Hormonal Morphogenesis of Mulberry Tree Mutant©

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Mulberry tree mutant 'Garyu' exhibited bending on its stem similar to dwarf mutant of trifoliate orange tree 'Hiryu'. Hormonal treatment by GA_3, (S)-(+)-ABA, and their mixture (GABA) showed that normal stem mulberry types, 'Ichibei' and 'Ryoumenguwa', responded to these hormones, whereas bending-stem mutant mulberry, 'Garyu', and hybrid mulberry, 'Shin-ichinose', did not respond to them. Through genetic analysis, we show that mulberry had hormonal related genes *GIA* and *BRI1* homolog genes.

INTRODUCTION

Mulberry trees (*Morus* sp.), species belonging to Moraceae family, have many uses. Sanchez (2002) noted that the uses of mulberry were for sericulture, fruit, wood, landscaping, and forage. However most of the mulberry production in Japan is to produce foliage for sericulture (rearing of silkworms for the production of raw silk).

Mulberry farm management is mostly designed to have high productivity of foliage and highly nutritive leaves for the silkworm. Tree pruning is one of the agronomic treatments frequently used to control the height of the mulberry tree so it will be easier for farmers to harvest the foliage. Hormonal control of tree developmental has been used for improvement in several plant species. Trifoliate orange tree is one example of a plant species that has been successfully used as dwarf-controllable stocks related to hormonal control in citrus tree breeding (Lliso et al., 2003).

Mulberry has many genetic variations in Japan (Machii et al., 2001). we studied the hormonal control of morphogenesis in the mulberry mutant 'Garyu', which has morphological growth similar to the trifoliate orange tree mutant 'Hiryu'.

MATERIALS AND METHODS

Four mulberry tree genotypes, 'Garyu', 'Ichibei', 'Ryoumenguwa', and 'Shin-ichinose', were used in our study. We first compared the distinctive morphological characteristics of 'Garyu' with the wild type 'Ichibei'. Next we carried out gibberellic acid and abscisic acid hormonal treatments and observed the effects of these treatments on their morphological characteristics. We also performed genetic analysis on mulberry 'Garyu' and 'Kokuso 16' to determine the hormonal-related genes in mulberry. Steps in genetic analysis were PCR, cloning, and sequencing.

RESULTS

Morphological Observation. Observation on 'Garyu' showed that it exhibited stem bending similar to that of the trifoliate orange tree mutant 'Hiryu' (Figs. 1A and B). Distinctive stem characters on mulberry were measured in the mid-summer. Distinctive shoot characters observed were: length, internode diameter, thickest internode diameter, the thinnest internode diameter, internode number, internode distance, the longest internode, and the shortest internode. Morphological

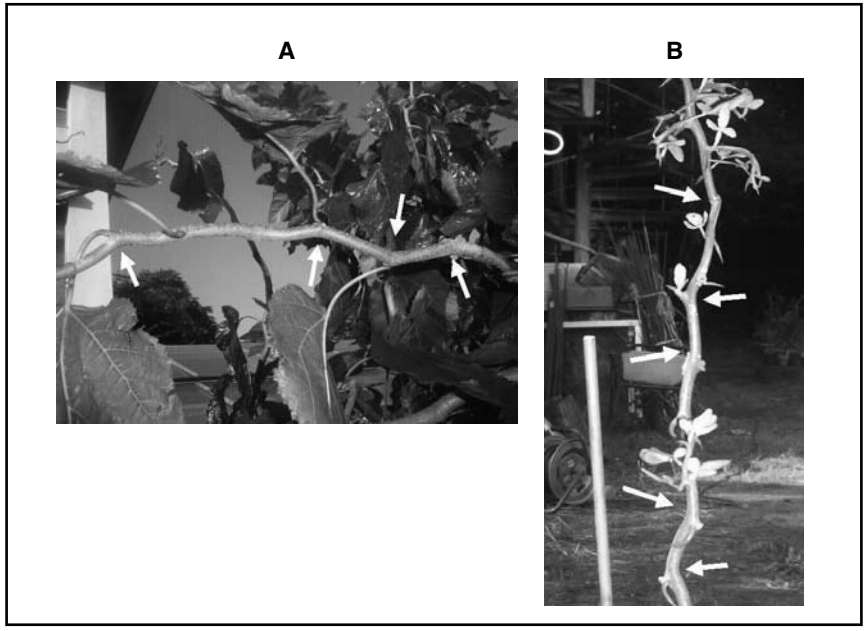

Figure 1. Stem morphology of mulberry 'Garyu' in field-grown (a) and trifoliate orange tree 'Hiryu' (b). Arrows showed some of the nodes position that exhibit bending stem characters.

characteristics that were found to be significantly difference between 'Garyu' and 'Ichibei' were only internode distance and the longest internode of the branch (Table 1). Internode distance of 'Garyu' was shorter than the wild type 'Ichibei'. These internodal distance measurement results are in agreement with the list of mulberry genetic resources that show mulberry 'Garyu' had a relatively short internode distance among 260 mulberry genotypes (Machii et al., 2001). The average of the longest internode distance was also significantly shorter in 'Garyu' than 'Ichibei' (Table 1).

Hormone Treatment Experiment. Treatment with GA_3 and (S)-(+)-ABA did not significantly affect the observed characters in the mutant 'Garyu'. Similarly, in the mulberry hybrid 'Shin-ichinose' the observed characters were not significantly affected by treatment with GA_3 and (S)-(+)-ABA. Responsive effects were found in wild type mulberry 'Ichibei' and the leaf developmental mutant 'Ryoumenguwa' (Table 2).

Mulberry wild type 'Ichibei' was grown dominantly to the vegetative phase (Fig. 2A); on the other hand, mulberry 'Garyu' was grown dominantly to generative phase (Fig. 2B).

Genetical Analysis. Two bands indicating gene fragments were found in agarose gel electrophoresis result of mulberries PCR product using *GAI*-gene-generated primers and in case of PCR result using *BRI1*-gene-generated primers on mulberries, there were three bands indicating gene fragments appeared (data not shown). We cloned a band of each PCR product and sequenced them. We found that from

Table 1. Variables that were measured at shoot in mulberry mutant 'Garyu' and wild-type 'Ichibei' in mid-summer.

Variables	N	Measurement 'Garyu'	Measurement 'Ichibei'	unit	t value
Shoot length	6	139.67 ± 43.72	178.33 ± 31.42	cm	-1.7594
Internodes diameter	6	8.80 ± 0.49	9.22 ± 1.50	mm	-0.6555
Thickest internodes	6	15.61 ± 1.92	15.70 ± 3.78	mm	-0.0529
Thinnest internodes	6	2.44 ± 0.57	3.19 ± 0.79	mm	-1.8870
Internodes number	6	38.8 ± 11.7	33.0 ± 7.4		(0.8221)
Internodal distance	6	3.74 ± 0.45	5.46 ± 0.46	cm	-6.4959*
Longest internodes	6	6.57 ± 0.52	9.60 ± 1.35	cm	-5.1387*
Shortest internodes	6	1.08 ± 0.44	1.82 ± 0.69	cm	-2.1858

Asterisk mark (*) on t value column indicates that there is a significance difference in 95% confidence interval among variable measurement in mulberry mutant 'Garyu' and wild-type 'Ichibei'. Minus value on t value column indicate that variable measurement of mulberry mutant 'Garyu' is lower than wild-type 'Ichibei'. Number in parentheses on t value column indicated that the t value was calculated by using logarithmic transformed data. n = number of samples that were measured for each genotype.

Table 2. Effect of hormones treatment to mulberry (4 days after treatment).

Mulberry genotype	Number of Flower		Number of Leaf		Remark.
'Garyu'	2.35	ns	0.33	ns	No effect
'Ichibei'	6.45	*	0.59	ns	Effect
'Ryoumenguwa'	3.79	*	2.61	ns	Effect
'Shin-ichinose'	0.83	ns	1.88	ns	No effect

* Significant; ns, not significant

mulberry 'Kokuso16' the first PCR product had homology to members of DELLA protein (Table 3) and from 'Garyu' the second PCR product had homology to conserved region of *BRI1* gene, LRR (leucine-rich repeat) (Table 4).

DISCUSSION

'Garyu' has a bending stem character. The bending character was similar to trifoliate orange tree 'Hiryu'. Experiment with GA_3 and (S)-(+)-ABA treatments on 'Garyu' showed that it was not affected by the application of either hormone. 'Garyu' did not respond to GA_3, (S)-(+)-ABA or the mixture GA_3 and (S)-(+)-ABA in both vegetative and generative phase.

Phytohormone GA_3 and growth inhibitor (S)-(+)-ABA have roles in development of morphological stem characters. The GA can enhance internodal cell elongation that further will promote stem elongation and plant height in rice (Kende et al., 1998). The ABA can reduce cell elongation and further reduce de-etiolation in

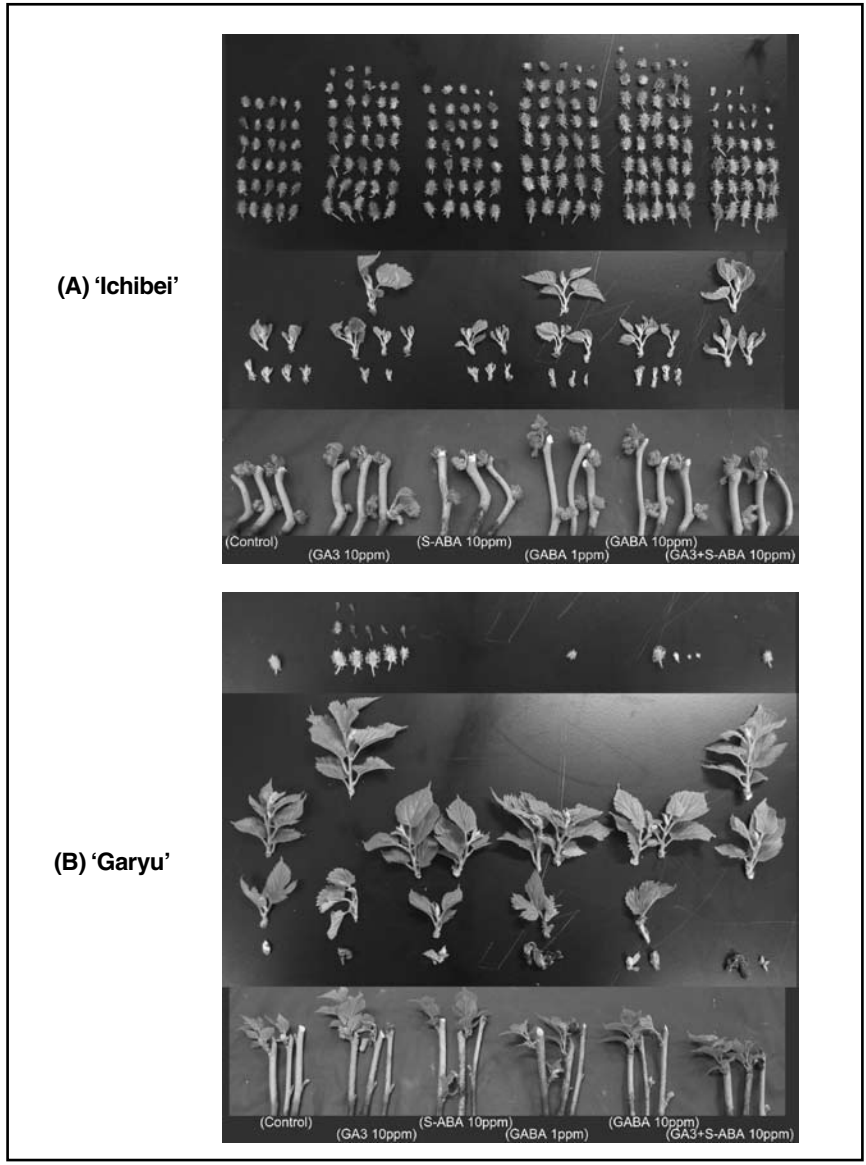

Figure 2. Morphological growth conditions of mulberry wild type 'Ichibei' (a) and bending stem mutant 'Garyu' (b). Label f indicates generative phase (flowers) and label l indicates vegetative phase (leaves at shoots). Each photo of mulberry hormones treatment responses were arranged as follow: Control, GA_3 10 ppm, S-(+)-ABA 10 ppm, GABA 1 ppm, GABA 10 ppm and mixture GA_3+S-(+)-ABA 10 ppm.

Table 3. Homology of *GAI* gene fragment clone sequence of mulberry 'Kokuso 16' with other plants.

Plants	Homology (%)	Description	Gene bank accession	Sequences hit number
Brassica rapa	87	DELLA protein (RGA2) gene	AY928550.1	178/207
Arabidopsis thaliana	86	RGA1 (repressor of GA1-3 1); transcription factor (RGA1) mRNA	NM_126218.2	105/122
Vitis vinifera	82	GAI-like protein 1 (GAI1) gene	AF378125.1	136/164
Cucurbita maxima	83	Gibberellic acid insensitive phloem (GAIP) mRNA	AY326306.1	178/214

Table 4. Homology of *BRI1* gene fragment clone sequence of mulberry 'Garyu' with other plants

Plants	Homology (%)	Description	Gene bank accession	Sequences hit number
Mangifera indica	85	Putative leucine-rich repeat protein gene	AY776277.1	265/308
Capsella rubella	86	ORF1, ORF2, ORF3, ORF4, ORF5 and ORF6	AJ303349.1	314/379
Arabidopsis thaliana	82	BRL1 (BRI 1 LIKE); kinase BRL1 mRNA	NM_104437.1	355/453
Daucus carota	81	LRR-S/T-RLK mRNA for putative leucine-rich repeat-type serine/threonine receptor-like kinase	AB178084.2	312/386

tomato (Fellner et al., 2000). Although they have contrary functions, the mixed application of GA and ABA has been shown to promote the generative phase in long-day plants (Kamuro et al., 2001).

In dwarf rootstock of orange tree as offspring of dwarf trifoliate orange tree, GA_3 takes a role in regulation of vegetative and generative phase. Competition among generative and vegetative phases as a result of GA regulation developed dwarf-type plants (Lliso et al., 2003). In the case of 'Garyu' in our experiment, the developmental phase during GA_3 and (S)-(+)-ABA application whole seedlings were dominantly developed into the generative phase and completed the vegetative phase. This case resulted in ineffective response of GA_3 and (S)-(+)-ABA application.

Muangprom et al. (2005) showed that gibberellic acid promoted stem growth by causing degradation of DELLA proteins via the ubiquitin-proteasome pathway. The most widely utilized dwarfing alleles in wheat (*Triticum aestivum*; e.g., *Rht-B1b* and *Rht-D1b*) encoded gibberellin-resistant forms of a DELLA protein that function as dominant and constitutively active repressors of stem growth. The other members of DELLA proteins were *RGA1* of *Arabidopsis thaliana*, *GAI* of *Vitis vinifera*, and *GAIP* of *Cucurbita maxima*, *GRAS* of *Musa* × *paradisiaca*, dwarf 8 of *Zea mays*, *SLN1* of *Hordeum vulgare*, and *rht-D1a*.

We found the homolog of *GAI* gene fragment in mulberry 'Kokuso 16' and also conserved region of *BRI1* gene fragment, which was leucine-rich repeat (LRR) in mulberry 'Garyu' in our genetical analysis. Zhou et al. (2004) mentioned that *BRI1*-like receptor kinase (*BRL1*) was identified as an extragenic suppressor of a weak *bri1* allele, *bri1-5*, in an activation-tagging genetic screen for novel brassinosteroid (BR) signal transduction regulators. The *BRL1* encodes a LRR receptor-like protein kinase (LRR-RLK). They showed that in *A. thaliana* sequence alignment revealed that *BRL1* is closely related to *BRI1*, which is involved in BR perception.

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