulatory pathways. Furthermore, abnormal periclinal divisions were observed in the blade initiation sites of leaf primordium developed at 20°C (Fig. 6e). However, how these abnormal periclinal divisions in the blade initiation sites affect the abnormal leaf development of cv. Sy-2 remains unclear. At 20°C, asymmetric true leaves developed and, in some cases, ectopic lamina (Fig. 2c) or wrinkled and/or lobed lamina (Fig. 2d) were formed. The mechanism whereby these characteristics are controlled remains largely unknown (Tsukaya 2006). In conclusion, our study suggests that changes in cell proliferation and abnormal periclinal cell divisions are, at least in part, related to the abnormal leaf development of cv. Sy-2 at temperatures below 24°C.

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