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This year three Japanese scholars – Profs. Yoichiro Hoshino (Hokkaido University), Eriko Yasunaga (University of Tokyo) and Tomonori Kawano (University of Kitakyushu) – joined the editorial team of Advances in Horticultural Science (AHS). This is surely a milestone in the century-long history of this traditional journal of Italian origin, which is on its way to a drastic shift towards successful internationalizations. To boost the link between European horticultural science communities, chiefly in Florence, Italy, and those in Asian counties, firstly Japan, we held a memorial workshop entitled "**First Advances in Horticultural Science-supported Workshop in Asia – Plant-sensing Technologies for Agricultural Application**s" in February 2014, with aid from the West Japan Industry and Trade Convention Association. The workshop took place at **Kitakyushu Science and Research Park** in Kitakyushu, Japan, where the first international branch of the **International Laboratory of Plant Neurobiology** (**LINV**-DiSPAA, Department of Agri-Food and Environmental Science, University of Florence) was established two years ago (known as LINV@Kitakyushu).

This special issue which has attracted environmental and horticultural specialists based in Japan, is one such effort aimed at enhancing an exchange of knowledge among scientific communities world-wide, especially between Italian and Japanese horticultural science specialists and environmental science specialists. Responding to our invitation, a number of submissions were received. Amongst them, 10 articles selected after the peer-review process are collected in this special issue; additional contributions (13) from Japanese institutions will appear in the next forthcoming issue (4, 2014) of this journal.

Here, as special issue guest editor, I since rely express my deepest acknowledgement to all contributing authors, editorial team members, and colleagues in Florence for enabling the release of this memorial issue. I hope it may stimulate further discussion and international research collaborations among European and Asian scientific communities.

Tomonori Kawano

Key speakers at *First Advances in Horticultural Science-supported Workshop in Asia.* Prof. Y. Hoshino, Hokkaido University (top, left); Prof. E. Yasugnaga, University of Tokyo (top, right); Dr. D. Comparini, LINV@Kitakyushu (middle, left); Dr. E. Masi, University of Florence (middle, right); Prof. S. Mancuso, University of Florence (bottom, left); Prof. T. Kawano, University of Kitakyushu and LINV@Kitakyushu (bottom, right).

Adv. Hort. Sci., 2014 28(2): 53-63

Molecular cloning and characterization of ABCG/PDR-type ABC transporter in grape berry skin

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Key words: full-size ABCG transporter, gene expression, grape, resveratrol, UV.

Abstract: Grape (*Vitis vinifera* L.) skin contains the phenolic compound resveratrol which is important not only for resistance to biotic and abiotic stresses but also for human health. However, little is known about resveratrol transport in plant cells. ABC (ATP binding cassette) transporters are well-known transporters responsible for secondary metabolite accumulation in plants. Previous reports speculated that the full-size ABCG transporter pleiotropic drug-resistant (PDR) is involved in resveratrol transport in fungi and plants. In this paper, all full-size ABCG transporters found in the grape genome database are listed and focus is placed on *VvABCG44/VvPDR14* as a candidate resveratrol transporter. The full-length cDNA of *VvABCG44* was cloned by RT-PCR using mRNAs extracted from grape berry skin. *VvABCG44* expression was induced by UV irradiation, and the expression pattern of *VvABCG44* in various grape organs was similar to that of stilbene synthase (STS), a key enzyme in resveratrol synthesis. Resveratrol content in grape berry skin increased after UV irradiation. These results suggest that VvABCG44 functions as a resveratrol transporter in grape.

1. Introduction

Grape (*Vitis vinifera* L.) is an economically important fruit crop, served fresh and used for wine production. Grape is one of the most studied fruit crops, given that the grape genome sequence is available (Jaillon *et al.,* 2007; Velasco *et al.,* 2007). Grape skin contains several phenolic compounds, such as anthocyanin, resveratrol, and catechin, which are important not only for resistance to biotic and abiotic stresses but also for berry qualities such as color, astringency, and human health benefits (Kader, 2002; Steyn, 2009). Resveratrol, a stilbenoid accumulating in the grape berry, is a key compound in the "French paradox" (Renaud and De Lorgeril, 1992) and is attracting attention in medicine and food science.

ATP binding cassette (ABC) transporters are well-known transporters responsible for secondary metabolite accumulation in plants (Yazaki, 2006). They form a large gene family and are found in all living organisms (Rea, 2007). Plants have much larger numbers of ABC transporters than animals or microorganisms: *Arabidopsis* and rice have more than 120 ABC proteins (Rea, 2007; Yazaki *et al*., 2009; Kretzschmar *et al*., 2011).

ABC transporters have a transmembrane domain (TMD) and a nucleotide-binding domain (NBD) comprising ATP-binding Walker A and B motifs (Martinoia *et al*., 2002). ABC transporters are classified into eight subfamilies (ABCA-H) according to their structure and sequence similarity. Half-size ABC transporters contain one TMD and one NBD, whereas full-size ABC transporters contain two repeats of the structure of half-size ABC transporters, two TMDs and two NBDs (Verrier *et al*., 2008).

The substrate specificity of ABC transporters is broad, and plant ABC transporters have been reported to transport various compounds, such as secondary metabolites, heavy metals, lipids, chlorophyll catabolites, xenobiotics, and plant hormones (Rea, 2007; Yazaki *et al*., 2009). ABC transporters show different localizations, such as the plasma membrane, vacuole, ER, Golgi apparatus, mitochondrion, and peroxisome; subcellular localizations of ABC transporters in the same subfamily are not always the same (Yazaki *et al*., 2009; Kretzschmar *et al*., 2011).

The ABCG subfamily is a major ABC transporter subfamily. It contains both half-size transporters, called the

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white-brown complex (WBC) subfamily, and full-size transporters, called the pleiotropic drug-resistant (PDR) subfamily. One of the best studied full-size ABCG subfamily members is yeast PDR5 (Lamping *et al*., 2010; Prasad and Goffeau, 2012). PDR5 is an exporter in yeast plasma membrane and is associated with multidrug resistance (Decottignies and Goffeau, 1997; Golin *et al*., 2007). In plants, full-size ABCG transporters have been reported to transport phytoalexins (Banasiak *et al*., 2013), abscisic acid (ABA) (Kang *et al*., 2010), strigolactone (Kretzschmar *et al*., 2012), and other compounds.

A strain of the plant pathogenic fungus *Botrytis cinerea*, lacking a full-size ABCG transporter BcatrB, is sensitive to resveratrol (Schoonbeek *et al*., 2001). On the other hand, after treatment with an elicitor, cyclodextrin, grape culture cells accumulated resveratrol and full-size ABCG transporter genes were induced in the cells (Zamboni *et al*., 2009). These results suggest that full-size ABCG transporters are associated with resveratrol transport in fungi and plants.

In this study, we listed all full-size ABCG transporters (PDRs) found in the grape genome database and focused on *VvABCG44/VvPDR14* as a candidate resveratrol transporter. We cloned the full-length cDNA of *VvABCG44* and determined its gene expression in various organs and after UV irradiation. *VvABCG44* expression was induced by UV irradiation and the expression pattern of *VvABCG44* in various grape organs was similar to that of stilbene synthase (STS), a key enzyme in resveratrol synthesis. To the best of our knowledge, this is the first report of an ABCG transporter in grape.

2. Materials and Methods

Plant material and treatments

Vitis vinifera "Pinot Noir" was harvested in the vineyards of the AZUMI Apple Corporation in Nagano Prefecture and of Nagoya University in Aichi Prefecture, Japan. For molecular cloning and gene expression analysis, young leaves, mature leaves, tendrils, stems, seeds, pulp, and berry skin were harvested in June and July. For molecular cloning, the skin of the berries after UV irradiation was used. For UV irradiation and ABA treatment analyses, the grape berry clusters were harvested in June and July, before the veraison stage. UV irradiation and ABA treatment were performed as described below.

Berry clusters were irradiated using a UV-C lamp (253.7 nm, GL-15, TOSHIBA, Japan) at a 50-cm distance for 1 h. Control samples (dark) were covered with a box and placed beside the sample receiving UV irradiation. For RNA extraction, the skin of the berries was collected immediately after UV irradiation. For measurement of resveratrol content, after UV irradiation, berry clusters were maintained for 23 h in the dark at room temperature and then the skins of the berries were collected.

Berry clusters were sprayed with 960 mM ABA containing 0.05% (v/v) Tween 20 and maintained in the dark at room temperature for 48 h. Control samples (water) were sprayed with water containing 0.05% (v/v) Tween 20 and placed beside the ABA-treated samples. After treatment, the skins of the berries were collected.

Three biological replicates were assayed for each treatment.

Identification of full-size ABCG transporter genes in the 12× version 1 of Vitis vinifera *genome*

Full-size ABCG transporters in grape were searched with BLAST (Basic Local Alignment Search Tool) at NCBI (http://www.ncbi.nlm.nih.gov/) against the predicted protein sequence dataset of the 12× version 1 (v1) of CRIBI (http://genomes.cribi.unipd.it/grape/) using the NpPDR1 protein sequence (CAC40990) as a query. Because the average full-size ABCG protein comprises 1,400 amino acids (Rea, 2007), only sequences comprising more than 400 amino acids were taken into account. These nomenclatures were represented according to Çakır and Kılıçkaya (2013). The sequences corresponding to full-size ABCG transporters confirmed that there was at least one PDR motif.

Molecular cloning of VvABCG44

The genome sequence corresponding to the partial cDNA sequence of a grape full-size ABCG transporter [tentative consensus sequence- TC76318, the grape gene index database (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=grape)], induced by cyclodextrin (Zamboni *et al*., 2009), was searched in the NCBI database (http://www.ncbi.nlm.nih.gov/) by BLAST. A genome sequence (accession number AM449250.2), provided by the IASMA Research Center (http://genomics.research.iasma.it/), was matched. The open reading frame (ORF) of the gene was predicted by Softberry (http://linux1.softberry.com/berry.phtml) and primers to amplify the entire ORF were designed (Table 1).

Total RNA was extracted from the berry skin by hot borate method (Wan and Wilkins, 1994). The full-length cDNA of *VvABCG44* was amplified using the PrimeScript High Fidelity RT-PCR kit (TaKaRa, Japan) according to the manufacturer's instructions. Three motifs (Walker A, Walker B, and ABC signature) were confirmed according to van den Brûle and Smart (2002). TMD was predicted by PHD (NPS@) (Rost and Sander, 1993, 1994). Sequence data of *VvABCG44* have been deposited in DDBJ under accession number AB910387.

Gene expression analysis

Total RNA from grape tissues was extracted by the technique described above. Total RNA was reverse-transcribed using a PrimeScript RT reagent Kit with gDNA eraser (perfect real-time) (TaKaRa) according to the manufacturer's recommendations.

Transcript levels were determined by quantitative RT-PCR using SYBER Premix EX Taq II (perfect real-time) (TaKaRa) and Thermal Cycler Dice Real Time System TP800 (TaKaRa) software ver. 3.00D. Primers for *VvABCG44*, *STS*, and *actin* are shown in Table 1. Reaction conditions for thermal cycling were as follows: after enzyme

Table 1 - Primers used in this study

Primer name	Purpose	Primer sequence(5'-3')
Take2_Forward	Cloning	CAC CAT GGC GAC GGC TGA AAT TTA TAR AG
Take2_Reverse	Cloning	TCG CCT TTG GAA GTT CAA TGC
VvPDR14_exp_Fw	Gene expression	TAG GAG TGG TTG CAG CTG TG
VvPDR14_exp_Rv	Gene expression	TTT TGC TCC GTG TGA CTT CTT
VvSTS_exp_Fw	Gene expression	GGG TCA CTA AGA GCG AGC AC
VvSTS_exp_Rv	Gene expression	GCT CCT CAA GCA TTT CTT CG
VvACT_Fw	Gene expression	TCC TGT GGA CAA TGG ATG GA
VvACT_Rv	Gene expression	CTTGCA TCC CTC AGC ACC TT

activation at 95°C for 10 s, amplification was performed in a two-step PCR with 40 cycles of 5 s at 95°C for denaturation and 30 s at 60°C for annealing/extension. Transcript levels were calculated using a standard curve, and normalized against *actin* as described by Reid *et al*. (2006). All reactions were performed in triplicate with three biological replicates.

Measurement of resveratrol content

Extraction of resveratrol (CAS number 501-36-0) and its analysis using an LC-Q-TOF/MS system equipped with an ESI interface (HPLC: Waters Acquity UPLC system; MS: Waters Xevo G2 Q-Tof , Waters, Germany) were performed according to Tamura *et al*. (2014). Identification, determination, and semi-quantification were compared with a 100 µM chemical reference standard. 10-camphorsulfonic acid was used as the internal control. Three samples of biological replicates were divided into two aliquots and a total of six samples were analyzed for each treatment.

3. Results

Zamboni *et al*. *(*2009*)* reported the partial sequence of a grape full-size ABCG gene that was induced by cyclodextrin. To obtain the full-length cDNA clone of the gene, we searched the genome sequence corresponding to the gene and successfully amplified a full-length cDNA using primers designed from the genome sequence data. The gene was designated *VvABCG44 or VvPDR14*. *VvABCG44* had a 4,350 bp coding region and was predicted to encode a protein of 1,450 amino acids (Fig. 1A) with two TMDs and two NBDs (Fig. 1B).

A phylogenetic tree of plant full-size ABCG transporters including VvABCG44 and all full-size ABCG transporters in *Arabidopsis* (Fig. 2) shows that NtPDR1 (BAD07483), NpPDR1 (CAC40990), and MtABCG10 (AES68070) are the closest homologues to VvABCG44. NtPDR1 (Crouzet *et al*., 2013) and NpPDR1 (Jasiński *et al*., 2001) were reported to transport diterpenes including sclareol, whereas MtABCG10 (Banasiak *et al*., 2013) transported isoflavonoids. A close homologue of VvABCG44, SpTUR2 (O24367) (van den Brûle and Smart, 2002), transported sclareol. Other close homologues transport different compounds; AtABCG40 (AAF71978) (Kang *et al*., 2010) and PaPDR1 (JQ292812) (Kretzschmar *et al*., 2012) transport ABA and strigolactone, respectively. The substrate range of VvABCG44 homologues is very broad, and it is not easy to identify the substrate of VvABCG44.

To determine the tissues in which *VvABCG44* is expressed, quantitative RT-PCR analyses were performed

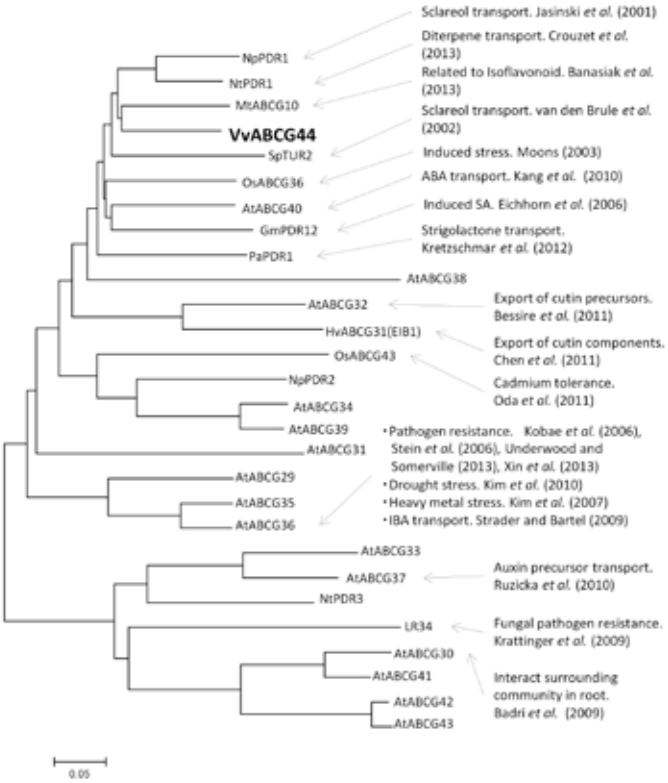
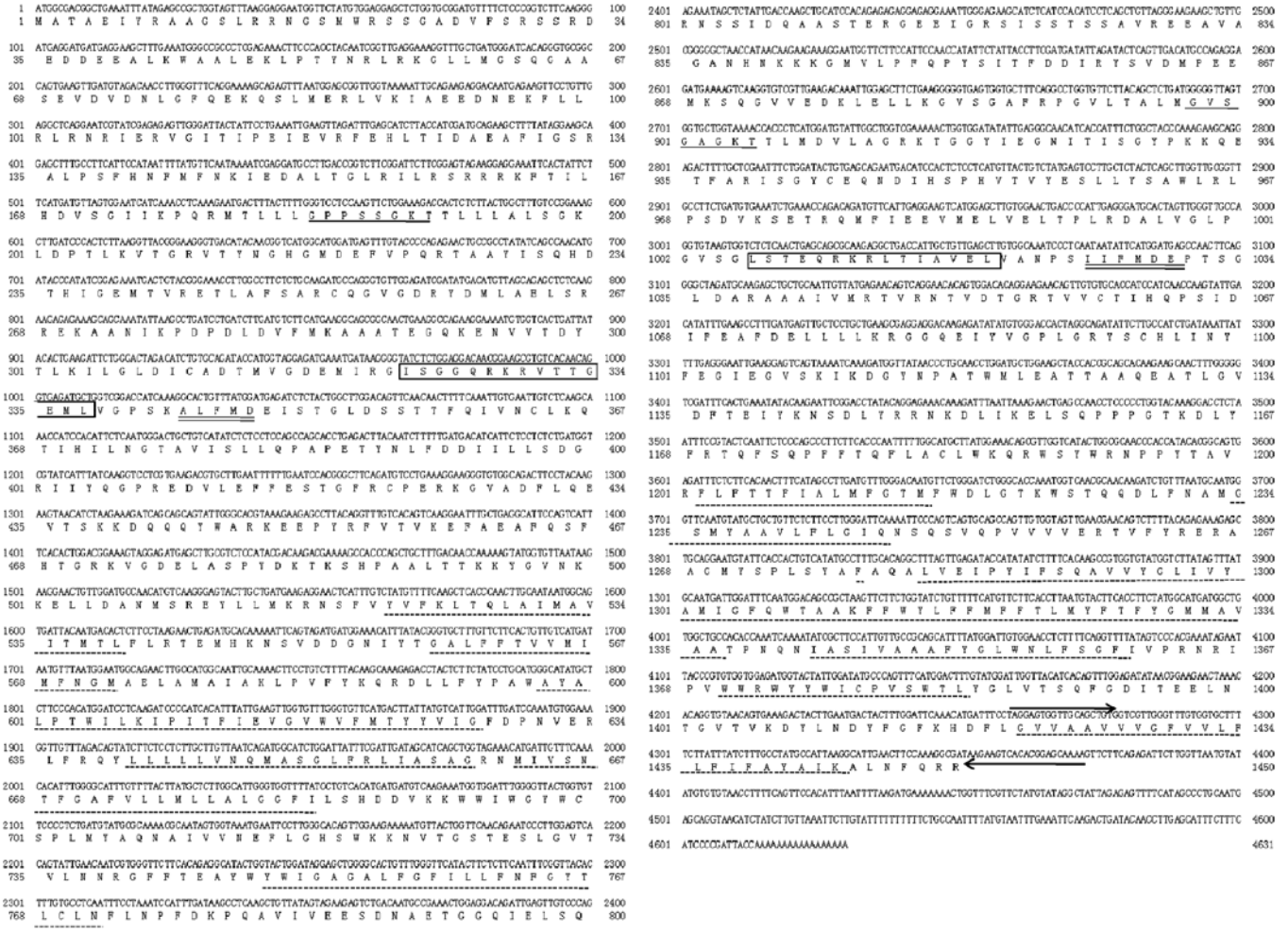


Fig. 2 - Phylogenetic tree of all full-size ABCGs in *Arabidopsis*, *VvABCG44*, and characterized full-size ABCGs from various plant species. NpPDR1 and NpPDR2 from *Nicotiana plumbaginifolia*, NtPDR1 and NtPDR3 from tobacco, SpTUR2 from *Spirodella polyrhiza*, OsABCG36 and OsABCG43 from rice, HvABCG31 from barley, PaPDR1 from Petunia, Lr34 from wheat, and GmPDR12 from soybean. The neighbor-joining tree was constructed with MEGA5 (Tamura *et al*., 2011).

A



B

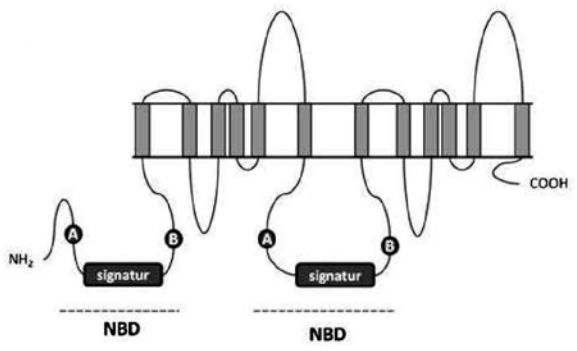


Fig. 1 - Nucleotide sequence, amino acid sequence, and topology of VvABCG44 A: Nucleotide sequence of *VvABCG44* and the deduced amino acid sequence. Walker A motifs are underlined. ABC signature motifs are boxed. Walker B motifs are double underlined. Transmembrane domains are dotted-lined. Arrows indicate the primers for quantitative PCR analysis. B: Putative topology of VvABCG44. The protein is composed of two halves and each half harbors TMD (gray boxes) and NBD (dashed lines), which contains an ABC signature and Walker A and B motifs.

(Fig. 3). The highest expression of *VvABCG44* was observed in mature leaves, which was 9.6 times higher than that in young leaves. *VvABCG44* expression in tendril and stem was higher than that in young leaves, but was not as high as that in mature leaves. *VvABCG44* expression was relatively low in the grape berry*VvABCG44*, where it was highest in the skin and lowest in seeds. We also determined the gene expression of stilbene synthase (STS), a key enzyme in resveratrol synthesis. The expression pattern of *STS* in various grape organs is similar to that of *VvABCG44* (Fig. 3), suggesting a relationship between VvABCG44 and resveratrol synthesis.

Later, we determined the induction of *VvABCG44* in grape berry skin by UV irradiation and by ABA treatment. Expression of *VvABCG44* was upregulated 2.7 times by UV irradiation, and the STS gene was strongly induced by UV irradiation. Furthermore, resveratrol content in the grape berry skin increased 159 times after 23 h of incubation following UV irradiation (Fig. 4). These results suggest a relationship between VvABCG44 and resveratrol accumulation. On the other hand, the expression of *VvABCG44* was not induced by ABA treatment in the grape berry skin (Fig. 5).

4. Discussion and Conclusions

There are few reports of grape ABC transporters, although comprehensive analyses, such as transcriptomics and proteomics, report the expression of ABC transporters in grape. Recently, Çakır and Kılıçkaya (2013) identified all ABC proteins using whole genome sequencing with 12× coverage and Francisco *et al*. (2013) identified an ABCC transporter of grape as a vacuolar anthocyanin

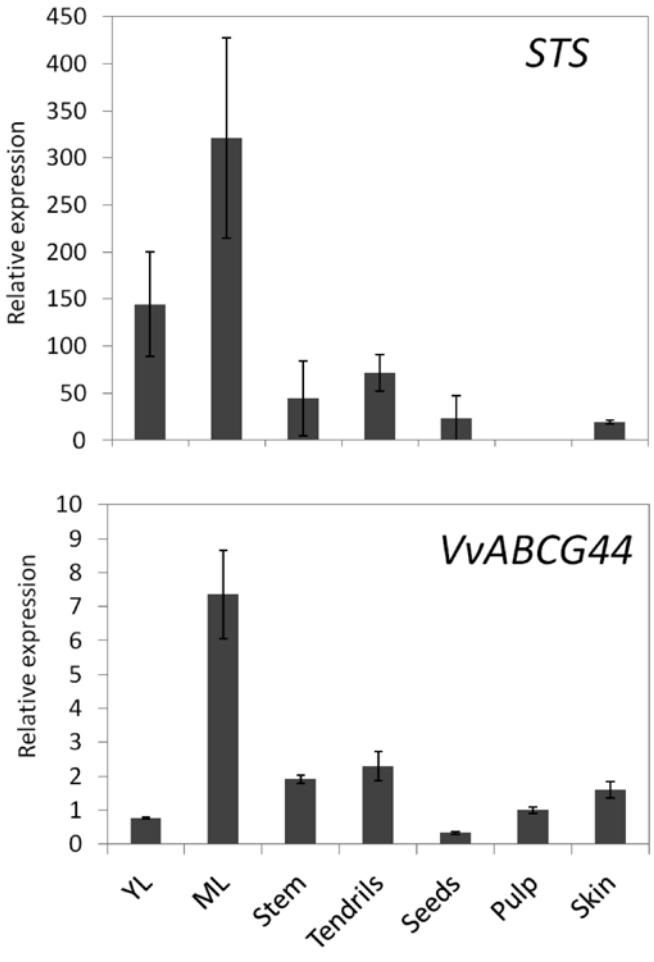


Fig. 3 - Gene expression of STS andVvABCG44 in various grape organs [YL (young leaves), ML; (mature leaves), stem, tendrils, seeds, pulp, and skin]. mRNA levels of STS and VvABCG44 were detected by quantitative PCR. Actin was used as an internal control. Each value represents mean ± SE of three independent experiments.

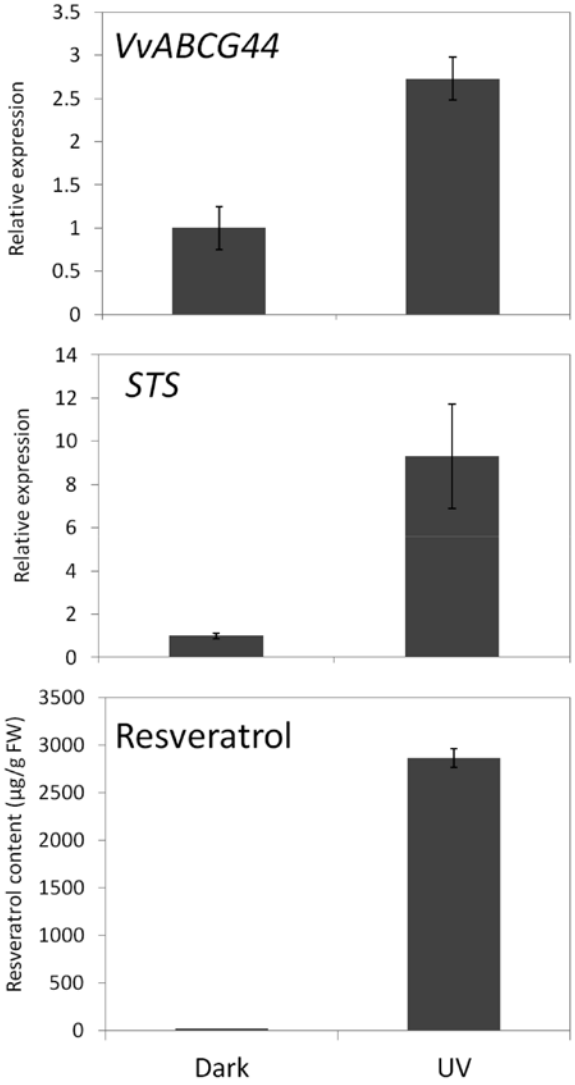


Fig. 4 - Expression of STS,VvABCG44 and resveratrol content in the grape berry skin after UV irradiation. mRNA levels of STS and VvABCG44 were detected by quantitative PCR. Actin was used as an internal control. Each value represents mean ± SE of three independent experiments. Resveratrol content was assayed by LC-ESI-Q-TOF/MS system in negative ion mode. Each value represents mean ± SE of six independent measurements.

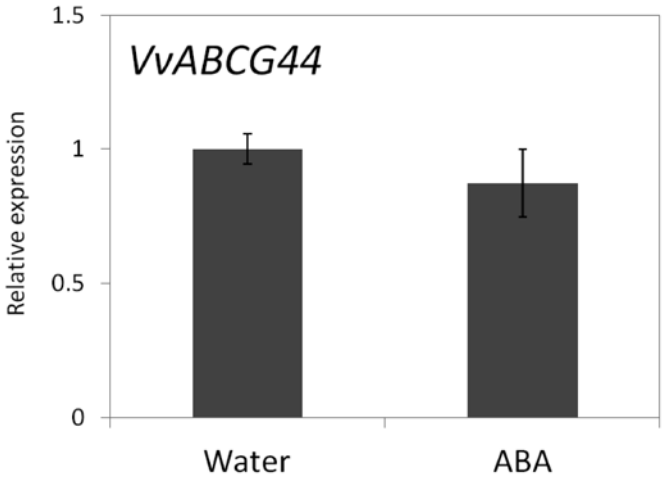


Fig. 5 - Expression of VvABCG44 in the grape berry skin after ABA treatment. mRNA level of VvABCG44 was detected by quantitative PCR. Actin was used as an internal control. Each value represents mean ± SE of three independent experiments.

transporter. To the best of our knowledge, this is the only characterized full-size ABC transporter in grape.

Why have such few full-size ABC transporters been studied? This is because of the difficulty in cloning full-length cDNA encoding full-size ABC transporters, particularly full-size ABCG transporters. One of the reasons for this difficulty is the very large size (ca. 4,000 bp) of full-size ABCG transporter cDNAs. Another reason is the frequently observed low growth rate of *Escherichia coli* harboring full-size ABCG transporter cDNA. The reason for this low growth rate of *E. coli* is unclear. Therefore, few or no full-length cDNA clones encoding full-size ABCG transporters are found in public cDNA databases or resources, and should be cloned.

Although 15 full-size ABCG transporters are present in *Arabidopsis* (van den Brûle and Smart, 2002), only five of them, AtABCG40, AtABCG37, AtABCG36, AtABCG32, and AtABCG30, have been characterized (Campbell *et al*., 2003; Lee *et al*., 2005; Ito and Gray, 2006; Kobae *et al*., 2006; Stein *et al*., 2006; Kim *et al*., 2007; Badri *et al*., 2009; Strader and Bartel, 2009; Kang *et al*., 2010; Kim *et al*., 2010; Růžička *et al*., 2010; Bessire *et al*., 2011; Underwood and Somerville, 2013; Xin *et al*., 2013). In other plant species, only two full-size ABCG transporters (OsABCG36, OsABCG43) in rice (Moons, 2003; Oda *et al*., 2011) and five full-size ABCG transporters (NpPDR1, NpPDR2, NtPDR1, NtPDR3, and ABCG5/PDR5) in tobacco family plants have been studied (Jasinski *et al*., 2001; Sasabe *et al*., 2002; Schenke *et al*., 2003; Ducos *et al*., 2005; Stukkens *et al*., 2005; Trombik *et al*., 2008; Bultreys *et al*., 2009; Navarre *et al*., 2011; Bienert *et al*., 2012; Seo *et al*., 2012; Crouzet *et al*., 2013).

In this study, we successfully cloned the full-length cDNA of *VvABCG44* using the primers designed from the grape genome sequence data, using a high-grade enzyme for PCR reactions and optimized *E. coli* culture conditions (culture at lower temperature and in higher volume). This appears to be the first report of a grape full-size ABCG transporter.

Two different data sets of grape genome sequences have been disclosed to the public. First, the Pinot Noir clone ENTAV115 was released by an Italian group, IASMA Research Center (http://genomics.research.iasma.it/) (Velasco *et al*., 2007). We used this information for cDNA cloning of *VvABCG44*. Second, the Pinot Noir-derived inbred PN40024 was sequenced by the French-Italian public consortium (Jaillon *et al*., 2007) (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/). The latter data set was updated from 8× to 12× and is now widely used. Recently the 12× version1(v1) has been made available by an Italian group, CRIBI (Grimplet *et al.*, 2012) (http://genomes.cribi.unipd.it/grape/). Therefore, we used v1 to find all full-size ABCG transporters in the grape genome (Table 2).

Fifteen and 23 full-size ABCG transporters were found in *Arabidopsis* (van den Brûle and Smart, 2002) and rice (Moons, 2008), respectively. In the grape genome data, we found 34 full-size ABCG transporters (Table 2, Fig. 6). This number is much larger than that in *Arabidopsis* and rice, suggesting a diversity of roles of full-size ABCG transporters in grape. As mentioned above, substrate and subcellular localization of full-size ABCG transporter cannot be determined from sequence similarity. However, full-size ABCG transporters are responsible for transport of secondary metabolites, plant hormones, cutins, and heavy metals (Fig. 2) and should have an important role in grape berry.

Recent reports showed that plant full-size ABCG transporters, transport plant hormones or their precursors, such

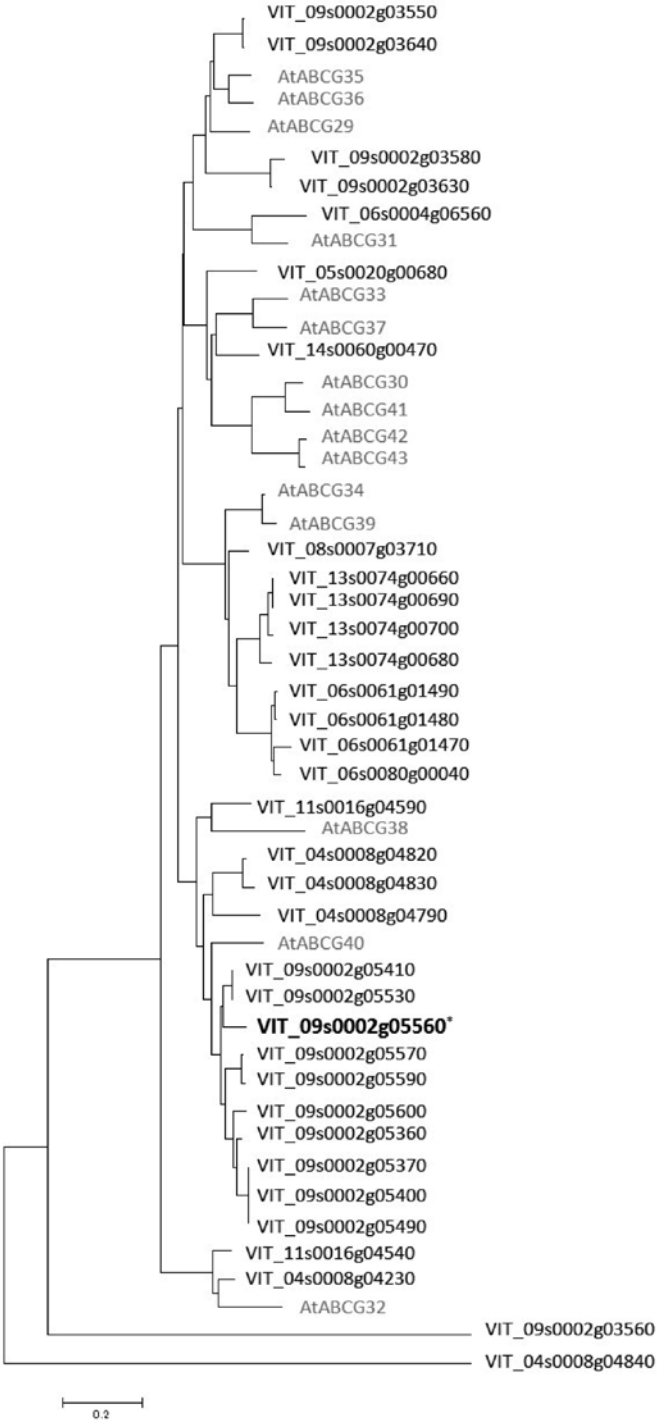


Fig. 6 - Phylogenetic tree of all full-size ABCGs in Arabidopsis and VvABCG44. A neighbor-joining tree was constructed with MEGA5 (Tamura *et al*., 2011).

Table 2 - Full-size ABCG transporters in grape (Vitis vinifera). Columns contain the *Vitis Vinifera* 12× V1 ID, chromosome location, protein length, PDR signatures, annotated description by Tair10, protein acronym (Name) and *Vitis Vinifera* 12× V0 ID for each gene are given

12X V1 ID	Chromosome location				Protein	PDR signatures**			Description of Tair10		Sanchez-	HGNC***	12X V0 ID
	Chr	Strand	Start	End		LLLGPP	GLDSST	GLDARA-	AGI code	Short description	Subfamily name	Subfamily name	
VIT_11s0016g04540	11	+	3825506	3837079	1422	+	+	+	AT2G26910.1	pleiotropic drug resistance 4	VvPDR1	VvABCG31	GSVIVT01015456001
VIT_11s0016g04590	11	-	3891367	3898727	1478	+	+	-	AT1G15520.1	pleiotropic drug resistance 12	VvPDR2	VvABCG32	GSVIVT01015461001
VIT_09s0002g03550	9	+	3229012	3242582	649	+	+	-	AT1G15210.1	pleiotropic drug resistance 7	VvPDR3	VvABCG33	GSVIVT01016991001
VIT_09s0002g03560	9	+	3242583	3244574	427	-	-	+	AT3G16340.1	pleiotropic drug resistance 1	VvPDR4	VvABCG34	GSVIVT01016992001
VIT_09s0002g03580	9	+	3246544	3252734	691	+	+	-	AT3G16340.1	pleiotropic drug resistance 1	VvPDR5	VvABCG35	GSVIVT01016993001
VIT_09s0002g03630	9	-	3318732	3327354	1411	+	+	+	AT1G59870.1	ABC-2 and Plant PDR ABC-type	VvPDR6	VvABCG36	GSVIVT01016998001
VIT_09s0002g03640	9	-	3328212	3336626	1494	+	+	+	AT3G16340.1	pleiotropic drug resistance 1	VvPDR7	VvABCG37	GSVIVT01016999001
VIT_09s0002g05360	9	-	5099146	5114849	1490	+	+	+	AT1G15520.1	pleiotropic drug resistance 12	VvPDR8	VvABCG38	GSVIVT01017184001
VIT_09s0002g05370	9	-	5115505	5122760	1422	+	+	+	AT1G15520.1	pleiotropic drug resistance 12	VvPDR9	VvABCG39	GSVIVT01017185001
VIT_09s0002g05400	9	-	5146167	5160090	1565	+	+	+	AT1G15520.1	pleiotropic drug resistance 12	VvPDR10	VvABCG40	GSVIVT01017187001
VIT_09s0002g05410	9	-	5169125	5176189	1438	+	+	+	AT1G15520.1	pleiotropic drug resistance 12	VvPDR11	VvABCG41	GSVIVT01017188001
VIT_09s0002g05490	9	-	5216536	5223507	1280	+	+	+	AT1G15520.1	pleiotropic drug resistance 12	VvPDR12	VvABCG42	GSVIVT01017196001
VIT_09s0002g05530	9	-	5259175	5266314	1460	+	+	+	AT1G15520.1	pleiotropic drug resistance 12	VvPDR13	VvABCG43	GSVIVT01017198001
VIT_09s0002g05560*	9	-	5281296	5288255	1455	+	+	+	AT1G15520.1	pleiotropic drug resistance 12	VvPDR14	VvABCG44	GSVIVT01017201001
VIT_09s0002g05570	9	-	5294437	5301677	1455	+	+	+	AT1G15520.1	pleiotropic drug resistance 12	VvPDR15	VvABCG45	GSVIVT01017202001
VIT_09s0002g05590	9	-	5316144	5323420	1455	+	+	+	AT1G15520.1	pleiotropic drug resistance 12	VvPDR16	VvABCG46	GSVIVT01017204001
VIT_09s0002g05600	9	-	5336090	5343699	1451	+	+	+	AT1G15520.1	pleiotropic drug resistance 12	VvPDR16	VvABCG46	GSVIVT01017204001
VIT_05s0020g00680	5	+	2548762	2557921	1438	+	+	+	AT3G53480.1	pleiotropic drug resistance 9	VvPDR17	VvABCG47	GSVIVT01017676001
VIT_06s0004g06560	6	+	7284901	7297724	1274	+	+	+	AT2G29940.1	pleiotropic drug resistance 3	VvPDR18	VvABCG48	GSVIVT01024743001
VIT_14s0060g00470	14	+	439701	448696	1449	+	+	-	AT3G53480.1	pleiotropic drug resistance 9	VvPDR19	VvABCG49	GSVIVT01031314001
VIT_06s0061g01490	6	-	1,9E+07	1,9E+07	1455	+	+	+	AT2G36380.1	pleiotropic drug resistance 6	VvPDR20	VvABCG50	GSVIVT01031377001
VIT_06s0061g01480	6	-	1,9E+07	1,9E+07	1461	+	+	+	AT2G36380.1	pleiotropic drug resistance 6	VvPDR21	VvABCG51	GSVIVT01031378001
VIT_06s0061g01470	6	-	1,9E+07	1,9E+07	1123	+	+	+	AT1G66950.1	pleiotropic drug resistance 11	VvPDR22	VvABCG52	GSVIVT01031380001
VIT_08s0007g03710	8	+	1,8E+07	1,8E+07	1452	+	+	+	AT2G36380.1	pleiotropic drug resistance 6	VvPDR23	VvABCG53	GSVIVT01033804001
VIT_13s0074g00660	13	-	8818113	8827874	1473	+	+	-	AT2G36380.1	pleiotropic drug resistance 6	VvPDR24	VvABCG54	GSVIVT01034741001
VIT_13s0074g00680	13	-	8859786	8867047	1477	+	+	-	AT1G66950.1	pleiotropic drug resistance 11	VvPDR25	VvABCG55	GSVIVT01034745001
VIT_13s0074g00690	13	-	8876000	8883078	1379	+	+	-	AT2G36380.1	pleiotropic drug resistance 6	VvPDR26	VvABCG56	GSVIVT01034746001
VIT_13s0074g00700	13	-	8897688	8904965	1481	+	+	-	AT2G36380.1	pleiotropic drug resistance 6	VvPDR27	VvABCG57	GSVIVT01034748001
VIT_04s0008g04230	4	-	3596683	3605452	1422	+	+	-	AT2G26910.1	pleiotropic drug resistance 4	VvPDR28	VvABCG58	GSVIVT01035715001
VIT_04s0008g04790	4	-	4227017	4234518	1437	+	+	+	AT1G15520.1	pleiotropic drug resistance 12	VvPDR29	VvABCG59	GSVIVT01035780001
VIT_04s0008g04820	4	+	4258541	4265241	1420	+	+	+	AT1G15520.1	pleiotropic drug resistance 12	VvPDR30	VvABCG60	GSVIVT01035784001
VIT_04s0008g04830	4	+	4282425	4286094	764	+	+	-	AT1G15520.1	pleiotropic drug resistance 12	VvPDR31	VvABCG61	GSVIVT01035785001
VIT_04s0008g04840	4	+	4286954	4295631	1120	-	-	+	AT1G15520.1	pleiotropic drug resistance 12	VvPDR32	VvABCG62	GSVIVT01035786001
VIT_06s0080g00040	6	+	2E+07	2E+07	1507	+	+	-	AT2G36380.1	pleiotropic drug resistance 6	VvPDR33	VvABCG63	GSVIVT01036184001

*VIT_09s0002g05560 shown with bold letters is corresponded to VvABCG44.

**PDR signatures were reported by van den Brûle and Smart (2002).

***Sanchez-Fernandez and HGNC subfamily names were reported by Çakır and Kılıçkaya (2013).

as ABA (Kang *et al*., 2010), strigolactone (Kretzschmar *et al*., 2012), and auxin (Ruzicka *et al*., 2010). One of the closest homologues of VvABCG44, AtABCG40, was reported to transport ABA (Kang *et al*., 2010) (Fig. 2). In grape berry, ABA accumulates just before maturation, called "veraison", and ABA works as a trigger of berry maturation (Coombe and Hale, 1973; Davies *et al*., 1997). After veraison, both sugar and anthocyanin accumulate considerably in the grape berry (Coombe, 1992; Davies *et al*., 1997; Deluc *et al*., 2007). In this study, we determined *VvABCG44* induction by ABA in the berry skin before veraison. However, no induction was observed (Fig. 5).

Many plant full-size ABCG transporters have been suggested to be associated with biotic and abiotic stress resistance, particularly resistance against pathogens, and some have been observed to transport secondary metabolites that function as phytoalexins (Fig. 2). Therefore, *VvABCG44* is considered to be associated with biotic and abiotic stress resistance and transports phytoalexins. *VvABCG44* was first found as an elicitor-induced gene in grape culture cells and the induction of *VvABCG44* corresponded to resveratrol accumulation in the cells (Zamboni *et al*., 2009).

It is known that UV irradiation induces resveratrol accumulation in the grape berry skin (Douillet-Breuil *et al*., 1999; Adrian *et al*., 2000; Versari *et al*., 2001; Takayanagi *et al*., 2004). Therefore, we determined the effect of UV irradiation on *VvABCG44* expression together with STS expression, a key enzyme for resveratrol synthesis and resveratrol accumulation. A clear induction of *VvABCG44* by UV irradiation, though not large compared with that of *STS* expression and resveratrol accumulation, *VvABCG44* was observed (Fig. 4). A similar pattern in the gene expression of *VvABCG44* and *STS* in various grape organs was also observed (Fig. 3). These results suggest a relationship between *VvABCG44* and resveratrol accumulation.

Close homologues of *VvABCG44*, NtPDR1 (Crouzet *et al*., 2013), NpPDR1 (Jasiński *et al*., 2001), MtABCG10 (Banasiak *et al*., 2013), SpTUR2 (van den Brûle and Smart, 2002), AtABCG40 (Kang *et al*., 2010), and PaPDR1 (Kretzschmar *et al*., 2012), transport diterpenoids, isoflavonoids, ABA, and strigolactones (Fig. 2). These functions are surprising because their molecular structures are completely different. Resveratrol is a compound belonging to the stilbenoids and both stilbenoids and flavonoids belong to the phenylpropanoids. The closest homologue of *VvABCG44*, MtABCG10, transports isoflavonoids (Banasiak *et al*., 2013). Although no direct evidence of stilbenoid transport activity of full-size ABCG transporter has been reported, it was observed that *B. cinerea*, lacking a full-size ABCG transporter, BcatrB, was more sensitive to resveratrol than the wild-type strain (Schoonbeek *et al*., 2001). This result suggests that BcatrB is an exporter of resveratrol in *B. cinerea.* It can be concluded that *VvABCG44* may work as a resveratrol transporter in grape.

We attempted to express *VvABCG44* in yeast lacking eight ABC transporters (Kang *et al*., 2010) and measure resveratrol transport activity. However, this attempt was unsuccessful because heterologous expression of plant full-size ABCG transporters is difficult not only in *E. coli* but also in yeast.

We identified 34 full-size ABCG transporters in the grape genome, including *VvABCG44*. It is assumed they transport key compounds for plant growth and stress resistance, including secondary metabolites, plant hormones, cutins, and heavy metals, and have important roles in grape. Further study on full-size ABCG transporters in grape is warranted.

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Adv. Hort. Sci., 2014 28(2): 64-72 *Review paper*

Progress on studies for seedless breeding of citrus in Japan

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Key words: female sterility, male sterility, protoplast fusion, self-incompatibility, triploid.

Abstract: Seedlessness is a desirable characteristic for both fresh and processed citrus markets and one of the most important breeding objectives. In this paper, progress on studies for seedless breeding of citrus in Japan is reviewed. Among the several types of male sterility, anther abortion is the strictest male sterility in citrus and was shown to be controlled by both nuclear and cytoplasmic genes. Several seedless cultivars with male sterility have been developed. The mechanism and inheritance of the strictest female sterility derived from 'Mukaku Kishu' (*Citrus kinokun*i hort. ex Tanaka) were clarified and seedless cultivar and parental lines with this female sterility have been released. Some self-incompatible cultivars show seedlessness when coupled with parthenocarpy. *S* (self-incompatibility) genotypes of several cultivars have been estimated. Tetraploid plants, as parents of triploid offspring, were obtained from nucellar seedlings, and by ploidy mutation, colchicine treatment, and protoplast fusion. Triploid plants were produced from the combination of not only tetraploid and diploid crosses but also diploid and diploid crosses. New triploid seedless cultivars were bred by programmed cross-breeding and protoplast fusion.

1. Introduction

Seedlessness is a desirable characteristic for both fresh and processed citrus markets (Vardi *et al*., 2008). In fact, major citrus cultivars on a global level such as 'Valencia,' navel orange [*Citrus sinensis* (L.) Osbeck] and 'Marsh' grapefruit (*C. paradisi* Macfad.) are seedless (Reuther, 1988). Although a major early-maturing citrus, satsuma mandarin (*C. unshiu* Marcow.), is seedless, almost all mid- and late-maturing citruses, such as natsudaidai (*C. natsudaidai* Hayata), iyo (*C. iyo* hort. ex Tanaka), and hassaku (*C. hassaku* hort. ex Tanaka), are seedy in Japan (Iwamasa, 1988). Thus, breeding new seedless cultivars is an urgent issue to develop the Japanese citrus industry.

To develop seedless citrus at the diploid (2x=18) level, utilization of sterility is essential (Iwamasa, 1966). Sterility can be divided into three types: male sterility, female sterility, and self-incompatibility. In addition, selection of triploid (3x=27) individuals is useful for breeding seedless cultivars (Ollitrault *et al*., 2007). Therefore, various kinds of investigations on the mechanism and genetic factors underlying seedlessness in citrus have been conducted and many breeding works have been carried out to develop new seedless cultivars, many of them carried out in Japan over the last few decades. In this review, the progress on studies for seedless breeding of citrus in Japan, with regard to 1) seedlessness at the diploid level and 2) ploidy manipulation for seedless breeding, is discussed.

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2. Seedlessness at the diploid level

Male sterility

The degree of male (pollen) sterility is variable in citrus, and usually pollen sterile accessions produce seedless or low-seedy fruits when cultivated in solid blocks. Male sterility couples with parthenocarpy to produce seedless fruits when cross-pollination is prevented. Even in mixed planting with pollen fertile accessions, male sterility reduces seed production and increases the percentage of seedless fruits because those accessions have a smaller chance of fertilization than male fertile ones (Yamamoto *et al*., 1993, 1995).

Iwamasa (1966) and Ollitrault *et al*. (2007) summarized the various levels of male sterility at the diploid level in citrus (Table 1). Chromosome aberration was one of the most important phenomena causing pollen sterility. Asynapsis in 'Mukaku Yuzu' (*C. junos* Sirbold ex Tanaka) is genetically controlled, while that in 'Eureka' lemon (*C. limon* (L.) Burm. f.) and 'Mexican' lime (*C. aurantifolia* (Cristm.) Swingle) is induced by low temperature (Nakamura, 1943; Iwamasa and Iwasaki, 1962; Iwamasa, 1966). Reciprocal translocation is found to cause pollen sterility of 'Valencia' orange (*C. sinensis*) (Iwamasa, 1966). Inversion is the cause of partial pollen sterility of 'Mexican' lime (*C. aurantifolia*) (Iwamasa, 1966). Male sterility that is not caused by chromosome aberration is also well known. Anther abor-

Table 1 - Diagramatic representation of various kinds of the male-sterility in citrus, according to the sequential order of development (Modified from Iwamasa, 1966)

Developmental stage	Nature of sterility	Cultivar or hybrid	Reference
Initiation of anther development	Anther abortion	Satsuma mandarin × Sweet orange, etc.	Iwamasa, 1966
↓			
Archesporial stage			
↓			
Resting stage	Degeneration of PMCs	Washington Navel	Osawa, 1912
		Tahiti lime	Uphof, 1931
		Lemon × Valencia, etc.	Frost, 1948
↓		Satsuma mandarin × Trifoliate orange	Iwamasa, 1966
Meiosis			
I-division	Asynapsis (genic)	Mukaku Yuzu	Iwamasa, 1966
	Asynapsis (by low temp.)	Eureka lemon	Nakamura, 1943
		Mexican lime	Iwamasa and Iwasaki., 1962
	Translocation	*C. assamensis*	Naithani and Raghuvanshi, 1958
		Valencia orange	Iwamasa, 1966
	Inversion	*C. assamensis*, etc	Raghuvanshi, 1962 a
↓		Mexican lime	Iwamasa, 1966
II-division	Failure of spindle	Marsh grapefruit	Raghuvanshi, 1962 b
↓			
Liberation from tetrad	Degeneration	Jaffa orange	Oppenheim and Frankel, 1929
↓			
Mitotic division	Degeneration	Satsuma mandarin	Nakamura, 1943
↓			
Mature pollen grain			

tion in satsuma mandarin (*C. unshiu*) hybrids is the strictest male sterility in citrus (Iwamasa, 1966). The sterile stamen appears only as the filament, and no pollen grains are produced (Fig. 1). Male sterility of 'Washington' navel (*C. sinensis*), 'Tahiti' lime (*C. latifolia* Tanaka), and some other hybrids is due to early degeneration of pollen mother cells (PMCs) (Osawa, 1912; Uphof, 1931; Frost, 1948; Iwamasa, 1966). Pollen sterility of satsuma mandarin (*C. unshiu*) is caused by plural sterility such as abnormal behavior and degeneration of pollen grains (Nakamura, 1943; Yang and Nakagawa, 1969, 1970).

To develop new seedless cultivars efficiently, genetic analysis of male sterility has been conducted. Among these studies, genetic analysis of anther abortion has progressed remarkably. This male sterility is due to gene-cytoplasmic interaction [satsuma mandarin (*C. unshiu*), 'Encore' mandarin (*C. nobilis* Lour. × *C. deliciosa* Ten.), yuzu (*C. junos*), and lemon (*C. limon*) possess sterile cytoplasm] and is probably controlled by more than one major gene (Iwamasa, 1966; Yamamoto *et al*., 1992 a, b, 1997; Nakano *et al*., 2001; Dewi *et al*., 2013 a). Dewi *et al*. (2013 a) postulated that a dominant nuclear fertility-restoring gene system comprising one epistatic gene and two complementary genes controls the restoration of male

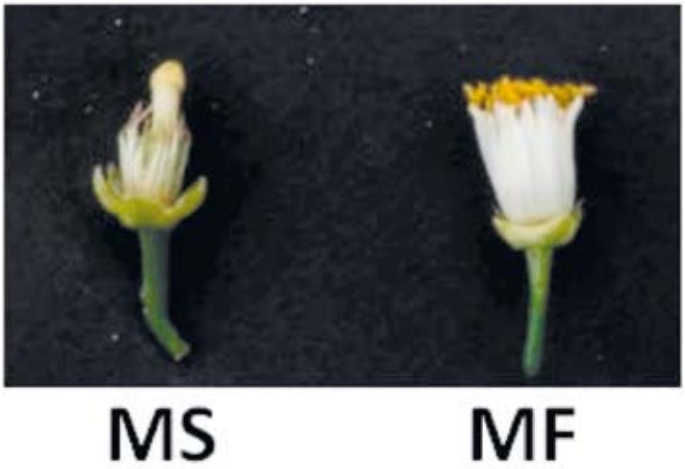

Fig. 1 - Flowers of male-sterile (aborted anthers, MS) and male-fertile (normal anthers, MF) citrus.

fertility and male-sterile anther size in citrus plants with sterile cytoplasm. Nakano *et al*. (2000) found DNA markers linked to aborted anther for juvenile screening of male-sterile plants. Male-sterile (aborted anther) progenies were also determined at the early period of seedling growth to exhibit precocious flowering, a phenomenon in which very young seedlings have flowers (Dewi *et al*., 2013 b). Another type of male sterility, inheritance of pollen fertility/sterility, was also studied (Ueno, 1986). Some pollen-sterile progenies arose from two pollen fertile parents.

In citrus, not only somatic hybrids but also cybrids were produced by symmetric protoplast fusion (Saito *et al*., 1993; Moriguchi *et al*., 1996; 1997; Tokunaga *et al*., 1999). Yamamoto and Kobayashi (1995) produced a cybrid having the sweet orange (*C. sinensis*) nuclear genome and satsuma mandarin (*C. unshiu*) cytoplasmic genome by fusion between satsuma mandarin protoplasts isolated from embryogenic callus and sweet orange mesophyll protoplasts. The cybrid is useful for seedless breeding because it has sterile cytoplasm derived from satsuma mandarin.

Several new seedless cultivars with male sterility were released in Japan (Nishiura *et al*., 1983; Okudai *et al*., 1991; Matsumoto *et al*., 1991, 2003; Yoshida *et al*., 2005 c) (Fig. 2, Table 2). All cultivars with aborted anther possess cytoplasm derived from satsuma mandarin (*C. unshiu*).

Female sterility

Female sterility is a very important trait which is closely related to seedlessness. Yamamoto *et al*. (1995) reported that the degree of female fertility/sterility is rated on the basis of the average number of seeds per fruit obtained through hand pollination. A high positive correlation (r = 0.93**) was found between the number of seeds of hand-pollinated fruits and that of open-pollinated fruit. This result indicated that female sterility is directly related to seediness. Female sterility estimated by the above-mentioned method was revealed to be a heritable characteristic (Yamamoto *et al*., 2001).

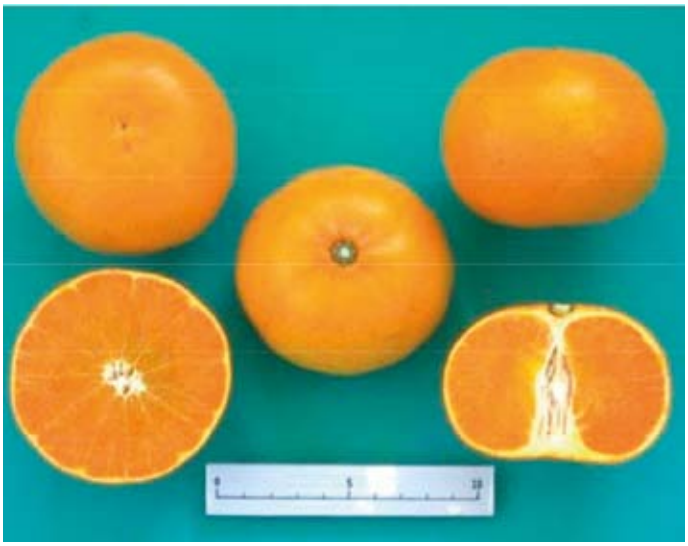

Fig. 2 - Male-sterile seedless citrus 'Setoka'.

Table 2 - Seedless cultivars and parental lines of citrus released in Japan

	Cultivar or Parental line	Cross combination	Note
Male sterility	Kiyomi	Miyagawa wase (*Citrus unshiu*) × Trovita (*C. sinensis*)	
	Seiho	Kiyomi × Minneola (*C. paradisi* × *C. tangerina*)	
	Tsunokaori	Kiyomi × Okitsu wase (*C. unshiu*)	
	Amaka	Kiyomi × Encore (*C. nobilis* × *C. deliciosa*)	
	Setoka	(Kiyomi × Encore) No. 2 × Murcott (probably tangor)	
	Harehime	E647 (Kiyomi × Osceola) × Miyagawa wase (*C. unshiu*)	
	Tsunokagayaki	(Kiyomi × Okitsu wase) No. 14 × Encore (*C. nobilis* × *C. deliciosa*)	
Female sterility	Southern Yellow	Tanikawa Buntan (*C. maxima*) × Mukaku Kishu (*C. kinokuni*)	
	Citrus parental line Norin No. 5	Lee (Clementine × Orlando) × Mukaku Kishu (*C. kinokuni*)	
	Citrus parental line Norin No. 6	King mandarin (*C. nobilis*) × Mukaku Kishu (*C. kinokuni*)	
Self-incompatibility	Ariake	Seike navel (*C. sinensis*) × Clementine (*C. clementina*)	
Triploid	Puchimaru	Oval kumquat (*Fortunella margarita*) × tetraploid Meiwa kumquat (*C. crassifolia*)	
	White Love	Cybrid having lemon (*C. limon*) nuclear and sudachi (*C. sudachi*) cytoplasmic genome + Haploid clementine (*C. clementina*)	Somatic hybrid
	Tokushima 3X No. 1	Tetraploid sudachi HS4 (*C. sudachi*) × sudachi Ryokuko-kei (*C. sudachi*)	
	Yellow Bell	Open-pollinated seedling of diploid Michitani-line Villafranca (*C. limon*)	

'Mukaku Kishu', a bud variant of the seedy kinokuni mandarin (*Citrus kinokun*i hort. ex Tanaka), is completely seedless and considered to have the strictest female sterility in citrus. Yamasaki *et al*. (2007, 2009) studied the mechanism of expression of seedlessness derived from 'Mukaku Kishu'. In fruits of 'Mukaku Kishu', specific very small and swollen seeds called "type A seeds" were observed. The expression of 'Mukaku Kishu'-type seedlessness is characterized by formation of "type A seed" with an immature seed coat and an embryo arrested at an early stage. However, arrested embryo development in the " type A seed" is not caused by endosperm abortion. This female sterility is controlled by two major genes: sterility and fertility are dominant and recessive, respectively (Nesumi *et al*., 2001). New seedless cultivar and parental lines with this sterility were bred in Japan (Yoshida *et al*., 2005 a, b) (Fig. 3, Table 2).

Navel orange (*C. sinensis*) and satsuma mandarin (*C. unshiu*) have strong female sterility; only a few seeds were developed when they were hand-pollinated (Miki, 1921; Nagai and Tanikawa, 1926; Nishiura and Iwasaki, 1963; Yamamoto *et al*., 1995). Osawa (1912) observed degeneration of the embryo sac in both navel orange and satsuma mandarin. Nesumi *et al*. (2000) assumed that the female sterility of satsuma mandarin is controlled by two major genes: sterility and fertility are recessive and dominant, respectively, and they were mapped on a linkage map (Omura *et al*., 2000).

Self-incompatibility

Self-incompatibility is a genetically controlled phenomenon preventing seed set in self-pollinated plants producing functional gametes. Self-incompatibility in citrus is a very important trait for fruit production. Without parthenocarpy, it requires cross pollination to achieve stable fruit production (Nagai and Tanikawa, 1926; Miwa, 1951). However, its coupling with parthenocarpy could produce seedless fruit (Iwamasa and Oba, 1980; Yamamoto *et al*., 1995; Yamamoto and Tominaga, 2002). Thus, much research has been conducted to determine self-incompatibility of many accessions (Nagai and Tanikawa, 1926; Miwa, 1951; Nishiura and Iwasaki, 1963; Iwamasa and Oba, 1980; Yamamoto and Tominaga, 2002; Yamamoto *et al*., 2006, 2012).

The incompatibility system of citrus is of the gametophytic type and Soost (1965, 1969) proposed *S* (self-incompatibility) genotypes of some accessions. Since then, the source of *S* genotypes has been less well elucidated. Recently, however, research has progressed remarkably *via* certain methods. Pollen tube growth was strongly inhibited in incompatible pollination; those pollen tubes exhibited abnormal behaviors, namely twisted and heavy and irregular callose deposition. On the other hand, in compatible pollination, many normal pollen tubes penetrated into the style (Ngo *et al*., 2001). Self-incompatibility *S* genotypes of several citrus cultivars were estimated by the observation of pollen tube behavior in the styles after controlled pollination with a restricted number of pollen grains on their stigmas (Ngo *et al*., 2010). Cross-incompatible, cross-semi-compatible, and cross-full-compatible relationships were clarified based on the results of the number of pollen tubes that reached the base of the style. From these results, *S* genotypes of several cultivars were estimated. The *S* genotype could be estimated with the aid of allozymes produced by the glutamate oxaloacetate transaminase isozyme gene (*Got-3*), which appeared to be linked to the *S* gene (Ngo *et al*., 2011). The most efficient way to determine the *S* genotype is considered to be pollination with pollen homozygous for the *S* genotype. Kim *et al*. (2010, 2011) revealed the *S* genotype of some cultivars by pollination of homozygous S_1 seedlings of 'Hirado buntan' [*C. maxima* (burm.) Merr.] and 'Banpeiyu' (*C. maxima*). There are no differences in estimated *S* genotypes among the three above-mentioned methods. Table 3 shows the estimated *S* genotypes of several accessions.

Incompatibility *S* alleles are distributed widely, not only in self-incompatible accessions but also self-compatible ones such as satsuma mandarin (*C. unshiu*), grapefruit (*C. paradisi*), and 'Dancy' (*C. tangerina* hort. ex Tanaka) (Soost, 1965, 1969; Vardi *et al*., 2000). Thus, self-incompatible individuals can be produced from cross combinations between two self-

Fig. 3 - Female-sterile seedless citrus 'Southern Yellow'.

Table 3 - Estimated S genotype of citrus accessions (Kim *et al*., 2011; Ngo *et al*., 2010, 2011)

Accession	Latin name	Estimated *S* genotype [z]
Banpeiyu	*Citrus maxima* (Burm.) Merr.	S_1S_2
Tosa Buntan	*C. maxima* (Burm.) Merr.	S_1S_3
Iriki Buntan	*C. maxima* (Burm.) Merr.	S_1S_2
Kaopang	*C. maxima* (Burm.) Merr.	S_1S_2
Soyu	*C. maxima* (Burm.) Merr.	S_1S_2
Hassaku	*C. hassaku* hort. ex Tanaka	S_4S_5
Yuge-hyokan	*C. yuge-hyohan* hort. ex Yu. Tanaka	S_6S_7
Shishiyuzu	*C. pseudogulgul* hort. ex Shirai	S_1S_6
Hyuganatsu	*C. tamurana* hort. ex Tanaka	S_1S_8
Tachibana No. 1	*C. tachibana* (Makino) Tanaka	SfS_8
Rough lemon	*C. jambhiri* Lush.	SfS_1
Zadaida	*C. aurantium* L.	SfS_1
Kinukawa	*C. glaberima* hort. ex Tanaka	SfS_2
Kawano Natsudaidai	*C. natsudaidai* hort. ex Tanaka	SfS_2

[z] Sf: self-compatible.

compatible parents, for example, 'Orlando' and 'Minneola' arose from 'Duncan' grapefruit and 'Dancy' combination (Swingle *et al*., 1931). In Japan as well, self-incompatible seedless 'Ariake' was bred by crossing self-compatible 'Seike' navel orange (*C. sinensis* (L.) Osbeck) and self-incompatible clementine (*C. clementina* hort. Tanaka) (Yamada *et al*., 1995; Yamamoto *et al*., 2006) (Fig. 4, Table 2).

Fig. 4 - Self-incompatible seedless citrus 'Ariake'.

3. Ploidy manipulation for seedless cultivar breeding

Although spontaneous triploid (3x=27) accessions were very rare in citrus (Krug, 1943; Krug and Bacchi, 1943; Noro and Kajimoto, 1955), many triploid hybrids have been produced by artificial hybridization. Since these triploids are seedless, it could be considered that producing triploids is a useful way to promote seedless breeding in citrus efficiently. Tachikawa *et al*. (1961) conducted one of the earliest programmed triploid breeding projects. First, they produced tetraploid materials (4x=36) by colchicine treatment and then triploid (2x=18) hybrids were obtained from these tetraploid and diploid cross combinations. As they showed, since triploids arise from tetraploid and diploid crossing, tetraploid plants are important for triploid breeding. Therefore, various tetraploid accessions were obtained by certain methods.

Oiyama *et al*. (1980) selected spontaneous autotetraploids from nucellar seedlings of polyembryonic cultivars. They revealed the leaf morphological characteristics of tetraploids: thick and broad leaves and reduced number of stomata per area. Kawase *et al*. (2005) obtained Meiwa kumquat (*Fortunella crassifolia* Swingle) autotetraploid from 500 seedlings. An autotetraploid also arose as a bud sport (Yamao *et al*., 1993). Colchicine treatment is useful for the production of autotetraploids in many higher plants. In citrus in particular, various tetraploids were produced by this treatment. Colchicine treatment of seeds was effective in polyembryonic cultivars (Yahata *et al*., 2004). However, this treatment of monoembryonic seeds is a problem because the tetraploids obtained by this treatment are not true-to-type. Oiyama and Okudai (1986) resolved this problem through a combination of colchicine treatment of isolated small buds and their micrografting. They successfully produced autotetraploids from three monoembryonic cultivars. Moreover, in another eight autotetraploids, monoembryonic citrus was produced using the same method (Kaneyoshi *et al*., 2008).

It has been revealed that although tetraploids arise from both diploid × tetraploid and tetraploid × diploid crosses, the latter combination is more effective (Cameron and Burnett, 1978; Kaneyoshi *et al*., 2008) and an unbalanced ploidy ratio between embryo and endosperm is considered to cause this phenomenon (Esen and Soost, 1973). Thus, artificially produced monoembryonic autotetraploids were important as seed parents for triploid breeding. On the other hand, triploid hybrids sometimes appeared from diploid-diploid crosses (Esen and Soost, 1971; Oiyama and Okudai, 1983; Yasuda *et al*., 2010); the appearance of triploids is due to the unreduced gametophyte of one parent (Esen and Soost, 1971).

Biotechnological methods such as protoplast fusion have contributed to the progress of citrus triploid breeding. Since somatic hybrids are tetraploids in general (Ohgawara *et al*., 1985), they are important parents for triploid breeding. Somatic hybrids derived from crosses between navel orange (*C. sinensis*) + satsuma mandarin (*C. unshiu*), grapefruit (*C. paradisi*), yuzu (*C. junos*) and 'Murcott' (artificial hybrid) were registered as parental lines (Kobayashi *et al*., 1995). Triploid hybrids could be produced directly by means of protoplast fusion. Somatic hybrids produced from diploid and haploid fusion became triploids (Kobayashi *et al*., 1997). The haploid parents were obtained by diploid × triploid cross (Oiyama and Kobayashi, 1993).

Table 2 shows the triploid cultivars released in Japan. Among the four cultivars, 'Puchimaru' (Yoshida *et al*., 2003) (Fig. 5), 'White love', Tokushima 3X No. 1' (Toku-

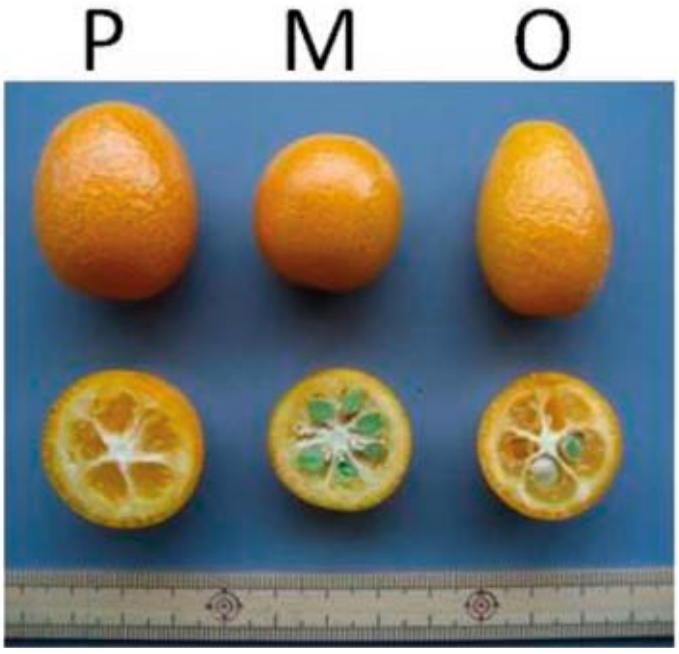

Fig. 5 - Triploid seedless kumquat 'Puchimaru' and diploid seedy Meiwa kumquat and Oval kumquat. P= Puchimaru, M= Meiwa kumquat, and O= Oval kumquat.

naga *et al*., 2005), and 'Yellow Bell' (Kaneyoshi *et al*., 2014) were derived from diploid × tetraploid cross, protoplast fusion between diploid and haploid, tetraploid × diploid cross, and diploid × diploid cross, respectively.

4. Conclusions

Seedless breeding of citrus has progressed rapidly in Japan over the last few decades. The production of new seedless cultivars is increasing in contrast to the decrease in production of seedy conventional cultivars such as 'Kawano Natsudaidai' (*C. natusdaidai*) and 'Miyauchi Iyokan' (*C. iyo*). Various kinds of cross combinations using cultivars or parental lines with sterility have been conducted actively and further polyploid breeding is being carried out to produce new triploid plants. In addition, methods to shorten the long juvenile period of citrus have developed in Japan (Okudai *et al*., 1980; Mitani *et al*., 2008), making it easier to breed, compared to a few decades ago, various types of new seedless cultivars.

Understanding the mechanism and hereditary mode of each type of sterility will contribute to produce seedless individuals efficiently and effectively. Biotechnological techniques are also very useful for producing seedless materials. The results of genome analysis have provided useful information for breeding new seedless citrus with female sterility (Garcia *et al*., 2000). Moreover, owing to the progress of recent DNA analysis technologies, the draft whole genome of sweet orange (*C. sinensis*) has already been reported (Xu *et al*., 2013). The combination of conventional breeding study, biotechnology, and genome analysis is considered to be essential to breed new superior seedless cultivars in citrus.

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Thermogenesis in skunk cabbage (*Symplocarpus renifolius*): New insights from the ultrastructure and gene expression profiles

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Key words: floral thermogenesis, inflorescence, low temperature, mitochondria, respiration, transcriptome.

Abstract: Floral thermogenesis has been found in several plant species. The spadix of one thermoregulatory plant, the Eastern Asian skunk cabbage (*Symplocarpus renifolius*), can maintain its temperature at approximately 22-26°C for several days, even when the ambient temperature falls below freezing. There are two major stages in skunk cabbage inflorescence development: the thermogenic female stage and the non-thermogenic male stage; in the former the spadix can produce massive amounts of heat, whereas in the latter, thermogenesis is undetectable. Based on previous studies, there is a positive correlation between heat production and the abundance of mitochondria in plant tissues and cells, and genes involved in cellular respiration and mitochondrial function are significantly enhanced at the female stage. Taken together, these findings suggest that the increased respiration or mitochondrial abundance observed in thermogenic tissues may be attributable to the high expression of specific genes. This review summarizes new insights into the changes in intracellular structures and gene expression profiles of skunk cabbage spadices during the female-male transition and proposes possible processes that are essential for each stage during floral development.

1. Introduction

Floral thermogenesis occurs in several plant taxa including gymnosperms (Cycadaceae), as well as eudicots (Nymphaeaceae) and monocots (Araceae). Thermogenesis begins when these plants bloom, and heat production terminates when pollen is released from the anthers. One thermoregulatory plant, the Eastern Asian skunk cabbage (*Symplocarpus renifolius*), can keep the spadix temperature between 22-26°C for several days even when the ambient temperature falls below freezing (Fig. 1A) (Knutson, 1974; Uemura *et al*., 1993; Seymour, 2004). Other thermoregulatory plants studied to date include *Phillodendron sellom* (Nagy *et al*., 1972; Seymour *et al*., 1983) and *Nelumbo nucifera* (Seymour and Schultze-Motel, 1998; Seymour *et al*., 1998). Many species, which are thermogenic but not thermoregulatory, are generally able to produce heat for only 24 h at best. The robust thermoregulation observed in *S. renifolius* and other species makes these plants great models for unraveling the mechanism underlying floral thermogenesis. In several species of Araceae, floral thermogenesis has been proposed to serve the physiological role of spreading odor to attract pollinators (Meeuse and Raskin, 1988), whereas thermoregulation in *S. renifolius* is not closely associated with cross-pollination (Seymour and Blaylock, 1999). *S. renifolius* produces only a faint aroma in early spring when few insects are active. Thus, heating may promote early flowering or protect the *S. renifolius* inflorescence from damage by freezing.

In *S. renifolius*, thermogenesis is closely associated with three stages of inflorescence development: female, bisexual, and male (Fig. 1B). At the female stage, which lasts until the stamens emerge from the surface of the spadix, the spadix can produce massive amounts of heat. At the bisexual stage, the stamens begin to release pollen and thermogenesis fluctuates. Finally, at the male stage, pollen is released from nearly all stamens and thermogenesis is undetectable. Microscopic analysis revealed that structural changes in the stamen are significant, and extensive anther development occurs during inflorescence development (Ito-Inaba *et al*., 2009 a). In addition to the structural changes in stamens, the ultrastructure of petals and pistils also significantly change. These tissues accumulate a larger number of mitochondria during the female stage than during the male stage. Also, large cytoplasmic vacuoles develop during the male stage. In our recent gene expression analysis, expression of genes involved in cellular respiration and mitochondrial function was significantly enhanced during the thermogenic female stage, whereas genes involved in stress responses and protein degradation were significantly up-regulated during the non-thermogenic male stage (Ito-Inaba *et al*., 2012 a). Therefore, changes in the intracellular structure observed in

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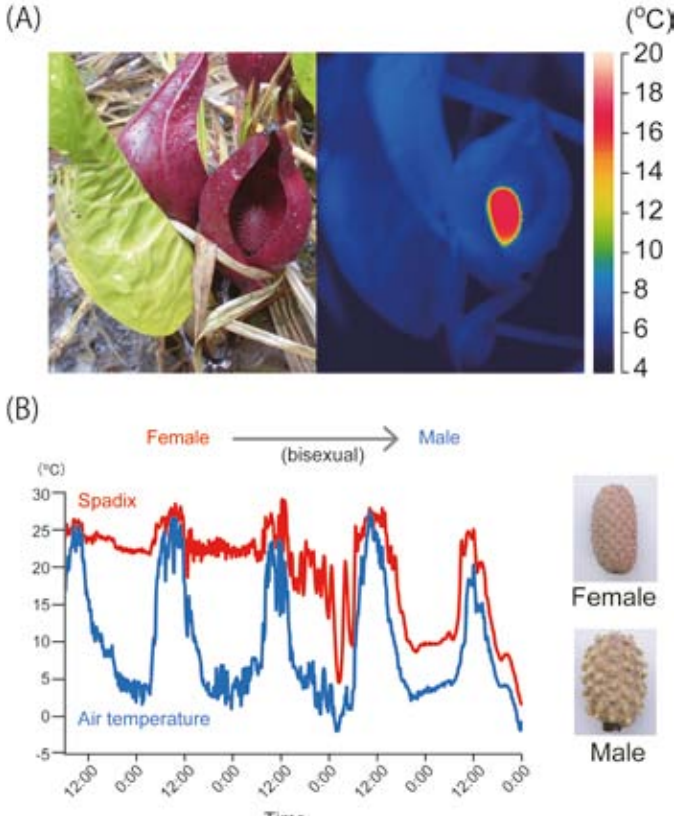

Fig. 1 - Thermoregulation in skunk cabbage (*S. renifolius*). (A) Skunk cabbages were photographed using a camera in the visible (left panel) and infrared spectra (right panel). The thermal image was taken with Thermotracer SC620 (FLIR). Heat production was observed in the spadix during the female stage of floral development. (B) The sequential changes in spadix (red) and air (blue) temperatures during floral development from the female to the male stage. Spadices at the female stage can maintain internal temperature at approximately 22-26°C, whereas spadices at the male stage cannot produce heat. Spadices at the bisexual stage between the female and male stages show unstable thermogenesis. Photographs of a female- and a male-stage spadix, are shown in the upper right and lower right panels, respectively. (B) was partially extracted from figure 1 in our previous paper (Ito-Inaba *et al*., 2009 a).

petals or pistils during the female-male transition are well supported by changes in the transcriptome during inflorescence development.

Two processes may be important for thermoregulation in skunk cabbage (Ito-Inaba *et al*., 2012 a). First, short-term mechanisms that depend on increased cellular respiration with the help of energy dissipating proteins, such as alternative oxidase (AOX) or uncoupling protein (UCP), may play an essential role in which AOX may have a more major function than UCP. Secondly, long-term effects of mitochondrial biogenesis on the number and structure of mitochondria probably are also involved. Following much effort to characterize the activity or expression of AOX during floral development, the pivotal role of this enzyme in floral thermogenesis was revealed (Watling *et al*., 2006; Grant *et al*., 2008; Wagner *et al*., 2008; Ito-Inaba *et al*., 2009 b; Miller *et al*., 2011). However, the presence of additional genes that are co-expressed with AOX and that may function directly or indirectly in thermogenesis remains to be clarified. In addition, although it has been hypothesized that heat-producing floral tissues contain many mitochondria, quantitative and comparative studies on mitochondrial content are lacking. In this review, we summarize our recent progress in describing changes in the ultrastructure and gene expression profiles during skunk cabbage floral development.

2. Thermogenesis and mitochondrial abundance

In mammalian cells, the positive correlation between metabolic activity and the number and size of mitochondria within a tissue is well established (Ghadially, 1988). Mammalian brown adipose tissue (BAT), which is the main site for non-shivering thermogenesis, contains considerable numbers of large mitochondria with abundant cristae. In contrast, these relationships are not well characterized in plants, and there are very few published papers that have examined the intracellular structure of thermogenic tissues by electron microscopy. In a well-known thermogenic plant, *Sauromatum guttatum* (voodoo lily), ultrastructural changes in the inflorescence during the transition from the pre- to post-thermogenic stages were extensively studied, and clear details of mitochondrial morphology were obtained (Skubatz *et al*., 1993). In addition, during the thermogenic stage of *S. guttatum* floral development, mitochondria accumulated osmophilic material between the inner and outer membranes (Skubatz and Kunkel, 2000). In another thermogenic plant, *Philodendron selloum*, large lipid bodies present in sterile florets before heating were progressively depleted during heat generation, and the mitochondria often contained enlarged cristae during maximum heating (Walker *et al*., 1983). However, there are no conclusive data indicating a relationship between heating in plant tissues and mitochondrial features, such as content or morphology.

We first analyzed the detailed changes in mitochondrial content and morphology during floral development of thermogenic skunk cabbage, *S. renifolius* (Ito-Inaba *et al*., 2009 a). As shown in figure 2A, petal cells at the female stage contained a large number of mitochondria. By contrast, petal cells at the male stage contained only a small number of mitochondria but had large central vacuoles. In the pistil cells, likewise, a large number of mitochondria were present at the female stage but few mitochondria persisted to the male stage. Furthermore, stamens at the female stage, especially in the microspore and plasmodium, had high densities of mitochondria. The sizes and morphologies of mitochondria observed in all tissues varied. To evaluate the mitochondrial content quantitatively between the female and male stages in each floral tissue, the average mitochondrial density (mitochondrial numbers μm^{-2} cytosol) in thin sections of cells were analyzed in five to 10 cells. These data also revealed that both petals and pistils at the female stage contained larger numbers of mitochondria compared with the male stage. Details of the ultrastructure and the quantitative

data on mitochondrial content are described in our previous paper (Ito-Inaba *et al*., 2009 a). We next compared the mitochondrial protein content recovered from thermogenic and non-thermogenic stages or tissues (Ito-Inaba *et al*.. 2009 a, b). As shown in figure 2B, the mitochondrial protein content of female-stage spadices (0.54 mg g^{-1}) was two-fold higher than that of males (0.29 mg g^{-1}), a value consistent with our electron microscopic data. In addition, mitochondrial protein content of non-thermogenic skunk cabbage, *Lysichiton camtschatcensis* (0.011 mg g^{-1}), was much lower than that of *S. renifolius*. Since *L. camtschatcensis* has no ability to produce heat but has a close relationship with *S. renifolius* in morphology and phylogeny, this result suggests that a lower mitochondrial content may correlate with the lack of thermogenesis in *L. camtschatcensis*. Taken together, these results reveal that there is a positive correlation between heat production and the abundance of mitochondria in plant tissues and cells. These are the first quantitative data indicating differences in mitochondrial content between thermogenic and non-thermogenic stages or tissues. Therefore, plants might produce the massive heat from their tissues by increasing their mitochondrial density in a manner similar to mammalian BAT.

3. The quantitative gene expression profile in female- and male-stage spadices of *S. renifolius*

To understand the molecular basis of floral thermogenesis, we examined the gene expression profiles of female- and male-stage spadices of *S. renifolius*. Since the complete genome sequence of *S. renifolius* is not available, we took advantage of the super serial analysis of gene expression (SuperSAGE) methodology as this method can provide quantitative and comprehensive gene expression profiles (Ito-Inaba *et al*., 2012 a). In our study, 26 bp tags (SuperSAGE tags) expressed from female- and male-stage spadices were prepared and sequenced using a 454 Life Sciences Genome Sequencer 20 System. Since the length of 26 bp tags is sufficient to identify the origin of a tag using cDNA databases (Matsumura *et al*., 2003, 2011), each 26 bp tag was annotated based on our cDNA database of the female-stage spadices using the BioEdit program. The gene expression profiles obtained were subjected to cluster analysis to identify candidate sets of co-regulated genes directly or indirectly associated with the process of female- and male-stage spadices, and were qualified as a group of female- or male-stage specific genes. To further assess the function of each gene, AGI codes of *Arabidopsis* orthologs corresponding to the identified genes were obtained from the database of The Arabidopsis Information Resource (http://www.arabidopsis.org/index.jsp), and the identified genes were classified based on Gene Ontology (GO) terms using the AGI codes. This analysis allowed us to predict the localization and function of the orthologs in *S. renifolius*. Each gene was weighted according to the number of corresponding SuperSAGE tags that reflected the expression level of each gene.

Based on these methods, transcripts were assigned to specific cellular components or biological processes (Ito-Inaba *et al*., 2012 a) and the major transcriptional changes are shown in figure 3. It was of particular interest that genes encoding mitochondrial proteins were actively transcribed in female spadices but not in male spadices (Fig. 3A). In addition, the activity of genes related to electron transport or energy pathways decreased significantly during the transition from the female to the male stage (Fig. 3B). These results suggest that mitochondrial function

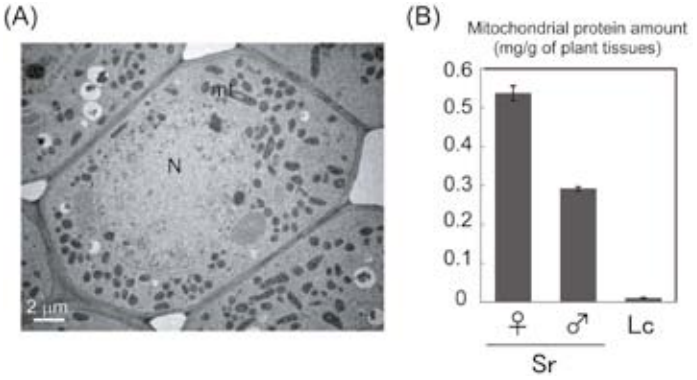

Fig. 2 - Abundant mitochondria are present in the spadix of thermogenic skunk cabbage. (A) Female spadix cells (petal tissues) contain many mitochondria. This photograph was adapted from Fig. 5A in our previous paper (Ito-Inaba *et al*., 2009a). In this study, large numbers of mitochondria were also observed in pistils and in several tissues in stamens. (B) Quantitative comparison of mitochondrial protein amount from thermogenic and non-thermogenic stages or tissues. Female-stage spadices (♀) in *S. renifolius* (Sr) contain 2-fold and 50-fold higher content of mitochondria than male spadices (♂) and or spadices from *L. camtschatcensis* (Lc), respectively. Data was extracted from Table 2 and Table 1 in our previous papers (Ito-Inaba *et al*., 2009 a, b, respectively).

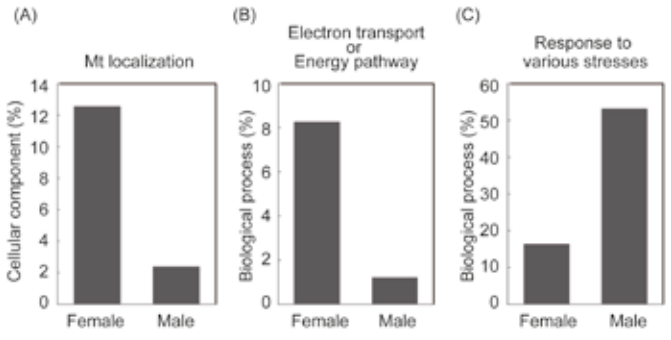

Fig. 3 - Examples of transcriptional changes of cellular components and biological processes of female- and male-stage spadices of *S. renifolius*. Genes encoding proteins localized in mitochondria (A) or that play roles in electron transport or the energy pathway (B) are highly expressed at the female stage, but not at the male stage of floral development. Genes encoding stress-responsive proteins (C) were highly expressed at the male stage, but not at the female stage. (A) was partially extracted from figure 3a (the cellular component data) in our previous paper (Ito-Inaba *et al*., 2012 a). (B) and (C) were also partially extracted from figure 3b (the biological process data) in the same paper.

and/or cellular respiration play a key role in floral thermogenesis. This finding is consistent with our electron microscopic observation that the thermogenic female-stage spadix accumulates a large number of mitochondria and has an increased oxygen consumption rate. Furthermore, genes classified as stress responsive were highly expressed in male spadices (Fig. 3C). Of these genes, a gene encoding a cysteine protease in *S. renifolius*, designated as Sr-CPA, was the most abundant transcript in the spadices, and levels increased significantly during the female-male transition (Ito-Inaba *et al*., 2012 b). This class of cysteine protease is involved in programmed cell death (Beyene *et al*., 2006) and stress responses (Stevens *et al*., 1996) in other organisms. Since our previous studies suggested that a parallel relationship exists between the increase in CP transcripts and vacuolar development in each of the various spadix tissues during the female-male transition, the high level of *SrCPA* expression may be correlated with vacuolar development in male-stage spadices. In addition, several stress-responsive genes and genes encoding degradative enzymes or ubiquitin-proteasome system components had increased expression levels at the post-thermogenic stage. Therefore, we hypothesize that cysteine protease and other degradative enzymes that leak from the vacuole may degrade mitochondria, thereby terminating thermogenesis at the male stage.

4. Conclusions and Perspectives

Our previous electron microscopic study revealed that intracellular structures within the individual tissues change significantly during the transition from the female- to the male-stage spadix in *S. renifolius*. The mitochondrial content is reduced, especially in the petals and pistils, whereas the vacuolar volume increases during the female-male transition. Consistent with this cellular change, gene expression profiles analyzed using SuperSAGE methods indicated that the genes involved in cellular respiration and mitochondrial function are up-regulated in female-stage spadices, whereas the genes involved in stress responses and protein degradation are up-regulated in male-stage spadices. These observations suggest that the maintenance and termination of floral thermogenesis in the female- and the male-stage spadices, respectively, may be explained as shown in figure 4. At the female stage, the high expression levels of genes related to cellular respiration and mitochondrial function induce significant oxygen consumption and mitochondrial biogenesis, and activate cellular metabolism leading to substantial heat production. In contrast, at the male stage, the high expression levels of genes related to protein degradation and vacuolar metabolism induce senescence, programmed cell death, and vacuolar development, leading to the termination of heat production. After thermogenesis, the expression of several stress response genes, such as cold-inducible genes, increase because the spadix cannot produce any heat. With exposure to the cold air, the spadix cells proceed to senescence.

More than 200 years ago, pioneering studies on floral thermogenesis were undertaken in the European Arum (Araceae) by Lamarck (1778). Since then, heat production by the reproductive organs of several plants has been investigated. We anticipate that the numbers of plants known to produce heat will increase in the future as the subtle temperature differences between the air and plant bodies can be measured by technical advances in temperature probes or thermography. To study the molecular mechanisms underlying floral thermogenesis, two energy dissipating systems, an alternative oxidase (AOX) and uncoupling protein (UCP), have been the principal subjects of investigation (Vanlerberghe and McIntosh, 1997; Vercesi *et al*., 2006; Zhu *et al*., 2011). Because of the correlation between heat production and AOX concentration, as well as activity in several thermogenic plants (Grant *et al*., 2008; Ito-Inaba *et al*., 2009 b; Miller *et al*., 2011), AOX rather than UCP has been assumed to control plant thermogenesis. Recently, the crystal structure of a trypanosomal AOX was reported (Shiba *et al*., 2013). Since the post-translational regulation of AOX has been hypothesized to regulate the thermogenic capacity of this protein (Grant *et al*., 2009), revealing the structural features of AOX may open the door to elucidating the mechanisms underlying the post-translational regulation of AOX. Furthermore, we anticipate that recent advances in next generation sequence (NGS) technology will uncover additional genes, besides AOX and UCP, that are involved in floral thermogenesis. In *S. renifolius*, the gene expression profile has already been studied using NGS technology combined with the SuperSAGE method and could provide valuable information to define the identity of female- and male-stage spadices at the molecular level (Ito-Inaba *et al*., 2012 a). As far as we know, this was the first study in which the molecular mechanism underlying floral thermogenesis was analyzed using NGS technology. Quite recently, the genome of the sacred lotus (*Nelumbo nucifera*), a well-known thermogenic plant, was sequenced (Ming *et al*., 2013). We also expect that this advance will accelerate study of the molecular mechanism underlying heat production in the reproductive organ development of

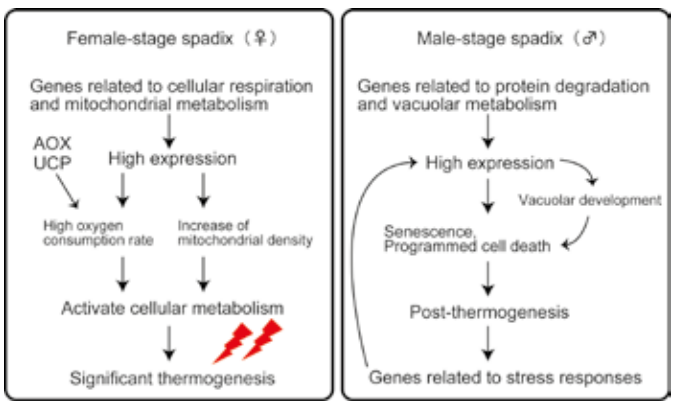

Fig. 4 - A proposed model of the possible processes in female- and male-stage spadices to maintain and terminate thermogenesis, respectively.

sacred lotus. *S. renifolius* is a monocot thermogenic plant, whereas *N. nucifera* is a eudicot thermogenic plant. Thus, comparative studies of these plants will reveal general and diverse aspects of floral thermogenesis.

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Development of *in vitro* propagation by node culture and cryopreservation by V-Cryo-plate method for *Perilla frutescens*

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Key words: aluminum plate, cryopreservation, *Perilla*, V-Cryo-plate method.

Abstract: A clonal propagation method by node culture of perilla (*Perilla frutescens* L. Britton) was investigated. Nodes were plated on solidified 1/2 MS medium with BA and optimum shoot elongation and propagation was obtained at BA 0.05 and 0.1 mg l^{-1}. Cryopreservation using an aluminum cryo-plate was successfully applied to *in vitro*-grown perilla shoot tips. Excised shoot tips from nodes were precultured on 1/2 MS medium with 0.3 M sucrose and embedded on an aluminum cryo-plate with alginate gel. The cryo-plate with shoot tips was osmo-protected with LS solution and dehydrated in PVS2 for 20 min at 25°C prior to immersion into liquid nitrogen. The recovery growth after cryopreservation was found to be about 80%. This new V-Cryo-plate method has many advantages and may facilitate the cryo-storage of other medicinal plants.

1. Introduction

Perilla (*Perilla frutescens* L. Britton) belongs to the *Lamiaceae* family and is cultivated in China and Japan (Pandey and Bhatt, 2008). The seeds yield oil and the leaves are used for medicine or garnish for fish (Nitta *et al.*, 2005; Hossain *et al.*, 2010). Dried red *Perilla* leaves are also used as 'Soyou' in Kampo medicine and it is one of the components of 'Saibokuto,' which is used to treat bronchial asthma (Homma *et al.*, 1992; Ueda *et al.*, 2002). The seeds of Perilla have been used for food for birds or humans, oil as a fuel or a cooking oil, and also the leaves are used as a potherb for medicine or food coloring and the foliage to produce an essential oil for flavoring (Brenner, 1993). Perilla has a variable chromosome complement (Brenner, 1993); a haploid chromosome count of fourteen plus zero to two beta chromosomes (Vij and Kashyap, 1976) and chromosome counts of both n=20 and 2n=38, and three distinguished chromosome sizes (Yamane, 1950). As the traditional crop landraces are facing danger of complete extinction in some areas, some of *Perilla frutescens* species are at the verge of extinction in the Central Himalaya (Negi *et al.*, 2011). Usually *Perilla* species are conserved in genebanks as plant genetic resources (Arora, 1997). Biotechnological tools are important to conserve the critical genotypes of medicinal plants like *Perilla* species. However, previous reports of plant regeneration for *Perilla* used seedlings segments such as the cotyledon and hypocotyl as material (Hou and Jia, 2005; Zhang *et al.*, 2005; Hossain *et al.*, 2010). Moreover, there are few reports about effective clonal propagation of *Perilla*. Cryopreservation techniques are now used for plant germplasm storage at several institutes around the world (Niino, 2006) and this method has become an important tool for the long-term storage of plant germplasm and of experimental materials that possess unique attributes, minimizing space and maintenance requirements without causing genetic alterations (Sakai, 1997; Matsumoto *et al.*, 2013). Recently, a vitrification protocol using the aluminum cryo-plate method (V-Cryo-plate method) has been reported (Sekizawa *et al.*, 2011; Yamamoto *et al.*, 2011 a, b; 2012). Niino *et al.* (2013) reported that the V-Cryo-plate method has two main advantages: a user-friendly procedure and very high cooling and warming rates of treated samples. As a result, very high regrowth was obtained after cryopreservation of the tested materials (Niino *et al.*, 2013).

In this study, we have developed effective clonal propagation by node culture and cryopreservation of *in vitro* grown shoot tips using the V-Cryo-plate method for *Perilla*.

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2. Materials and Methods

In vitro *propagation by node culture*

The material, seeds of *Perilla frutescens* L. Britton, was obtained from Tsukuba Division, Research Center for Medicinal Plant Resources, National Institution of Biomedical Innovation (Tsukuba, Japan). The seeds were surface sterilized in 70% ethyl alcohol for 30 sec then in 1% NaOCl for 15 min. After rinsing three times in sterile distilled water, they were placed on hormone-free half-strength solidified MS medium (Murashige and Skoog, 1962; termed 1/2 MS) and incubated at 25°C with 16 h light/8 h dark photoperiod under a light intensity of 50 µmol m^{-2} s^{-1} for germination. After one month, the axillary buds were placed on solidified 1/2 MS medium with 0, 0.01, 0.05, 0.1 and 0.5 mg l^{-1} BA and incubated. The shoot length, number of leaves, number of shoots, number of roots and longest root length were measured. Ten buds were used for each treatment with three replicates.

Cryopreservation by V-Cryo-plate method

Axillary shoot tips (1 mm size) were excised from *in vitro* grown plants of *Perilla* and used for cryopreservation by V-Cryo-plate method (Yamamoto *et al.*, 2011 a, b) with some modification. Figures 1 and 2 show the schematic diagram of the aluminum cryo-plate used and the V-Cryo-plate procedure. Cryo-plates used in this study were obtained from the National Institute of Agrobiological Sciences (Tsukuba, Japan). The following steps were performed:

1) Excised shoot tips were precultured on solidified 1/2 MS medium with 0.3 M sucrose for 1 day at 25°C to induce the osmo-protection.
2) An aluminum cryo-plate (Fig. 3) was placed in a petri-dish and 2.0-2.5 µl of 2% (w/w) Na-alginate solution with 0.4 M sucrose in 1/2 MS medium was poured in a well.
3) The precultured shoot tips were positioned in each well and 100 mM $CaCl_2$ solution with 0.4 M sucrose in 1/2 MS were added to the aluminum plate for 15 min for polymerization (Fig. 3).
4) After removing $CaCl_2$ solution, the cryo-plate with shoot tips was treated with LS solution (2 M glycerol + 0.6 M sucrose) (Matsumoto *et al.*, 1994; Yamamoto *et al.*, 2011 b) in a petri dish (8 cm in diameter) for 20 min at 25°C for osmo-protection.

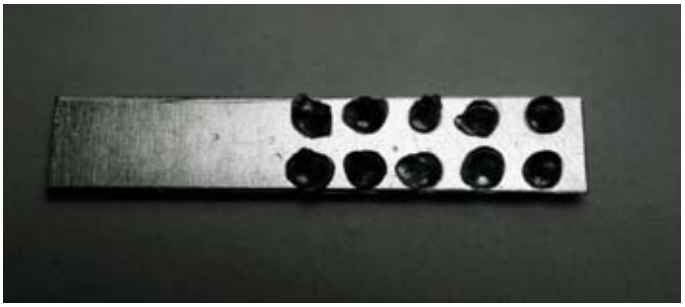

Fig. 1 - Aluminum cryo-plate with embedded shoot tips. Size: 7 mm × 37 mm × 0.5 mm with ten wells (diameter 1.5 mm. depth 0.75 mm).

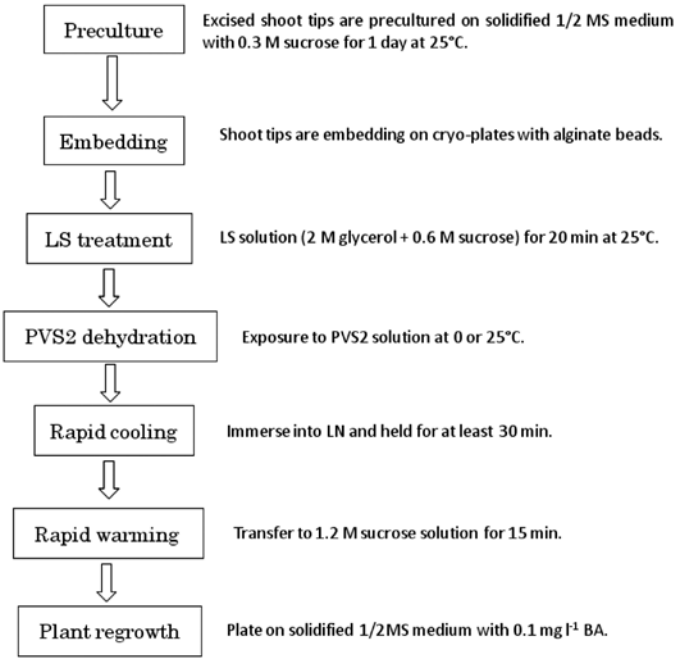

Fig. 2 - Procedure of V-Cryo-plate for cryopreservation of *Perilla* shoot tips.

Fig. 3 - Shoot formation by node culture after 2 months 1/2MS + BA 0.1 mg l^{-1}. Bar = 10 mm.

5) After LS treatment, the cryo-plate with shoot tips was dehydrated with PVS2 solution (Sakai *et al*., 1990) for 0 to 40 min at 0 C and for 0 to 20 min at 25°C.
6) The cryo-plate was then transferred to an uncapped 2 ml cryotube held on a cryo-cane and directly plunged into liquid nitrogen (LN) for at least 30 min.
7) For plant regeneration, the cryo-plate with shoot tips in LN was transferred to 1.2 M sucrose solution with 1/2 MS in a petri dish (8 cm in diameter) for 15 min at 25°C for rapid warming and unloading. Shoot tips were then plated on solidified 1/2 MS medium with 3% sucrose and incubated at 25°C under standard conditions.

Three replicates of 10 shoot tips were tested in each experiment. Statistical analyses were performed using Tukey's test and significant differences ($P<0.05$) were determined.

3. Result and Discussions

In vitro *propagation by node culture*

After incubation for 30 days on 1/2MS medium with different concentrations of BA, all nodes of *Perilla* formed shoots without callus formation. The optimum shoot elongation and propagation was obtained at BA 0.05 and 0.1 mg l^{-1} (Table 1). In previous reports, plant regeneration of *Perilla* was obtained from hypocotyl segment including apical bud (Hossain *et al.*, 2010), cotyledon, and hypocotyl (Zhang *et al.*, 2005). The shoot recovery rate of this node culture (100%) is higher than that of the previous reports (65 to 91%). Moreover, regenerated plants from cotyledon and hypocotyl segments are not genetically the same and the propagation systems are not suitable for clonal propagation. In this experiment, 1.6 shoots with 5.8 leaves (average of BA 0.05 and 0.1 mg l^{-1}) were obtained from one node after 30 days of incubation. Nodes were located basal respect to each petiole, and we found that one node produced 9.28 nodes (5.8 × 1.6) after 30 days incubation. This propagation efficiency (9.28) is considered to be as high as clonal propagation. Furthermore, no abnormalities were observed in the regenerated shoots (Fig. 3) indicating that this method can be considered a suitable propagation method for the material of cryopreservation.

Table 1 - Effect of BA concentration on shoot regrowth from nodes in Perilla

BA (mg l^{-1})	Shoot length (mm)	No of leaves	No of shoots	No of roots	Longest root (mm)	Re-growth (%)
0	6.7 ab	5.4 a	1.0 b	1.9 b	26.5 ab	100
0.01	10.1 a	5.1 ab	1.0 b	1.4 ab	30.7 a	100
0.05	11.3 a	6.0 a	1.5 a	2.2 b	16.3 ab	100
0.1	8.2 a	5.6 a	1.7 a	1.3 ab	10.8 ab	100
0.5	5.1 b	3.7 b	1.0 b	0.7 a	6.2 b	100

Cryopreservation by V-Cryo-plate method

In our previous reports of cryopreservation using vitrification (Matsumoto *et al.*, 1994; 1995), we demonstrated that the osmo-protection treatments (preculture of high sucrose medium, LS treatment of 2 M glycerol + 0.4 M sucrose solution) were necessary to produce high regrowth before PVS2 dehydration for shoot tips of most plant species. The V-Cryo-plate procedure was carried out according to Yamamoto *et al.* (2011 a, b) with these two osmo-protection treatments. Figure 4 shows the regrowth rates with different exposure times to PVS2 at 0 and 25°C. The highest regrowth rates obtained were about 55% for 30 min at 0°C and about 70% for 15 min at 25°C. In the vitrification protocol, direct exposure of less tolerant cells and meristems to highly concentrated PVS2 at 25°C was found to possibly be harmful due to osmotic stress or chemical toxicity (Matsumoto and Sakai, 2003). In this experiment, the regrowth rate at 0°C was 15% lower than that at 25°C. The reason of this result was not clear, but might be related to problems associated with sensitivity to PVS2 and/or dehydration. The high regrowth rate after cryopreservation by V-Cryo-plate method is due to rapid cooling (4,000-5,000°C min^{-1}) and warming (3,000-4,500°C min^{-1}) (Niino *et al.*, 2013). In vitrification-based procedures, damage can be caused by chemical toxicity of PVS2 and osmotic stress by excessive duration of PVS2 treatment (Engelmann, 1997; Sakai *et al.*, 2008). Our results suggest that this high regrowth rate with exposure to PVS2 at 25°C may be due to the rapid cooling and warming. For practical use for cryopreservation, the time for the cryogenic procedure should be short and the regrowth rate after cooling should be high (at least 70%). It is worth noting that no abnormalities were found in shoots developed from

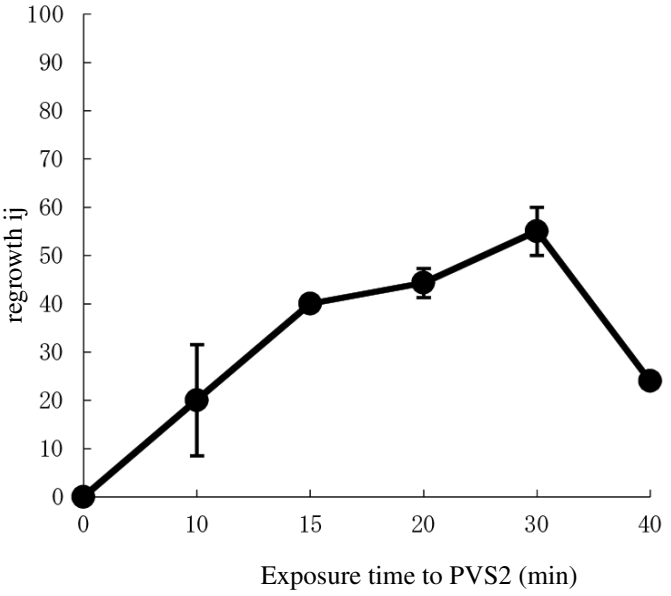

Fig. 4 - Effect of different exposure times and temperatures of PVS2 solution on regrowth of cryopreserved *Perilla* shoot tips using V-Cryo-pate method.

cryopreserved shoot tips using V-Cryo-plate method treated at 25°C. Thus, the PVS2 dehydration at 25°C in this V-Cryo-plate method is suitable for use with *Perilla*.

In conclusion, an efficient clonal propagation method for *Perilla* using node culture was successfully established. In addition, we demonstrated that the V-Cryo-plate procedure led to about 70% regrowth and this procedure was thus a very efficient and practical method for cryopreservation of *Perilla* germplasm. This protocol appears promising for cryopreservation of other medicinal plants, as well as other plant species and/or cultivars of horticultural interest after marginal modifications to the procedure.

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Present status and future outlook of plant factories in Japan

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Key words: environment control, fluorescent light, LED, lettuce, organic electroluminescence, plant factory, strawberry.

Abstract: Plant factories for the steady production of high-quality vegetables year round, and which can be divided into solar and artificial-light types, have recently been expanding in Japan as trigged by a report by a collaboration study group in 2009 and the Great East Japan Earthquake in 2011. Common solar-type plant factories with mulch-span roofs are often difficult to construct in the northern areas of Japan, especially along the Japan Sea coast, because of limited sunshine duration and heavy winter snowfall, while artificial light-type factories are more promising in this region although high running costs due to electricity bills for irradiating plants and cooling the room often hinder the promotion of such facilities. The use of LEDs has recently increased in artificial light-type plant factories, but fluorescent lights are still predominant for economic reasons. Generally only small plants can be grown commercially in artificial light-type factories and the light intensity reaching the lower leaves decreases continuously as the stem of the plant elongates, deteriorating light use efficiency. Flexible organic electroluminescent devices able to cover the whole plant when irradiation is required and that can easily be applied/removed like a plastic film are expected to be introduced in both types of plant factories.

1. Introduction

Highly systematized greenhouses for plant growth are called plant factories (PF) in Japan, and they have been rapidly increasing in recent years (Kobayashi, 2010; Nonami, 2010). PF are roughly divided into two types: the solar light-type (SPF) and artificial light-type (APF) (Murase and Fukuda, 2012; Kozai, 2013). Although both types are essentially the same as greenhouse cultivation, more advanced technologies have been adopted in PF to control growth environments. Japan's Ministry of Economy, Trade and Industry (METI) defines a PF as "a facility that aids in the steady production of high-quality vegetables all year round by artificially controlling the cultivation environment (e.g. light, temperature, humidity, carbon dioxide concentration, and culture solution), allowing growers to plan production" (METI, 2014).

The Netherlands is leading the world in the commercial use of SPF, and some well-known companies such as Hoogendoorn, Hortimax, and Priva export excellent cultivation systems to many countries, including Japan (Kozai, 2013). Compared to these advanced companies, the practical application of PF in Japan has just begun. Nevertheless, research papers and the use of PF have greatly expanded over the last few years. One of the triggers for the expansion of PF was the establishment of the "Plant Factory Working Group" of the "Agriculture, Commerce and Industry Collaboration Study Group in April 2009 in cooperation with MAFF (METI, 2009), who compiled a report describing issues to be addressed, as well as the support required for the promotion and diffusion of PF (METI, 2014).

The Japanese government appropriated approximately €100 million in the fiscal year (FY) 2009 as a supplementary budget to promote PF. Of this, approximately €36 million was spent on constructing and promoting advanced PF, and eight research facilities and 18 pilot plants were established in various areas of Japan, including seven universities (Kobayashi, 2010).

From a broad perspective, there are two ways to promote PF: one is to enhance the productivity of horticultural crops without relying on the experience of farmers, and the other is to allow the private sector to enter agro-industries.

2. Present economic status and agricultural production in Japan

Due to the impact of the financial crisis precipitated by the Lehman Brothers bankruptcy in 2008, the gross domestic product (GDP) of Japan decreased by approximately 7% during the five years from 2008 to 2012 (approximately € 3.7 and € 3.4 trillion, respectively) (Fig. 1) (IMF, 2013). However, Japan has been suffering from a long-term economic slowdown for an even longer period, often dubbed "the lost two decades." This long-term recession heavily impacted the private sector, for example

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construction industries, resulting in many unemployed workers.

Although agricultural production in Japan accounts for less than 2% of the GDP, production was sustained at a constant level during this period (approximately € 6.1 billion), showing that agriculture in Japan was not hit hard by the recession (Fig. 1).

Nevertheless, agriculture in Japan faces some difficult problems, such as a decline in the labor force due to a decreasing birthrate and the aging of farmers, limited farmland, high cost of production, and low productivity. However, such difficulties can also provide great opportunities for the private sector to enter into new business. Because of this, many companies in the private sector are now interested in entering agriculture, but it is also difficult for them to start new businesses due to legal restrictions hampering their entry into agriculture.

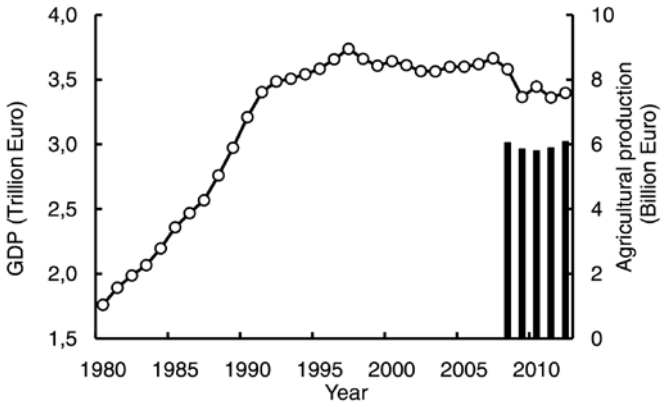

Fig. 1 - Figures for the GDP (1980-2012) and agricultural production (2008-2012) in Japan calculated using the IMF World Economic Outlook Database (2013) and statistics released by MAFF (2013), respectively.

3. Laws regulating the entry of the private sector into agriculture

Until 70 years ago, villages in Japan consisted of a few "zinushi" (landowners) who owned most of the farmland and a large number of "kosakunin" (tenant farmers) who worked on these lands (Council for Regulatory Reform, 2002). "Zinushi" lent the farmland to "kosakunin" to cultivate the land, and took some farm products, such as rice and beans, as "kosakuryo" (land rent). However, after World War II, farmlands in Japan were divided and distributed to each "kosakunin". This new policy allowed "kosakunin" to become land owners and saved Japan from food shortages during the postwar period. However, the area of distributed farmlands was too small for children to inherit. As the Japanese economy developed rapidly during the postwar period, many of these workers left the villages to find jobs in the cities, resulting in the aging of the farming population since the 1960's. According to statistics from Japan's Ministry of Agriculture, Forestry and Fisheries (MAFF), the total number of Japanese farmers in 2013 was 5.62 million, and 2.03 million of them (36.1%) were older than 65 years, which is higher than the percentage of over 65 years of the total population (24.1%) (MAFF, 2014).

Aging of the farming population rapidly decreases the production of agricultural products. Therefore, the Japanese government is now aiming to expand farm management by consolidating farmlands to develop efficient and stable large-scale farm management to improve agricultural productivity and stabilize production. However, such an integration of farmland has not yet given results because many farmers are still eager to possess their farmland as a property or as a means to earn capital gains by using it for alternative purposes (Council for Regulatory Reform, 2002). In addition, the "Agricultural Land Law" and "Agriculture Promotion Law" which were implemented in 1952 and 1969, respectively, work as a barrier when the private sector wishes to enter into agriculture, because they prohibit persons other than farmers from acquiring any farmland. Even if a farmer tries to repurpose his farmland, he has to send application forms with opinions from the Agricultural Committee located in each village, town, or city to the governor of each prefecture who is authorized to give permission through the committee. On the other hand, there is almost no restriction on the sale of horticultural crops, which will enable the private sector to enter agriculture if such companies grow horticultural crops on company-owned land, although the fixed property tax on farmland is considerably lower than that on commercial or industrial areas.

Another way for the private sector to enter agriculture is to finance agricultural corporations, but it will also be difficult to increase the flow of investment without improving the circumstances of corporate entry into agriculture, such as the further liberalization of corporate entry through farmland acquisition (Aritsubo, 2003).

For all these reasons, intensive cultivation of vegetables using company-owned factories and lands is possible for the private sector trying to enter agriculture. Also, chronic recession affecting the private sector associated with cutbacks in public investment leads to disused equipment, land, and available workers, all of which can also be directed by the private sector toward agriculture.

4. Characteristics of greenhouse production in Japan

In Japan, rice production was responsible for the highest sales among agricultural products until 1986, but both horticulture and animal husbandry increased thereafter as the price of rice decreased. At present, the sum of vegetable and flower production accounts for approximately 30% of the gross product, which is greater than rice production and almost the same as animal husbandry (Fig. 2).

Greenhouses have made a marked contribution to increasing the sales of these horticultural products, and the total area of greenhouses in Japan is now approximately

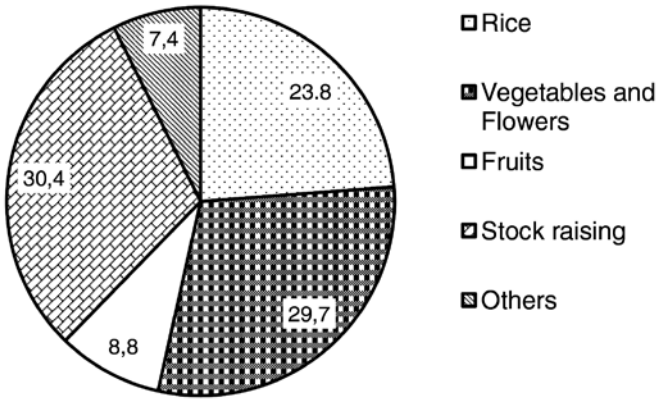

Fig. 2 - Proportion of each sector for total agricultural production in 2012. The pie chart was created using statistics released by MAFF (2013).

50,000 ha (Kozai, 2013). However, many of them are still plastic-film greenhouses smaller than 0.1 ha, and only 40% of them have a heating system installed. In addition, computerized environmental control systems have yet to sufficiently circulate commercially, which means that environmental control techniques in greenhouses still largely rely on the experience of farmers. As a result, the average yield of horticultural products from common plastic-film greenhouses still remains far lower compared to that of advanced greenhouses, where environmental management programs have been utilized to automatically optimize plant growth automatically. For example, the average yield of tomatoes in Japan is 60 $t \cdot ha^{-1}$, which is only 13% of that in the Netherlands (476 $t \cdot ha^{-1}$) (FAO, 2013). However, the introduction of computerized environmental management programs is not the only answer, cultivation of horticultural crops in Japan should be converted from empirical to computer-controlled methods to minimize the decrease in production due to the aging of farmers.

5. Introduction of SPF as a method to recover from the damage due to the Great East Japan Earthquake (GEJE)

The GEJE occurred on 11 March 2011, causing considerable damage to the country, including the agricultural sector. According to the data book of MAFF, the earthquake and subsequent tsunami affected approximately 24,000 ha of farmland in six prefectures located along the Pacific coast, which accounted for 2.7% of the total farmland in the region (Fig. 3, Table 1). Among the affected prefectures, the damage to Miyagi Prefecture was the greatest (10.7% of the total farmland), and it accounted for 60.6% of the total farmland area affected. In 2011, the Japanese government established the "Law on Special GEJE Reconstruction Areas" and special support programs to promote early recovery, such as the "GEJE Recovery Special Loan" program and "GEJE Recovery Emergency Guarantee" program. According to data from the Ministry of Finance (MAF), the central government spent approximately €137 billion on recovery from the GEJE during FY 2010-2012 (Sato, 2013). Miyagi Prefecture was a famous area for greenhouse production of horticultural crops such as strawberry, bell-pepper, and tomato, but many facilities were destroyed by the earthquake, and the subsequent tsunami caused serious salt damage to farmland, which resulted in only 33.3% restoration by 11 March 2012 (Table 1). Therefore, there is much expectation that the affected area will recover as a center for horticultural production

Fig. 3 - Major prefectures in Japan.

Table 1 - Distribution of farmland areas affected by the tsunami following the Great East Japan Earthquake on 11 March 2011 and their restoration

Prefecture	Farmland (ha)	Affected area (ha)	% of affected area	Restored area (ha)[z]	% of restored area
Aomori	156,800	107	0.1	101	94.4
Iwate	153,900	1,209	0.8	269	22.2
Miyagi	136,300	14,558	10.7	4,855	33.3
Fukushima	149,900	5,927	4.0	549	9.3
Ibaraki	175,200	1,063	0.6	958	90.1
Chiba	128,800	1,162	0.9	1,162	100.0
Total	900,900	24,026	2.7	7,894	32.9

[z] Restored area on 11 March 2012.
Table created from databases of MAFF 2011 and 2012.

by introducing large-scale SPF (Ito, 2012), and some facilities have already started running commercially (Fig. 4).

Fig. 4 - A solar-type plant factory constructed in the area affected by the Great East Japan Earthquake that occurred on 11 March 2011.

6. Present and future of SPF in Japan

The productivity of SPF largely depends on light conditions. In this regard, weather conditions in Japan are often a big barrier to introducing SPF. For example, the total duration of sunshine a Pacific coast area such as Kochi, Aichi, and Shizuoka Prefectures is 2,115-2,158 h per year (5.8-5.9 h per day), while that on the Japan Sea coast such as Akita, Aomori, and Yamagata Prefectures is 1,490-1,600 h per year (4.1-4.4 h per day), 69-76% of that on the Pacific coast (Fig. 3 and Table 2). When the duration is divided into summer (April to October) and winter (November to March), the former length does not differ much between the two regions (5.5-5.8 vs. 5.2-5.5 h per day for Pacific coast and Japan Sea coast, respectively), while the latter length on the Japan Sea coast (2.3-3.3 h per day) is only 36-56% of that on Pacific coast (5.8-6.4 ha per day). Moreover, the Japan Sea coast receives far more snowfall compared to the Pacific coast (377-669 vs. 0-16 cm per year on the Japan Sea coast and Pacific coast, respectively) (Table 2). Although SPF are usually multi-span type glasshouses with small roofs (Fig. 5), such structures of-

Fig. 5 - A multi-span type glasshouse with small roofs constructed in Tsukuba City, Ibaraki Prefecture.

Table 2 - Comparison of seasonal duration of sunshine and total amount of snowfall between Pacific coast and Japan Sea coast

Month	Monthly duration of sunshine (h)					
	Pacific coast			Japan sea coast		
	Kochi	Shizuoka	Aichi	Akita	Aomori	Yamagata
Jan	189.9	204.6	169.1	37.7	47.8	80.0
Feb	176.9	187.1	174.3	65.5	72.3	99.1
Mar	191.9	193.7	199.0	117.6	123.0	136.7
Apr	195.4	188.1	198.1	165.9	175.3	170.0
May	185.2	182.1	192.4	175.9	189.7	186.6
Jun	134.2	129.3	146.4	171.4	174.6	159.3
Jul	174.2	157.5	166.6	146.8	154.1	145.2
Aug	206.8	202.6	200.7	184.9	177.2	171.5
Sep	168.3	159.9	159.7	153.4	156.0	135.1
Oct	180.7	164.2	173.4	145.2	149.9	135.4
Nov	166.2	173.0	163.4	81.1	84.2	101.5
Dec	188.2	202.5	171.9	44.1	49.1	76.7
Jan-Dec	2157.9	2144.6	2115.0	1489.5	1553.2	1597.1
	Daily duration of sunshine (h)					
Apr-Oct	5.8	5.5	5.8	5.3	5.5	5.2
Nov-Mar	6.1	6.4	5.8	2.3	2.5	3.3
Jan-Dec	5.9	5.9	5.8	4.1	4.3	4.4
	Total amount of snowfall (cm per year)					
Jan-Dec	1	0	16	377	669	426

Table created from the database of the Japan Meteorological Agency in 2013.

ten cannot withstand the weight of heavy snow, especially in northern Japan. Therefore, APF and not SPF are more valuable from a commercial aspect along the Japan Sea coast of northern Japan.

7. Present and future of APF in Japan

Pioneering studies of plant growth using artificial light began as early as the 1920's (Harvey, 1922), and in the 1960's a useful book on artificial light for the production of horticultural crops was published (Canham, 1966). In Japan, vegetable production using artificial light has been studied since the 1970-80's, mainly in the research laboratories of MAFF and universities, and the results have largely contributed to clarifying the mechanisms of flowering, photosynthesis, carbohydrate partitioning, etc., under controlled environments (Nishizawa and Shishido, 2013). However, the results were not effectively applied commercially (Hashimoto, 2012). One of the pioneering examples of APS was the TS-farm of Kewpie Co., Ltd., constructed in Ibaraki Prefecture (Fig. 6), where leafy vegetables have been produced since June 1986 (Sekiyama, 1994). On this farm, triangle panels were arranged in an environmentally controlled facility and high-pressure sodium lamps and spraying hydroponics were used for cultivation. Another example of a pioneering APS was the "Rotary lettuce production facility" of Hitachi Co., Ltd., which was exhibited at the International Exposition, Tsukuba, Japan, 1985 (Takatsuji, 2009).

Although approximately 100 APF are functioning commercially in Japan now, many of them have been used for the cultivation of leafy vegetables such as leafy lettuce, while higher valued crops such as strawberry have been grown only in a few facilities (Kozai, 2013). Moreover, lighting systems of commercial APF still largely rely on fluorescent lights, which have the problem of heat accumulation in the plant during irradiance because of a low and high conversion efficiency from electrical energy to photons and heat, respectively (Hoshi *et al*., 2010; Takatsuji, 2010). Therefore, the cost for electricity to cool the facility is often more than 50% of that for irradiating the plants (Kozai, 2012; 2013).

Fig. 6 - TS-farm constructed by Kewpie Co., Ltd., in Ibaraki Prefecture.

Because there is marked potential to overcome the disadvantage of fluorescent lights by using LEDs (Massa *et al*., 2008), studies for the cultivation of vegetables using this light source have also expanded widely in Japan (Watanabe, 2011), but the commercial use of LEDs is still very limited mainly because of the high cost (Kozai, 2013). Even if the price of LEDs decreases to that of fluorescent lights, some difficulties still remain regarding the expansion of APF in terms of management. One of the intractable problems is that only small low-value-added crops are available to grow in such facilities. To solve this problem, studies to add value to the crops have been conducted, for example high-level antioxidative properties (Kozai, 2012; 2013), low potassium content (Suzuki, 2013), etc. by altering growth environments, or to cultivate more valuable crops such as seedlings (Yokoi *et al*., 2007), medical plants, genetically modified plants (Usami, 2011), etc.

However, such studies may come up against a limitation in the near future. While almost all horticultural crops can be grown in SPF, some which elongate longitudinally are still very difficult to grow in APF because the light intensity is theoretically inversely proportional to the square of the distance. Therefore, the light intensity reaching the lower leaves decreases continuously as the stem of the plant elongates. Longitudinally arranged fluorescent lights, comprising a line of corded LEDs in the plant canopy, and the utilization of laser diodes have been tested to solve this problem (Takatsuji and Mori, 2003), but these methods often aggravate working conditions for growers. One possible solution is to utilize organic light-emitting diodes (OLED) which emit light in response to an electric current using an emissive electroluminescent layer with organic compounds (Tang and VanSlyke, 1987). Although the light intensity of OLED is still lower than that of other illuminants, a high-performance white-light display of OLED has been newly developed at the Faculty of Engineering, Yamagata University in Japan (Kido *et al*., 1995), meaning that small horticultural crops such as leafy lettuce and strawberry can be grown (Fig. 7). Flexible OLED devices are theoretically possible to rewind like a negative film. Therefore, it may be possible to improve the efficiency of farmers' work by hanging film-type OLED devices from the ceiling of greenhouses and covering the whole plant only when irradiation is required. Such an illuminant can be utilized not only in APF but also in SPF.

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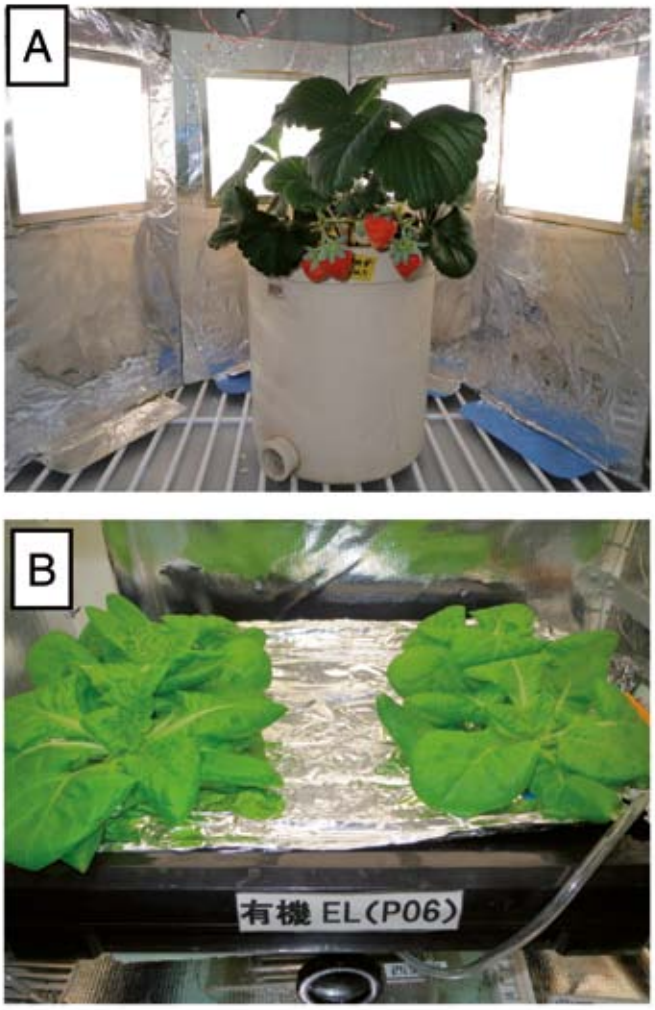

Fig. 7 - Strawberry (A) and leafy lettuce (B) grown under white-light organic electroluminescence display at the Faculty of Agriculture, Yamagata University.

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Physiological and psychological effects of olfactory stimulation with D-Limonene

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Key words: heart rate, heart rate variability, limonene, physiological relaxation, semantic differential method.

Abstract: Although D-Limonene can be considered an important component of nature-based stimuli, the physiological effects of olfactory stimulation with D-Limonene have not been completely clarified by scientific studies. The physiological and psychological effects of olfactory stimulation with D-Limonene were studied measuring heart rate variability (HRV), heart rate, and subjective evaluation using a modified semantic differential method; thirteen Japanese female university students (mean age±SD, 21.5±1.0 years) participated in the study. A concentration of 60 µL of D-Limonene was used as olfactory stimulant and room air as control. Subjects were exposed for 90 s while sitting with eyes closed. During D-Limonene inhalation: (1) the high-frequency (HF) value of HRV, a marker of parasympathetic nervous activity that is enhanced in relaxing situations, was significantly higher; (2) the heart rate was significantly lower; and (3) subjects reported feeling significantly more comfortable during D-Limonene administration than control. The results obtained clearly indicate that olfactory stimulation with D-Limonene induced physiological and psychological relaxation, providing important scientific evidence of the health benefits of D-Limonene.

1. Introduction

In the modern age, people are forced to lead busy lives and are exposed to a state of stress (Lederbogen *et al*., 2011). Thus, measures to prevent and relieve this stress state are urgently needed.

Recently, forest therapy has emerged as a method to address stress states, and much data on the physiological and psychological relaxing effects of forest environments have been accumulated. Previous studies have reported that viewing forest scenery or walking in forests can: increase parasympathetic nervous activity, which is enhanced in relaxing situations and suppresses sympathetic nervous activity which is increased in stress states (Tsunetsugu *et al*., 2007; Park *et al*., 2008; Lee *et al*., 2009; Park *et al*., 2009; Park *et al*., 2010; Lee *et al*., 2011; Park *et al*., 2012; Tsunetsugu *et al*., 2013; Lee *et al*., 2014); decrease cerebral blood flow in the prefrontal cortex (Park *et al*., 2007); and decrease salivary cortisol concentration of stress hormone (Tsunetsugu *et al*., 2007; Park *et al*., 2007; Park *et al*., 2008; Lee *et al*., 2009; Park *et al*., 2010). In addition, visiting a forest enhanced natural killer-cell activity and improved immune function (Li *et al*., 2007; Li *et al*., 2008 a, b, c) and the effect lasted 30 days (Li *et al*., 2008 b). In subjective evaluations, it was reported that people feel more "comfortable," "soothed," and "natural" when experiencing a forest environment (Park *et al*., 2007; Tsunetsugu *et al*., 2007; Park *et al*., 2008; Lee *et al*., 2009; Park *et al*., 2009; Lee *et al*., 2011; Park *et al*., 2011; Tsunetsugu *et al*., 2013; Lee *et al*., 2014), and that the "tension-anxiety," "depression," "anger-hostility," "fatigue," "confusion," and "vigor" of the mood state profile (McNair and Lorr, 1964; McNair *et al*., 1992; Yokoyama, 2005) improved (Li *et al*., 2008 a, b, c; Park *et al*., 2010; Lee *et al*., 2011; Park *et al*., 2011; Tsunetsugu *et al*., 2013; Lee *et al*., 2014). Unfortunately, many people living in cities find it difficult to access forest environments. Thus, much attention has been focused on nature-based stimuli, such as walking in an urban park (Song *et al*., 2013), viewing rooftop forests (Matsunaga *et al*., 2011), the presence of plants, including dracaena (Igarashi *et al*., 2014) or roses (Ikei *et al*., 2014), and physical contact with wood (Sakuragawa *et al*., 2008), and the relaxing effects of these stimuli have been reported.

Nature-based stimuli are intuitively perceived through the five senses. Of these five senses, the physiological effects of olfactory stimulation have been characterized

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in detail. Miyazaki *et al*. (1992) conducted a pioneering study which revealed that olfactory stimulation with *Chamaecyparis taiwanensis* essential oil significantly decreased blood pressure. Furthermore, inhalation of rose oil odor was shown to suppress sympathetic nervous activity and decrease adrenaline concentration (Haze *et al*., 2002). Lavender oil has been shown to induce deep sleep (Goel *et al*., 2005) and improve concentration (Sakamoto *et al*., 2005).

However, evidence-based research using the indices of autonomic nervous activity to clarify the effect of components of these essential oils is lacking.

The essential oil components of *Cryptomeria japonica* and *Pinus densiflora*, representative forest trees, have been reported (Cimanga *et al*., 2002; Hong *et al*., 2004; Cheng *et al*., 2009). These oils are composed of various volatile organic compounds, including D-Limonene, α-Pinene, β-Pinene. D-Limonene is the main component of citrus peel oil (Bernhard, 1960; Attaway *et al*., 1968; Shaw, 1979; Chiralts *et al*., 2002; Yoo *et al*., 2004).

The purpose of the present study was to investigate the physiological effect of olfactory stimulation with D-Limonene on autonomic nervous activity by measuring its effect on heart rate variability (HRV) (Camm *et al*., 1996; Kobayashi *et al*., 1999) and the heart rate.

2. Materials and Methods

Subjects

Thirteen Japanese female university students (age range, 21.5±1.0 years; mean±SD) participated in the study. Before beginning the experiment, a full explanation about the research aim, the experimental procedure, and all measured indices was provided. Informed consent was obtained from all subjects. This study was conducted in accordance with the regulations of the Ethics Committee of the Center for Environment, Health, and Field Sciences, Chiba University, Japan.

Study protocol

Physiological and psychological measurements were carried out in a chamber with an artificial climate maintained at 25°C with 50% relative humidity and 230-lux illumination. D-Limonene (>95.0% purity, Tokyo Chemical Industry Co., Ltd., Japan) was used as an olfactory stimulant, and room air was used as a control. A total of 60 μL D-Limonene was injected into a 24-L odor bag (polyethylene terephthalate film heat seal bag; NS-KOEN Co., Ltd., Kyoto, Japan) and the odors were presented to each subject by means of a device fitted on the chest and situated approximately 10 cm under the nose (Fig. 1). The flow rate of the odor was set at 3 L/min. Subjective sensitivity to the odor was determined in a preliminary investigation. The subjects were exposed to the odor for 90 s while sitting with their eyes closed. The order of presentation of D-Limonene and control was counterbalanced.

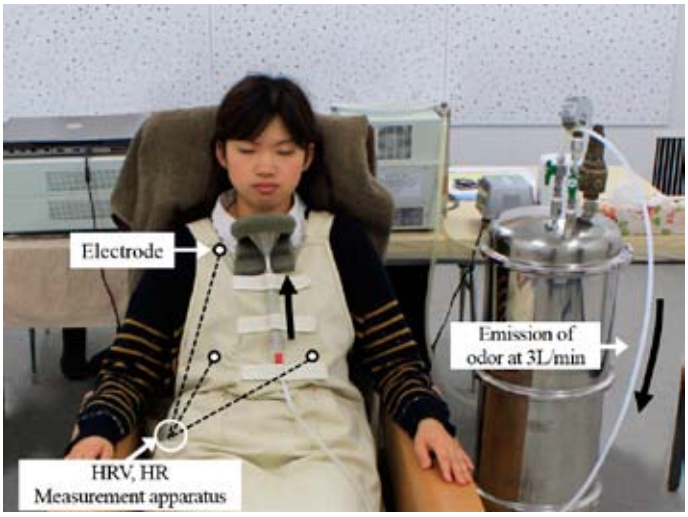

Fig. 1 - Olfactory stimulation setup.

Heart rate variability and heart rate

HRV was measured as the periods between consecutive R waves (R-R intervals) in an electrocardiogram recorded with a portable electrocardiograph (Activtracer AC-301A, GMS, Japan). In this study, two major spectral components of HRV, the low-frequency (LF; 0.04–0.15 Hz) band and the high-frequency (HF; 0.15-0.40 Hz) band were obtained by the maximum-entropy method (MemCalc/Win, GMS, Japan). The HF power was considered to reflect parasympathetic nervous activity, and the LF/HF power ratio was considered to reflect the sympathetic nervous activity (Camm *et al*., 1996; Kobayashi *et al*., 1999). Heart rate was also investigated using R-R interval data.

Semantic differential method

The subjects provided a subjective evaluation of the emotional impact of the odors according to a modified semantic differential (SD) method (Osgood *et al*., 1957). This method allowed the subject to assess a pair of adjectives, such as "comfortable-uncomfortable," using a 13-point scale. The SD method was performed after administration of each odor.

Statistical analysis

All statistical analyses were performed using Statistical Package for Social Sciences software version 20.0 (IBM Corp., Armonk, NY, USA). A paired t-test was used to compare differences in the physiological responses over the 90 s of exposure to D-Limonene and air. Wilcoxon signed-rank test was applied to analyze differences in psychological response between D-Limonene and air. A one-sided test was used in this study. In all cases, the significance level was set at $P < 0.05$.

3. Results

The results of the HRV data after exposure to D-Limonene and control were compared, and a significant differ-

ence was found in the HF value, which is a marker of parasympathetic nervous activity, as shown in Figure 2. The HF value increased 26.4% during D-Limonene administration (827.2±191.3 ms^2; mean±SE) compared with control (654.4 ±163.6 ms^2), indicating that parasympathetic nervous activity was significantly higher during D-Limonene administration (P<0.05). However, no significant difference was found in the LF/HF power ratio for the two stimuli.

Figure 3 shows a comparison of the heart rate during the administration of D-Limonene and control. Heart rate decreased during D-Limonene administration (72.8±2.3 bpm) compared with control (74.1±2.5 bpm), and this difference was significant (P<0.05).

Figure 4 shows the results for a "comfortable" feeling according to the subjective evaluation. Subjects reported significantly more comfortable ratings during D-Limonene administration than control (P<0.01).

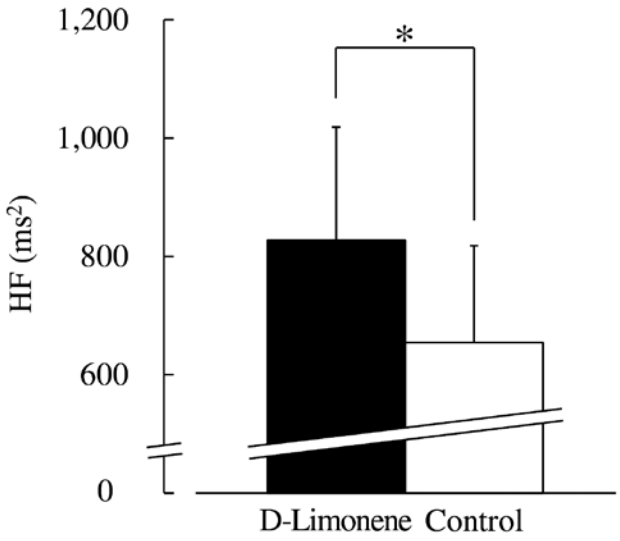

Fig. 2 - Comparison of high-frequency power levels of heart rate variability during olfactory stimulation with D-Limonene or control (air). Data are expressed as mean ± SE; n = 13. *P < 0.05 by paired t-test.

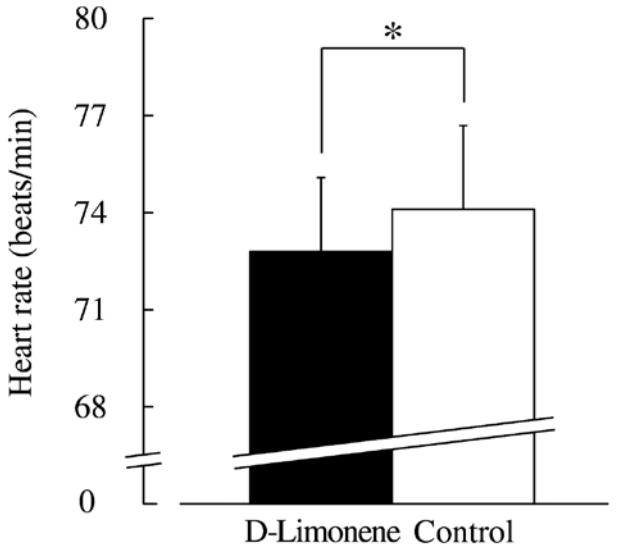

Fig. 3 - Comparison of the heart rate during olfactory stimulation with D-Limonene or control (air). Data are expressed as mean ± SE; n = 13. *P < 0.05 by paired t-test.

4. Discussion and Conclusions

D-Limonene is one of the most common volatile organic compounds in nature (Sun, 2007). It is a major component of various citrus oils, such as lemon, orange, grapefruit, and lime (Attaway *et al*., 1968; Bernhard, 1960; Chiralts *et al*., 2002; Shaw, 1979; Yoo *et al*., 2004), as well as essential oils from coniferous trees, such as *Pinus densiflora*, *Pinus koraiensis*, *Chamaecyparis obtusa*, and *Cryptomeria japonica* (Cimanga *et al*., 2002; Hong *et al*., 2004; Cheng *et al*., 2009). In addition, because of its citrus fragrance, D-Limonene is commonly added to perfumes, soaps, and cosmetics (Bakkali *et al*., 2008).

Although D-Limonene is an important component of nature-based stimuli, the physiological effect of olfactory stimulation with D-Limonene has not been completely clarified. Previously, Tsunetsugu *et al*. (2012) investigated the physiological effect of olfactory simulation with D-Limonene on blood pressure and showed that olfactory simulation with a concentration of 10 μL D-Limonene decreases subjects' systolic blood pressure. However, to our knowledge, no previous study has examined the physiological effect of olfactory stimulation with D-Limonene on HRV and heart rate.

The present study shows that olfactory stimulation with D-Limonene induced (1) a significant increase in parasympathetic nervous activities, (2) a significant decrease in the

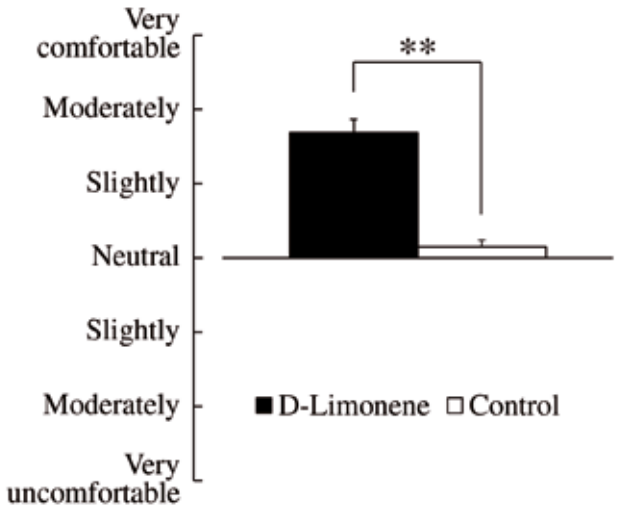

Fig. 4 - Subjective evaluation of "comfortable" measured by a modified semantic differential questionnaire after olfactory stimulation with D-Limonene or control (air). Data are expressed as mean ± SE; n = 13. **P < 0.01 by Wilcoxon signed-rank test.

heart rate, and (3) a significant increase in a "comfortable" feeling. These results agree with previous studies of other nature-based stimuli (Tsunetsugu *et al*., 2007; Park *et al*., 2008; Park *et al*., 2009; Park *et al*., 2010; Lee *et al*., 2011; Park *et al*., 2012; Song *et al*., 2013; Tsunetsugu *et al*., 2013; Ikei *et al*., 2014, Lee *et al*., 2014). Park *et al*. (2012) showed that the HF value of HRV was significantly increased while viewing scenery of forests using the results of field experiments at 35 forests in Japan. Ikei *et al*. (2014) reported that the HF component was significantly increased by viewing roses. Song *et al*. (2013) revealed that parasympathetic nervous activity was enhanced and the heart rate was significantly lower after walking in an urban park than walking in a city area. Our results support the hypothesis that olfactory stimulation with D-Limonene has a relaxation effect that is similar to other nature-based stimuli.

In conclusion, our results clearly indicate that olfactory simulation with D-Limonene induced physiological and psychological relaxation. And these finding provide important scientific evidence on the health benefits of D-Limonene exposure.

As all the participants in this study were healthy females in their twenties, further studies are needed to ascertain the effect in diverse groups, including males and different age groups. In addition, it is necessary to examine the effect using multiple indices, such as prefrontal cortex activity, stress hormones, and others.

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Characterization of chloroplast *matK* sequences of *Citrus tachibana* and *Citrus depressa*, two indigenous species in Japan

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Abstract: *Citrus tachibana*, *C. nippokoreana*, and *C. depressa* are indigenous mandarin species in Japan. We deduced their phylogenetic relationships from nucleotide sequences of the chloroplast *matK* gene. The results indicate that *C. tachibana*, *C. nippokoreana*, and *C. depressa* accessions can be classified into two types: type A, all sixteen *C. tachibana* and six *C. depressa*; type B, eleven *C. depressa* and one *C. nippokoreana*. Both type A and type B accessions of *C. depressa* were found on the Okinawa Islands, whereas only type B accessions of *C. depressa* were found on the Sakishima and Amami Islands. This cpDNA divergence seemed to indicate a polyphyletic origin of *C. depressa*. The *matK* genes of type A were found only in *C. tachibana* and some *C. depressa*. From these results, both species probably possess a characteristic chloroplast genome among various *Citrus* species.

1. Introduction

Citrus is one of the most important fruit crops in Japan and also worldwide. Various accessions of *Citrus* species are adapted to the southwest of Japan, and although they are cultivated in this region, almost all of them are non-native, that is, they were introduced from abroad, arose as chance seedlings, were selected from bud sports, and were bred by artificial pollination. Only two species, *Citrus tachibana* (Makino) Tanaka (Tachibana) and *Citrus depressa* Hayata (Shiikuwasha) were present in Japan before recorded history.

C. tachibana mainly grows indigenously on the Pacific side of the southwest of Japan's main islands (Kyushu, Shikoku, and Honshu). *C. tachibana* was recorded in "Kojiki", the oldest chronicle in Japan dating from the early 8th century. Its indigenous trees were also found on the Ryukyu Islands (islands including the Okinawa Islands, Sakishima Islands, and Amami Islands, which were ruled by Japan from the 17th to19th centuries) and Taiwan (Tanaka, 1931; Lin and Chen, 2006; Inafuku-Teramoto *et al*., 2010). *C. depressa* is indigenous to both the Ryukyu Islands and Taiwan (Tanaka, 1936; Lin and Chen, 2006). Compared to *C. tachibana*, *C. depressa* is considered to be adapted to a warmer climate; the former is usually used as an ornamental for gardens and its fruit is inedible. On the other hand, fruit of *C. depressa* is in much demand as an ingredient for food and drinks, to garnish dishes similar to a lemon or lime, to make juice and jam, and as an additive to soy sauce and distilled spirits. Recently, this fruit has attracted attention because it contains high levels of polymethoxyflavonoids, one of the most important health-promoting components of citrus (Inafuku-Teramoto *et al*., 2010).

We have investigated the phylogenetic relationships of *Citrus* and its relatives through the analysis of genes encoded in chloroplast DNA (cpDNA) (Tshering *et al*., 2010, 2013). In our recent study (Tshering *et al.*, 2013) in which various *Citrus* accessions were used as materials, we found that *C. tachibana* and *C. depressa* possess a characteristic cpDNA genome based on the sequences of the chloroplast *matK* genes, which encode a maturase involved in splicing type II introns from RNA transcripts (Hilu and Liang, 1997; Hilu *et al*., 2003; Olmstead and Palmer, 1994). There are many accessions in both species, and intraspecies diversity is found within each species (Hirai *et al*., 1990; Yamamoto *et al*., 1998; Kinjo, 2007; Inafuku-Teramoto *et al*., 2010; Yamamoto *et al*., 2011).

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However, a limited number of accessions were used in our previous study (Tshering *et al*., 2013).

Therefore, for the present work, we analyzed the *matK* gene sequences of a number of *C. tachibana* and *C. depressa* plants grown in various regions in Japan to reveal their characteristic profiles of the cpDNA genome. *C. nippokoreana* (Korai Tachibana), a *C. tachibana* relative indigenous to Hagi City, Yamaguchi Prefecture, Japan, and Cheju Island, Korea (Kimura and Taninaka, 1995), was also investigated.

2. Materials and Methods

Plant materials

Sixteen *C. tachibana*, one *C. nippokoreana*, 17 *C. depressa*, and 13 control accessions were used in this study. The sources of the materials are shown in Table 1 and Figure 1.

PCR amplification and DNA sequencing

Genomic DNA was extracted from leaves using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). By using this genomic DNA as a template, the *matK* gene was amplified by PCR using proofreading PrimeSTAR GXL DNA Polymerase (TAKARA BIO, Ohtsu, Shiga, Japan). The primers used for PCR amplification of the *matK* gene were matK1F (5′-ACCGTATCGCACTATGTATC-3′) and matK1R (5′-GAACTAGTCGGATGGAGTAG-3′). The amplified DNA fragments were purified using the NucleoSpin Gel and PCR Clean-up Kit (MACHEREY-NAGEL, Düren, Germany). The primers used for sequencing of the *matK* gene were matK1F, matK2F (5′-ACGGTTCTTTCTCCACGAGT-3′), matK3F (5′-GGTCCGATTTCTCTGATTCT-3′), matK1R, matK2R (5′-AGAATCAGAGAAATCGGACC-3′), and matK3R (5′-ACTCGTGGAGAAAGAACCGT-3′). The purified DNA fragments were sequenced in both directions in an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems) with a BigDye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (Applied Biosystems) as described previously (Platt *et al*., 2007). Sequence data were submitted to DDBJ/GenBank/EBI and were assigned accession numbers ranging from AB839905 to AB839932. The sequences of the accessions from No. 29 to No. 34 were deposited in our previous study (Tshering *et al*., 2013).

Fig. 1 - Collection sites of *Citrus tachibana*, *C. nippokoreana*, and *C. depressa* in the present study.

Phylogenetic analyses

The neighbor-joining (NJ) and maximum likelihood (ML) methods from the MEGA (version 5.2.1) program (Tamura *et al*., 2011) were used to create phylogenetic trees. The reliability of each branch was tested by bootstrap analysis with 1,000 replications.

3. Results and Discussion

We constructed multiple sequence alignments of 1,630-bp fragments containing the *matK* gene from different *Citrus* accessions. Each sequence contained a 1,530-bp protein-coding sequence and 100 bp of the 3′ UTR. One exception is the *matK* gene of trifoliate orange (*Poncirus trifoliata*), which has a 6 bp insertion at the 3′ UTR. Of these, 23 bases were variable and six bases were phylogenetically informative.

We created phylogenetic trees using the NJ and ML methods. The topologies of the different trees were identical (data not shown). Therefore, we present here only the ML tree (Fig. 2). *C. tachibana*, *C. depressa*, and *C. nippokoreana* accessions were classified into two types as follows:

Type A: all 16 *C. tachibana* and six *C. depressa* [Shiikuwasha-Okinawa#1 (No. 17), Shiikuwasha-Okinawa#3 (No. 19), Shiikuwasha-Okinawa#6 (No. 22), and Shiikuwasha-Oku (No. 26), Kabishi (No. 29), and Fusubuta (No. 31)].

Type B: Eleven *C. depressa* [Shiikuwasha-Taketomi (Nohara) (No. 11), Shiikuwasha-Taketomi (Takana) (No. 12), Shiikuwasha-Iriomote (No. 13), Shiikuwasha-Iriomote (Katoura) (No. 14), Shiikuwasha-Kohama (Ufudake) (No. 15), Shiikuwasha-Kohama (Omori) (No. 16), Ishikunibu (No. 28), Mikanguwa (No. 30), Kaachi (No. 32), Shiikunin (No. 33), and Shiikurubu (No. 34)] and one *C. nippokoreana*.

None of the control accessions belonged to type A, whereas all seven control mandarin accessions belonged to type B. The other control accessions were clearly distinguished from type A and type B. This finding is consistent with the results of our previous study (Tshering *et al*., 2013).

All 16 *C. tachibana* accessions carried an identical *matK* sequence. Previous studies (Hirai *et al.*, 1990; Yamamoto and Tominaga, 2003) reported that *C. tachibana* was genetically differentiated from *Citrus* species originating from all

Table 1 - *Citrus tachibana*, *C. nipponkoreana*, and *C. depressa* accessions used in the present study

No.	Accession	Latin name	Origin	Note
1	Tachibana-Dazaifu (uchi)	*Citrus tachibana* (Makino) Tanaka	Fukuoka, Kyushu	Planted tree
2	Tachibana-Dazaifu (soto)	*C. tachibana* (Makino) Tanaka	Fukuoka, Kyushu	Planted tree
3	Tachibana-Heian Jingu	*C. tachibana* (Makino) Tanaka	Kyoto, Honshu	Planted tree
4	Tachibana-Iwashimizu Hachimangu	*C. tachibana* (Makino) Tanaka	Kyoto, Honshu	Planted tree
5	Tachibana-Kitano Tenmangu	*C. tachibana* (Makino) Tanaka	Kyoto, Honshu	Planted tree
6	Tachibana-Toshijima (Mie)	*C. tachibana* (Makino) Tanaka	Mie, Honshu	Native tree
7	Tachibana-Matsuoyama (Kochi)	*C. tachibana* (Makino) Tanaka	Kochi, Shikoku	Native tree
8	Tachibana-Nangoku (Kochi)	*C. tachibana* (Makino) Tanaka	Kochi, Shikoku	Planted tree
9	Korai Tachibana	*C. nippokoreana* Tanaka	Kochi, Shikoku	Planted tree
10	Tachibana-Ishigakijima	*C. tachibana* (Makino) Tanaka	Ishigaki-jima, Sakishima	Native tree
11	Shiikuwasha-Taketomi (Nohara)	*C. depressa* Hayata	Taketomi-jima, Sakishima	Native tree
12	Shiikuwasha-Taketomi (Takana)	*C. depressa* Hayata	Taketomi-jima, Sakishima	Native tree
13	Shiikuwasha-Iriomote	*C. depressa* Hayata	Iriomote-jima, Sakishima	Native tree
14	Shiikuwasha-Iriomote (Katoura)	*C. depressa* Hayata	Iriomote-jima, Sakishima	Native tree
15	Shiikuwasha-Kohama (Ufudake)	*C. depressa* Hayata	Kohama-jima, Sakishima	Native tree
16	Shiikuwasha-Kohama (Omori)	*C. depressa* Hayata	Kohama-jima, Sakishima	Native tree
17	Shiikuwasha-Okinawa#1	*C. depressa* Hayata	Okinawa-honto	Native tree
18	Tanibuta-Okinawa#2	*C. tachibana* (Makino) Tanaka	Okinawa-honto	Native tree
19	Shiikuwasha-Okinawa#3	*C. depressa* Hayata	Okinawa-honto	Native tree
20	Tanibuta-Okinawa#4	*C. tachibana* (Makino) Tanaka	Okinawa-honto	Native tree
21	Tanibuta-Okinawa#5	*C. tachibana* (Makino) Tanaka	Okinawa-honto	Native tree
22	Shiikuwasha-Okinawa#6	*C. depressa* Hayata	Okinawa-honto	Native tree
23	Tanibuta-Okinawa#7	*C. tachibana* (Makino) Tanaka	Okinawa-honto	Native tree
24	Tanibuta-Okinawa#8	*C. tachibana* (Makino) Tanaka	Okinawa-honto	Native tree
25	Garagara	*C. tachibana* (Makino) Tanaka	Okinawa-honto	Native tree
26	Shiikuwasha-Oku	*C. depressa* Hayata	Okinawa-honto	Native tree
27	Tanibuta	*C. tachibana* (Makino) Tanaka	Okinawa-honto	Native tree
28	Ishikunibu	*C. depressa* Hayata	Okinawa-honto	Native tree
29	Kabishi	*C. depressa* Hayata	Okinawa-honto	Native tree
30	Mikanguwa	*C. depressa* Hayata	Okinawa-honto	Native tree
31	Fusubuta	*C. depressa* Hayata	Okinawa-honto	Native tree
32	Kaachi	*C. depressa* Hayata	Okinawa-honto	Native tree
33	Shiikunin	*C. depressa* Hayata	Tokuno-shima, Amami	Native tree
34	Shiikuribu	*C. depressa* Hayata	Okinoerabu-jima, Amami	Native tree
Control accessions				
	Satsuma mandarin ‘Aoshima’	*C. unshiu* Marcow.		
	Ponkan ‘Yoshida Ponkan’	*C. reticulata* Blanco		
	Mediterranean mandarin	*C. deliciosa* Ten.		
	Dancy	*C. tangerina* hort. ex Tanaka		
	Kinokuni ‘Hirakishu’	*C. kinokuni* hort. ex Tanaka		
	Sunki	*C. sunki* (Hayata) hort. ex Tanaka		
	Cleopatra	*C. reshni* hort. ex Tanaka		
	Yuzu ‘Yamane’	*C. junos* Siebold ex Tanaka		
	Sweet orange ‘Fukuhara’	*C. sinensis* (L.) Osbeck		
	Lemon ‘Eureka’	*C. limon* (L.) Burm. f.		
	Pummelo ‘Mato Buntan’	*C. maxima* (Burm.) Merr.		
	Citron ‘Maru Busshukan’	*C. medica* L.		
	Trifoliate orange ‘Standard’	*Poncirus trifoliata* (L.) Raf.		

other countries except Japan. The present study also confirmed that the *matK* sequence of *C. tachibana* was not identical to those of studied accessions originating from all other countries except Japan. However, we found that the *matK* sequence of *C. tachibana* was identical to those of some investigated *C. depressa* accessions that are indigenous to the Ryukyu Islands, Japan. This suggests that *C. tachibana* has been isolated from the mandarins elsewhere, and evolved in Japan in unique ways. We found no diversity within species. However, further study considering more accessions is needed since the materials used here did not cover the entire area where *C. tachibana* grows. The *matK* sequence of *C. nippokoreana* was not identical to that of *C. tachibana*, indicting genetic differentiation between the two species. Because it is considered that *C. nippokoreana* is related to *C. tachibana* (the Japanese name "Korai Tachibana" means "Tachibana from Korea"), this finding is interesting.

C. depressa accessions were divided into two types according to *matK* sequences. One was the same type as *C. tachibana* and the other was the same type as several mandarins such as *C. reticulata* and *C. sunki*. This result completely agrees with the results of our previous study (Tsheringet *al*., 2013). Differentiation of the cpDNA genome in *C. depressa* was also reported by Urasaki *et al*. (2005) and Yamamoto *et al*. (2013), who analyzed the *trnL-trnF* and *trnF-trnVr* regions, respectively. These results strongly suggest a polyphyletic origin of *C. depressa*. This divergence of *matK* genes was found in *C. depressa* accessions grown on Okinawa-honto (the main island of Okinawa Islands) but not in those grown on the Sakishima and Amami Islands. *C. depressa* possessing *C. tachibana*-type cpDNA (A type) was found only on Okinawa-honto. Similar results were reported by Yamamoto *et al*. (2013) who studied *C. depressa* on Okinawa-honto and the Amami Islands. However, Urasaki *et al.* (2005) found that *C. depressa* accessions possessed *C. tachibana*-type cpDNA (*trnL-trnF* sequence) on the Sakishima Islands. Thus, further study using many *C. depressa* accessions grown on various islands is necessary to resolve the distribution of each type.

There is a possibility that type A *C. depressa* is geneticallyclose to *C. tachibana* than type B. However, this hypothesis is not supported since the proportion of common bands from random amplified polymorphic DNA (RAPD) analysis between *C. depressa* of type A and *C. tachibana* was not so different from that of type B and *C. tachibana* (Yamamoto *et al*., 1998). Since the origin and/or relationship of *C. depressa* to *C. tachibana* cannot be elucidated only by cpDNA analysis, cpDNA analysis combined with nuclear genome analysis such as simple sequence repeat (SSR), sequence-related amplified polymorphism markers (SRAPs) (Barkley *et al*., 2006; Uzun *et al.*, 2009), and restriction site-associated DNA sequences (RAD-seq) (Baird *et al*., 2008) is considered to be necessary. For this purpose, structural analysis (Barkley *et al*., 2006) seems to be informative.

The present work demonstrates the characteristic profiles of the chloroplast genome of *Citrus tachibana* and *Citrus depressa*, two indigenous species in Japan, using a number of accessions grown in various regions based on the results of *matK* sequencing. Furthermore, the divergence of the cpDNA genome of *C. depressa* seems to indicate a polyphyletic origin of this species. These findings are a contribution to progress in the study of the genetic resources in *Citrus* and related genera.

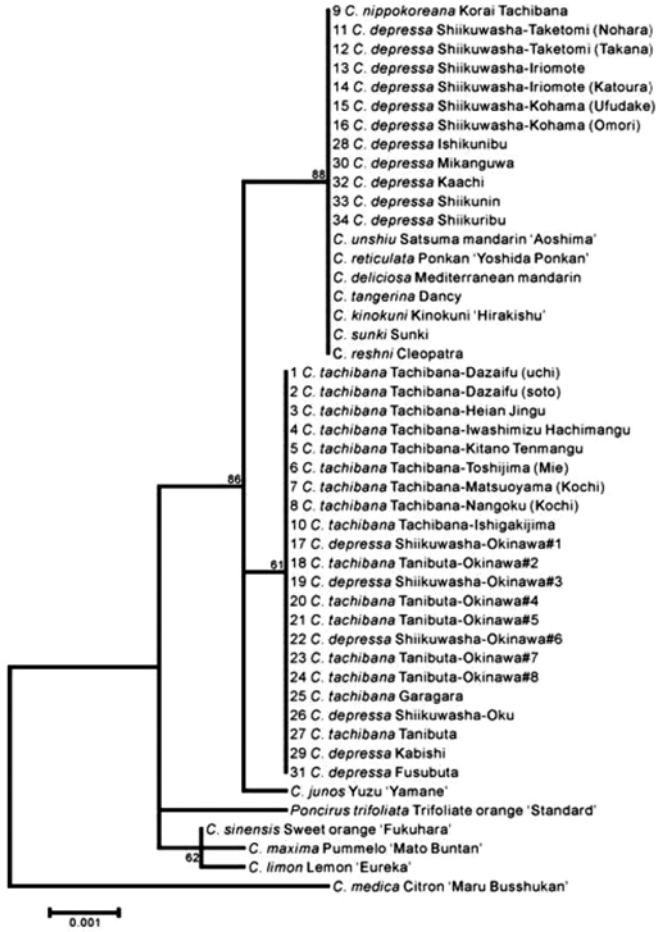

Fig. 2 - Maximum likelihood tree of the *matK* genes from *Citrus tachibana*, *C. nippokoreana*, and *C. depressa* and their control accessions. Numbers at the nodes indicate bootstrap values (% over 1000 replicates). The scale bar shows the number of substitutions per site.

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Potential marker proteins for ozone-induced yield reduction in rice

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Key words: grain yield, ozone stress, protein markers, 60-kDa chaperonin.

Abstract: Three proteins - a 60-kDa chaperonin (CPN-60), chloroplastic ATP synthase, and enolase 1 - were evaluated as potential markers of ozone-induced yield responses in six rice (*Oryza sativa* L.) cultivars ('Kirara 397', 'Koshihikari', 'Nipponbare', 'Takanari', 'Kasalath', 'Suphanburi 90') under ozone stress in laboratory-scale tests. The levels of all three proteins decreased after ozone exposure in cultivars identified as ozone-sensitive while they increased or remained constant after ozone exposure in tolerant cultivars, although ATP synthase tended to decrease. Furthermore, the protein level and grain yield in each cultivar exposed to ozone were significantly positively correlated for all three proteins. Thus, CPN-60 and enolase 1 are potential markers for chronic ozone stress in rice.

1. Introduction

Ozone is a major gaseous pollutant in the troposphere and ozone concentrations have in recent years increased rapidly in developing Asian countries. Indeed, the emission of anthropogenic nitrogen oxides (ozone precursors) in Asia under a no-further-control scenario was predicted to increase by 350% between 1990 and 2020 (Aunan *et al*., 2000).

An elevated ozone concentration will reduce the growth and yield of crop plants including rice, the most important food crop in Asia (Kobayashi *et al*., 1995; Yonekura *et al*., 2005). Many researchers have described the mechanisms responsible for visible injury on plant leaves by acute ozone exposure (reviewed by Kangasjarvi *et al*., 2005). The primary mechanism is oxidative damage caused by an increase in levels of reactive oxygen species (ROS). However, the cause for yield reductions under chronic ozone stress remains unclear. In a previous report we described how ozone sensitivity in evaluated rice cultivars, in terms of visible injury (chlorotic or necrotic lesions), did not coincide with that indicated by the grain yield reduction (Sawada and Kohno, 2009). In addition, conventional evaluation of chronic ozone effects relies on measurements such as growth and yield reductions, which require large-scale studies (e.g. in a field or greenhouse) and long time periods (e.g. about six months). A rapid and small-scale method for early evaluation of chronic ozone effects, such as the use of molecular markers, would make it faster, easier, and less expensive to select ozone-tolerant cultivars.

Kubo *et al*. (2011) reported that during ozone stress, sakuranetin, a flavonone in the phytoalexin family, appears to serve as a molecular marker of the stress response. Sakuranetin contents in rice leaves exposed simultaneously to ozone and high temperature increased only in the three cultivars whose grain yield was unaffected by ozone stress. However, their experiment was performed under both elevated ozone and elevated temperature, making it difficult to determine the separate effect of each factor. Moreover, the ozone concentration was 150 nl l^{-1} (ppb), much higher than ambient ozone levels. Therefore, more practical markers are needed.

Proteomic studies are useful to reveal protein markers associated with various stress tolerance (reviewed by Kosova *et al*., 2011). In a previous study, we conducted differential proteome analysis using three rice cultivars that showed different levels of ozone sensitivity (indicated by the reduction in grain yield) when exposed to elevated ozone during the cultivation season in open-top chambers (Sawada *et al*., 2012). In these cultivars, we observed significant changes in the size of spot that contained three proteins: a 60-kDa chaperonin (CPN-60), chloroplastic ATP synthase, and enolase 1. The change in size of this spot was proportional to their ozone sensitivity, measured as the reduction in grain yield. These results suggest that these proteins are closely involved in the mechanisms that underlie the yield reduction

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that occurs under elevated ozone levels, and therefore they have potential as molecular markers that can predict the ozone-induced yield loss. To clarify the usefulness of these proteins for use in laboratory-scale tests, we investigated the levels of these candidate proteins in the seedlings of six rice cultivars under short-term ozone exposure in growth chambers, and tested for a significant correlation between the protein levels and relative grain yield.

2. Materials and Methods

Chronic ozone exposure

Six rice (*Oryza sativa* L.) cultivars were used in this study: 'Kirara 397', 'Koshihikari', 'Nipponbare' (*japonica* cultivars), 'Takanari' (a hybrid *indica* cultivar), 'Kasalath', 'Suphanburi 90' (*indica* cultivars). Seedlings (n = 40) of each cultivar were grown in seedling boxes for three weeks in a glasshouse under ambient atmospheric conditions, then transplanted into pots (at four plants per pot with a 0.05-m^2 surface area and a 0.015-m^3 volume) in open-top chambers (OTCs; 3.6 × 3.6 m) at an experimental field of the Akagi Testing Center of the CRIEPI (Maebashi, Japan) in the late spring of 2007, 2008, and 2009. Fertilizer was supplied at a rate of N-P_2O_5-K_2O=15-15-15 g m^{-2}. The OTC fumigation system has been described previously (Frei *et al*., 2011). Ozone was added in the chambers using a mass-flow controller combined with a PID controlling system to maintain the designated concentrations. Three ozone-level treatments were established, from transplanting of rice plants into the pots to harvest, for three years with a regular diurnal pattern: charcoal-filtered air (CF), ambient ozone (Ozone ×1), and twice ambient ozone (Ozone ×2). Concentrations of ozone were continuously monitored in each chamber at 3-min intervals using a UV absorption ozone analyzer (ML9810, Monitor Labs, Englewood, CO, USA). Mean ozone concentration, air temperature and relative humidity in the different treatments are summarized in Table 1.

Measurement of the yield

The rice cultivars were harvested between September and November in 2007, 2008, and 2009. Harvesting of each cultivar was conducted when about 80% of the grains had turned yellow. After harvesting, grains were separated from the panicles and categorized into two groups (filled and unfilled grains) using an automatic seed-sorting machine (FV-459A, Fujiwara Seisakusho KK, Tokyo, Japan). The filled grains (rough rice) were weighed to determine the grain yield.

Short-term ozone exposure

Rice seedlings were grown in indoor growth chambers at 28/23°C (day/night), photosynthetic photon-flux density of 400 μmol m^{-2} s^{-1}, with a 12-h photoperiod, and a relative humidity of 60±5%. After two weeks, the 'Kirara 397', 'Koshihikari', and 'Takanari' seedlings were exposed to three levels of ozone (12 h/day) for three days in three individual replicates: CF, ambient ozone (40 ppb), and twice ambient ozone (80 ppb). Similarly, 'Nipponbare', 'Kasalath', and 'Suphanburi 90' seedlings were exposed to CF and 40 ppb of ozone. Ozone was generated with a silent electrical discharge in dry oxygen. The concentration of ozone in the chambers was monitored continuously during exposure with a UV absorption ozone detector (Model 1150, Dylec Inc., Tokyo, Japan). At the end of the exposure, we removed the third leaves, immediately froze them in liquid nitrogen, and stored them in -80°C until the immunoblot analysis was performed.

Immunoblot analysis

Leaves (100 mg) were homogenized in sodium dodecyl sulfate (SDS) buffer (10% (w/v) glycerol, 5% (v/v) β-mercaptoethanol, 2.3% (w/v) SDS, and 62.5 mM Tris-HCl, pH 6.8). Equal amounts of protein samples were separated using 15% SDS-polyacrylamide gel electrophoresis (PAGE). After the SDS-PAGE, the protein samples were transferred onto a polyvinylidene fluoride membrane or they were stained by Coomassie brilliant blue (CBB).

Table 1 - Ozone concentrations and environmental conditions in the open-top chambers during the cropping seasons of rice

		Ozone concentration (ppb)			Temperature (°C)	Relative humidity (%)
		12 h mean	24 h mean	Mean daily Maximum	24 h mean	24 h mean
2007	CF	3.1	2.1	4.1	–	–
	Ozone x1	37.6	31.1	61.3	–	–
	Ozone x2	68.6	56.3	101.7	–	–
2008	CF	4.7	3.9	6.5	21.1	83.4
	Ozone x1	40.4	27.5	57.6	21.3	83.5
	Ozone x2	82.7	57.0	118.3	21.3	81.7
2009	CF	5.1	5.0	9.7	20.6	78.7
	Ozone x1	35.1	27.9	56.9	20.7	78.9
	Ozone x2	73.5	54.7	110.2	20.9	77.4

Measurements of environmental conditions and ozone concentrations were recorded at 3 and 10-minute interval throughout the experiment, respectively. Average values of the two replicate chambers per treatment are shown. 12 h means were calculated for the period from 6:00 to 17:59 hours. The temperature and relative humidity were not measured in 2007.

The blotted membrane was blocked for 1 h in TBS-T (20 mM Tris-HCl, pH 7.6, 150 mM NaCl and 0.1% v/v Tween-20) containing 5% (w/v) nonfat milk (Skim milk; Difco, Sparks, MD, USA). The membrane was subsequently incubated with the monoclonal antibody anti-heat-shock protein 60 (Acris Antibodies GmbH, Herford, Germany), with the polyclonal antibodies anti-ATP synthase β-subunit (AntiProt, Pullach i. Isartal, Germany), and anti-enolase (Aviva system biology, San Diego, CA, USA) at 1:5000 dilutions for 1 h at room temperature. As secondary antibodies, we used anti-mouse or anti-rabbit IgG with conjugated HRP (Bio-Rad Laboratories Inc., Hercules, CA, USA). After incubation for 1 h with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies, we detected the immunoblot signals using the ECL plus western blotting detection kit (GE Healthcare, Piscataway, NJ, USA) following the manufacturer's protocols and the results were visualized using an LAS-3000 luminescent image analyzer (Fujifilm, Tokyo, Japan). The relative intensities of the bands were calculated using PD-Quest software (version 8.0.1, Bio-Rad).

3. Results and Discussion

After chronic ozone exposure during three years of growing seasons (from 2007 to 2009) each cultivar showed a similar yield response to ozone in all years of the experiment. The grain yields of 'Kirara 397', 'Takanari' and 'Kasalath' decreased significantly by 15 to 36%, 10 to 21%, and 12 to 19%, respectively, under twice the ambient ozone level (about 80 ppb treatment, daily 12-h mean concentration), although the grain yields did not differ significantly from CF under ambient ozone level (about 40 ppb treatment), except for 'Kirara 397' and 'Takanari' in 2007 (Fig. 1, P <0.05). The grain yields of 'Koshihikari', 'Nipponbare', and 'Suphanburi 90' did not decrease significantly with ozone stress. On this basis, we defined 'Kirara 397', 'Takanari', and 'Kasalath' as ozone-sensitive cultivars, and 'Koshihikari', 'Nipponbare', and 'Suphanburi 90' as ozone-tolerant cultivars.

To confirm whether CPN-60, ATP synthase, and enolase 1 can be used as markers for ozone-induced rice yield loss in laboratory-scale tests, we analyzed the levels of these proteins (Fig. 2A). Levels of CPN-60 decreased significantly after three days of exposure to 40 (the ambient concentration) and 80 ppb (twice the ambient concentration) of ozone in 'Kirara 397' and 80 ppb of ozone in 'Takanari' (Fig. 2B, P<0.05). Levels of ATP synthase and enolase 1 tended to decrease after ozone exposure, although not significantly (except for 'Takanari' exposed to 80 ppb of ozone), in both 'Kirara 397' and 'Takanari'. These cultivars also showed lower grain yield under ozone exposure (Fig. 1). In contrast, levels of CPN-60 and enolase 1 in 'Koshihikari' exposed to 40 ppb of ozone increased significantly and remained the same compared with the levels in CF (P <0.05). Moreover, enolase 1 production also remained constant in 'Koshihikari' at 80 ppb ozone exposure. The level of ATP synthase tended to decrease after ozone exposure in 'Koshihikari'. Because the levels of CPN-60 and enolase 1 decreased and increased at 40 ppb ozone exposure in ozone-sensitive and ozone-tolerant cultivars, respectively, 'Kasalath', 'Nipponbare', and 'Suphanburi 90' seedlings were exposed to CF and

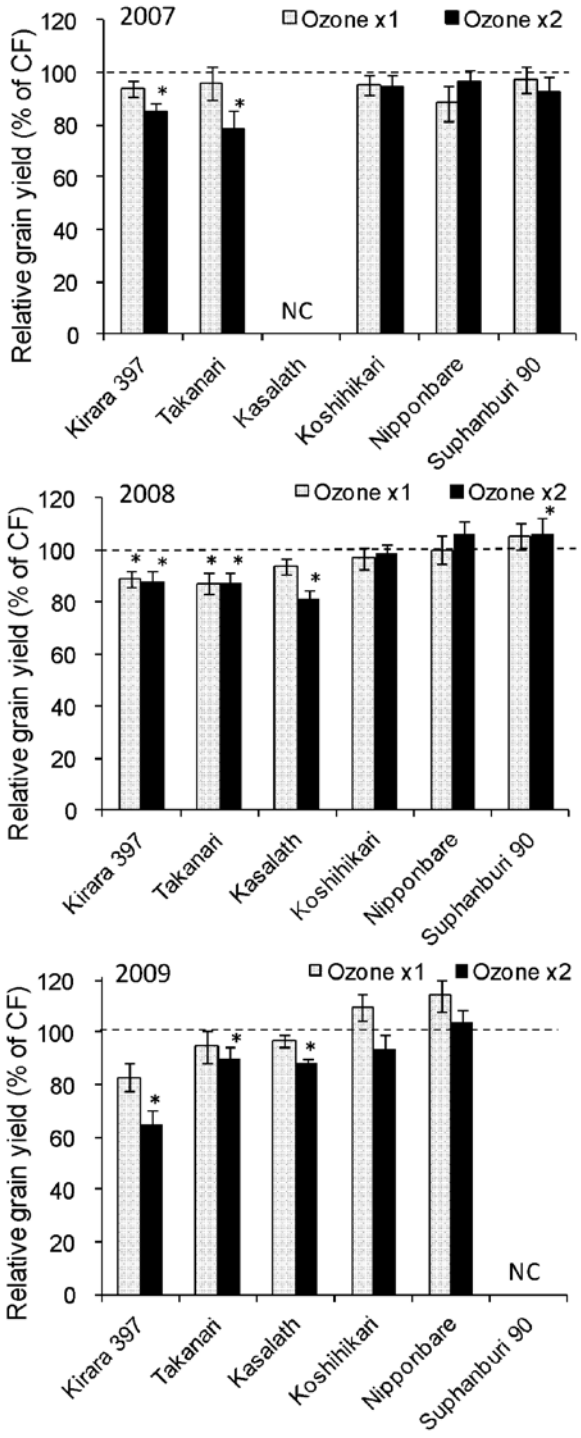

Fig. 1 - Effects of chronic ozone exposure on the grain yields of six rice cultivars in 2007, 2008, and 2009. Values are mean ± SE (n= 40). Asterisk indicates a significant difference compared with CF according to Dunnett's test (P<0.05). 'Kasalath' and 'Suphanburi 90' were not cultivated in 2007 and 2009, respectively, and yields are shown as "NC".

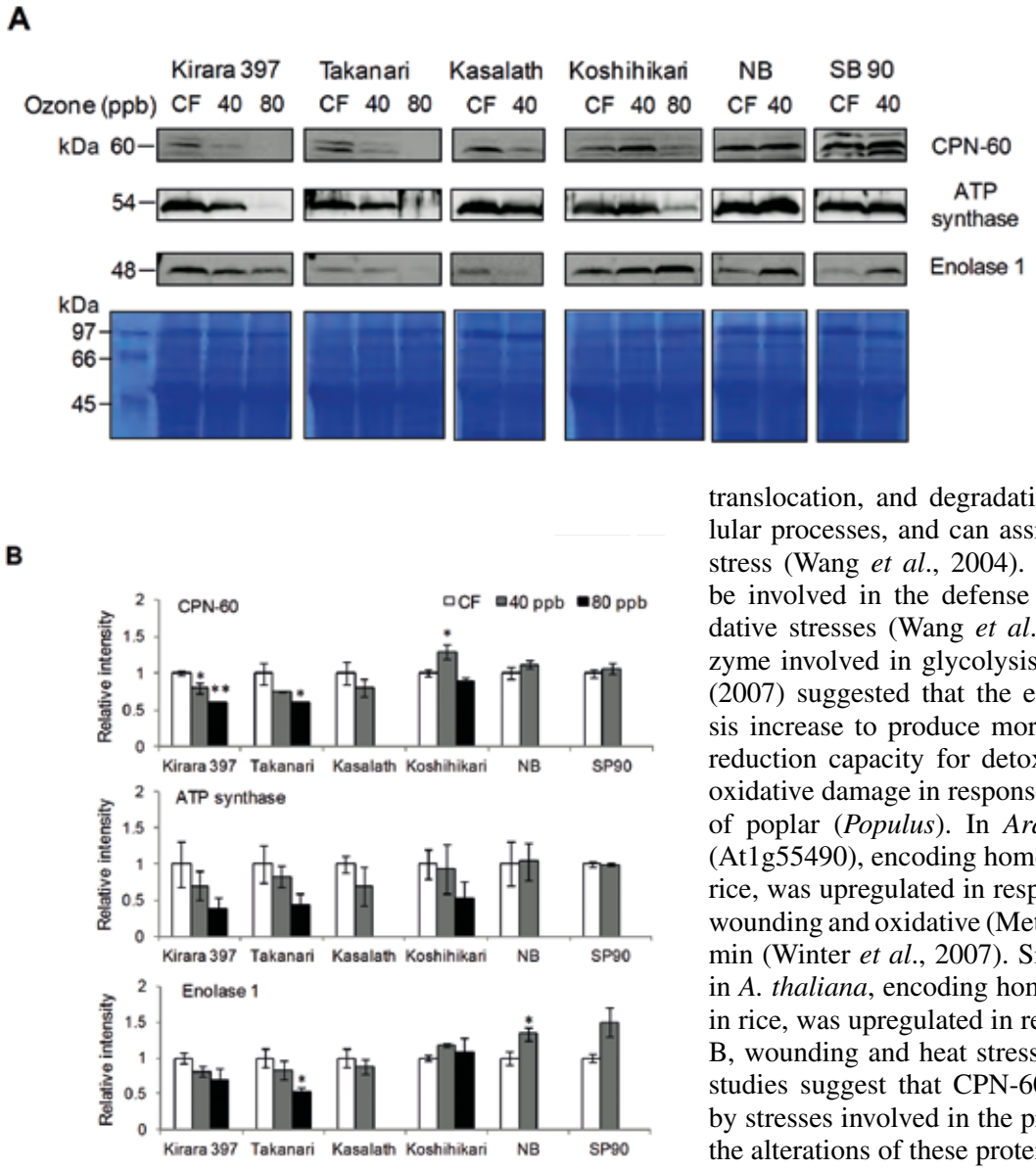

Fig. 2 - (A) Immunoblot analysis of CPN-60, ATP synthase, and enolase 1 in leaves of rice seedlings exposed to charcoal filtered air (CF), or to 40 or 80 ppb of ozone. Immunodetection was performed with antibodies specific to these proteins. (bottom panel) CBB-stained SDS-PAGE showing the quality and loading quantity of the protein samples. (B) Relative intensity for each protein estimated from the immunoblot analysis in panel (A). Values are mean ± SE (n = 3). Asterisk indicates a significant difference compared with CF according to Dunnett's test or *t*-test ($P<0.05$). NB, Nipponbare; SP90, Suphanburi 90.

40 ppb of ozone. The levels of all three proteins increased significantly or were maintained under 40 ppb of ozone in 'Nipponbare' and 'Suphanburi 90', but tended to decrease in 'Kasalath'. Therefore, the levels of CPN-60 and enolase 1 differed between the ozone-sensitive and ozone-tolerant cultivars: they decreased and increased, respectively at least at 40 ppb.

CPN-60 is a molecular chaperone. Many molecular chaperones were originally identified as heat-shock proteins (HSPs), which function in protein folding, assembly, translocation, and degradation during many normal cellular processes, and can assist in protein refolding under stress (Wang *et al*., 2004). CPN-60 (HSP60) appears to be involved in the defense response that mitigates oxidative stresses (Wang *et al*., 2011). Enolase 1 is an enzyme involved in glycolysis in the cytosol. Bohler *et al*. (2007) suggested that the enzymes involved in glycolysis increase to produce more energy and to increase the reduction capacity for detoxification of ROS and repair oxidative damage in response to ozone stress in the leaves of poplar (*Populus*). In *Arabidopsis thaliana*, *CPN60B* (At1g55490), encoding homologous protein to CPN-60 in rice, was upregulated in response to drought, UV-B, heat, wounding and oxidative (Methyl viologen) stress within 30 min (Winter *et al*., 2007). Similarly, *ENO2* (At2G36530) in *A. thaliana*, encoding homologous protein to enolase 1 in rice, was upregulated in response to cold, drought, UV-B, wounding and heat stress (Winter *et al*., 2007). These studies suggest that CPN-60 and enolase 1 are induced by stresses involved in the production of ROS. Therefore, the alterations of these protein levels with ozone exposure might result in ozone-derived ROS rather than ozone itself. However, there has been no report describing whether CPN-60 and enolase 1 influence grain production in crops under environmental stress, although these proteins might not be specific markers to ozone. Further studies will be needed to clarify the relationship between the reduction in grain yield and decreased production of these proteins by ozone-sensitive cultivars.

In order to compare the relative levels of each protein upon short-term ozone exposure with the relative grain yields under chronic ozone exposure we performed a linear regression analysis (Fig. 3). We found significant positive correlations between the levels of CPN-60, ATP synthase, and enolase 1 and the relative grain yield (i.e. yield decreased as the protein concentrations decrease). Therefore, the three proteins may serve as potential markers for chronic ozone stress in rice, although further experiments will be required for ATP synthase that also tended to decrease in 'Koshihikari' at 40 ppb ozone exposure (Fig. 2B). The level of CPN-60 had the highest goodness of fit ($R^2 = 0.786$) with the grain yield. This suggests that the potential ozone-induced yield reduction can be evalu-

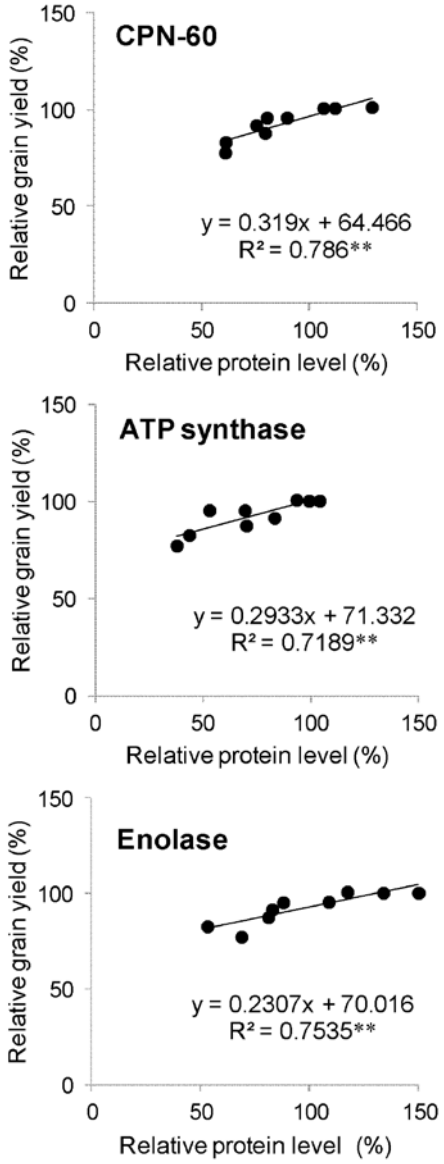

Fig. 3 - Regression analysis for the relative levels of CPN-60, ATP synthase, and enolase 1 in rice seedlings exposed to 40 and 80 ppb ozone, plotted as a function of relative grain yield. The grain yields are relative to the values for six cultivars grown in open-top chambers. Significance levels: **, $P< 0.01$; *, $p< 0.05$.

ated using the level of CPN-60 at the seedling stage in laboratory-scale tests. Moreover, the protein markers that we identified in this study may be useful in crop breeding to quickly select ozone-tolerant rice varieties. Vincent *et al*. (2007) indicated that the inhibition of shoot growth was best correlated with the level of CPN-60 in two wine grape cultivars exposed to salinity and water deficit stress, suggesting that the protein marker is also applicable to other plant or crops.

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Evaluation of local eggplant cultivars in terms of the suitability as materials for "Yakuzen" dishes

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Key words: functionality, local cultivars, property and taste, *Solanum melongena.*

Abstract: "Yakuzen" is a form of medicinal cooking based on the theories of Oriental medicine. To prepare Yakuzen dishes, in-season materials with appropriate properties and tastes, "Sei-Mi," are selected according to the health status and constitution of each person. In this study, the suitability of eggplant (*Solanum melongena* L.) cultivars for Yakuzen was evaluated by sensory tests and by analysis of the functional constituents considered to be closely related to the taste and functionality of Yakuzen dishes. Twenty-two eggplant cultivars including 21 Japanese and 1 Italian cultivar, and a Thai species (*Solanum xanthocarpum* Schrad & Wendl.) were evaluated. Principal component analysis (PCA) was used for comprehensive evaluation among the cultivars. From the PCA, many of the cultivars with round or oval fruit were characterized as juicy and sweet and considered easy to eat; most of the long-fruit cultivars were characterized as having higher specific amino acid contents. The small and round fruit cultivar Dewako and the Thai species (Makhuea pro) were considered to contain many functional ingredients, such as ash, polyphenols, and specific amino acids, and to have higher suitability for Yakuzen dishes.

1. Introduction

In recent years, malignant neoplasm (cancer), cerebrovascular disease and heart disease have become major causes of death among Japanese people, with approximately 75% of deaths in Japan caused by these diseases (Japanese Ministry of Health, Labour and Welfare, 2009 a). Such diseases are generally called lifestyle diseases because they are thought to be strongly related to a lack of exercise and high intake of fat and salt. Therefore, emphasis has shifted from early diagnosis (secondary prevention) to lifestyle improvement (primary prevention) (Japanese Ministry of Health, Labour and Welfare, 2009 b). In this context, research on foods that have pharmacological effects or physiological functions, such as disease prevention and health maintenance, has become more important (Namba, 1999; Tokui *et al*., 2003). Regarding the pharmacological effects of food, there is a form of medicinal cooking called "Yakuzen" which is based on the philosophy of Oriental medicine and is intended to maintain good health and improve physical condition. The preparation of Yakuzen dishes draws from the theory of "Yaku-shoku Dou-gen", which means that the same principle underlies the daily diet and medical treatment, and on the yin-yang theory, the five-phase theory in Oriental medicine. As a result, Yakuzen has attracted considerable attention for the prevention of lifestyle diseases.

To prepare Yakuzen dishes, in-season materials with appropriate properties and tastes are selected according to the health status and constitution of each person (Namba, 1995; Lan *et al*., 2002; Tokui *et al*., 2003). These properties and tastes are called "Sei-Mi" in Yakuzen theory. Sei-Mi consists of four properties (making the human body hot, warm, cool, or cold) and five tastes (salty, bitter, sweet, pungent, and sour), and each is considered to have its own function in the human body (Namba, 1999; Tokui *et al.*, 2003). If the concept of Sei-Mi can be applied to vegetables, a cultivar that has a strong flavor and a high content of functional constituents related to the properties and taste is considered to have strong Sei-Mi and is suitable for Yakuzen dishes.

However, the inherent flavor of vegetable cultivars has been weakened by breeding because priority has been given to ease of consumption for consumers or ease of production for growers. In the case of eggplant (*Solanum melongena* L.), popular cultivars in Japan today are F_1 (first filial generation) cultivars derived from a parental line with oval fruit, a deep purple pericarp, and high yield; these cultivars have improved fruit quality with less unpleasant or harsh taste.

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However, eggplant was introduced into Japan more than 1,260 years ago, and it is mentioned in Nara-period documents (The Shõsõin documents) edited in 750 A.D. (Yoshida, 2010). Therefore, many cultivars have been developed over the long history of cultivation, and even today there are many cultivars with local origins. The chemical composition, flavor, and texture of the edible parts of plants vary among cultivars. Local cultivars grown for many years may have retained more flavor, and some of them may be more suitable for Yakuzen dishes than the currently popular cultivars.

Regarding the functionality of foods, many studies have focused on certain ingredients and have discussed the relationship between the amounts of those ingredients and the functionality of the food. However, it is important to consider the functionality of foods comprehensively in Yakuzen theory. In previous studies, we used principal component analysis (PCA) to comprehensively evaluate the functionality of local cultivars, and we demonstrated that two local cultivars of Japanese radish (*Raphanus sativus* L.), two of carrot (*Daucus carota* L.), and one of bitter melon (*Momordica charantia* L.) were more suitable for Yakuzen dishes than the widely used F_1 cultivar (Saito *et al.*, 2010; Tsukagoshi, *et al.*, 2011 a, b).

In this study, two F_1 cultivars and 21 local cultivars of eggplant were evaluated for their suitability as materials for Yakuzen dishes according to their taste and content of functional constituents.

2. Materials and Methods

Plant materials and growing condition

Twenty-one Japanese eggplant cultivars with different fruit shapes were selected for this study (Table 1). Most of them were purebred cultivars, but two F_1 cultivars were included. In addition, an Italian cultivar and a Thai species (*Solanum xanthocarpum* Schrad & Wendl.) were used. All cultivars were grown at the Center for Environment, Health and Field Sciences, Chiba University. Seeds of all cultivars were sown in 9-cm plastic pots filled with upland soil on 17 April 2012, and the seedlings were raised in a glasshouse. On 5 June seedlings were transplanted to the open field at a spacing of 50 cm between plants and 100 cm between rows. Fruits were harvested when they reached the regular size for each cultivar. Harvest began on 31 July and ended on 14 September. All other management was carried out according to the conventional methods in Japan (Chino, 2001).

Sensory test

Fruits were harvested on 7 and 9 August and the sensory test was conducted the following day. The fruits were washed, cut to a size of 1.5×1.5×3.0 cm, and steamed at 100°C for 5 min. The characteristics of steamed samples were evaluated by six panelists. The panelists passed recognition tests for five tastes (sweetness, umami, saltiness,

Table 1 - Eggplant cultivars used for the experiment

Fruit shape (country)	Cultivar	Abbreviation	Fruit length, weight and color	Remark
Small, Round (Japan)	Dewako	De	3-8 cm, 10-15 g, deep purple	
	Minden	Min	3-8 cm, 10-15 g, deep purple	
Round (Japan)	Aizu maru	AM	8-10 cm, 200-300g, deep purple	
	Kamo nasu	KN	12-15 cm, 200-350 g, deep purple	
	Tonosama	To	approx. 15 cm, 300-450 g, deep purple	
	Yamatoyo maru	YM	10-12 cm, 250-350g, deep purple	
Money pouch (Japan)	Nagaoka kinchaku	NK	8-10 cm, 300-350g, deep purple	
(swelling toward the bottom)	Saitama ao daimaru	SA	approx. 15 cm, 300-450 g, green	
Oval (Japan)	Heta murasaki	HM	approx. 5 cm, 30 g, deep purple	
	Senryo 2 gou	Se2	10-12 cm, 80-90 g, deep purple	F_1, Control cultivar in this experiment
	Se2 (Control)	SK	10-11 cm, 150-180 g, deep purple	
	Wase shinkuro	WS	10-12cm, 80-120 g, deep purple	
	Yamashina	Ya	10-12cm, 80-120 g, deep purple	
Long (Japan)	Chikuyou	Chi	20-25 cm, 120-150 g, deep purple	F_1
	Hakata naga	Hak	40-45 cm, 200-300 g, deep purple	
	Hhogo naga	Hho	17-18 cm, 85-90 g, deep purple	
	Himo nasu	HN	25-30 cm, 150-200 g, pale purple	
	Kitta chunaga	KC	10-12cm, 80-120 g, deep purple	
	Kurume oh naga	KO	30-35 cm, 250-300 g, deep purple	
	Shikon sendai naga	SS	8-10 cm, 20-30 g, bluish purple	
	Shin nagasaki naga	SN	35-40 cm, approx. 250 g, deep purple	
Big, Oval (Italy)	Zebra	Ze	20-25 cm, 300-400 g, purple and white stripes	
Small, Round (Thailand)	Makhuea pro	MP	4-5 cm, approx.10 g, green	Solanum xanthocarpum

bitterness, and sourness) and the discrimination tests for four solutes (sucrose, sodium chloride, tartaric acid, and sodium glutamate). In addition, they had more than one year experience in evaluating vegetables and were classified as expert assessors (Japanese Society for Sensory Evaluation, 2009). The characteristics listed in Table 2 were evaluated on a scale of -5 (weaker) to 5 (stronger) compared to Se2, the control cultivar in this study.

Taste sensor analysis and amino acid content

Approximately 50 g of fresh fruit was homogenized in 100 mL of water in an ice bath, then filtered through cotton cloth. Although filtrates were prepared separately from three or four fruit samples, the filtrates were mixed to obtain the quantity necessary for measurements. Bitterness, astringency, acridity, and pungency of the filtrates were measured using a taste sensor system (SA402B, Intelligent Sensor Technology, Kanagawa, Japan). Each value was expressed relative to the control cultivar (Se2), which was set at zero. A portion of each filtrate was filtered again through a 0.45-µm filter (DISMIC-25CS, Advantec, Tokyo, Japan) and the amino acid content was measured using an amino acid analyzer (JLC-500/V, JEOL, Tokyo, Japan).

Soluble solids content

Fresh fruit was cut into small pieces and pressed in gauze to extract the juice. The soluble solids content of the juice was measured using a refractometer (PAL-1, ATAGO, Tokyo, Japan) and expressed as percent Brix.

Ash and polyphenol contents

The harvested fruit was stored at -30°C until use. Fresh-frozen fruit samples were freeze-dried and ground into a fine powder. Ash content was determined using the dry ashing method. Briefly, 0.3 g of the powder was put in a crucible and ashed at 550°C for 24-48 hr. After cooling, the weight of the residue was measured. Polyphenol content was determined using iron tartrate spectrophotometry. First, 0.2 g of the powder was mixed with 10 mL of distilled water, and shaken for 10 min at 80°C. After cooling, the sample was centrifuged at 3,000 rpm for 15 min. Then, 3.2 mL of the supernatant was mixed with 1.6 mL of iron tartrate reagent (0.1% (w/v) ferrous sulfate and 0.5% (w/v) potassium sodium tartrate) and 3.2 mL of phosphate buffer (0.1 M, pH7.5). The absorbance at 540 nm was then measured using a spectrophotometer (U-2000, Hitachi, Tokyo, Japan). The polyphenol content was calculated from a

Table 2 - Evaluation of the taste of eggplant cultivars by sensory test [z]

Fruit shape	Cultivar	Aroma		Softness		Juiciness	Sweetness	Bitterness	Astringency
		Good	Grassy	Pericarp	Flesh				& Acridity
Small, Round	De [y]	0 [x]	0	-1	0	0	0	1	1
	Min	1	1	0	-1	0	-1	2	1
Round	AM	0	1	0	-1	0	0	0	1
	KN	1	1	0	-1	0	0	0	0
	To	0	0	-1	0	0	0	1	0
	YM	0	0	0	1	1	1	-1	0
Money pouch	NK	0	2	-3	-2	-1	-1	0	0
	SA	0	2	-4	0	-1	0	0	1
Oval	HM	0	0	0	1	0	0	0	0
	Se2 (Control)	0	0	0	0	0	0	0	0
	SK	0	2	0	1	2	1	0	0
	WS	0	0	-1	0	-1	-1	0	0
	Ya	1	0	-1	0	0	0	0	0
Long	Chi	0	0	0	1	0	-1	0	0
	Hak	0	0	1	1	-1	0	-1	0
	Hho	0	0	0	0	0	0	0	0
	HN	0	1	-1	1	-1	-1	0	0
	KC	0	0	-2	1	0	-1	0	1
	KO	0	0	-2	1	-1	-1	0	0
	SS	0	1	-1	0	0	-1	0	1
	SN	0	2	0	1	-2	0	-1	0
Big, Oval	Ze	2	2	0	-2	0	-1	0	2
Small, Round	MP	0	3	0	-1	0	-1	0	1

[z] Eggplant fruit was cut to the size of 1.5 x 1.5 x 3.0 cm, then steamed at 100 degree C for 5 min before the test.
[y] Amino acid which is considered to be important for the functionality of eggplant in Yakuzen theory.
[x] Tastes were evaluated on a scale of -5 (weaker) to 5 (stronger) as compared to Senryo 2 gou (Se2).

standard curve of ethyl gallate.

Principal component analysis and characterization of cultivars

Data were analyzed by principal component analysis (SPSS for Windows version 13), and the characteristics of cultivars were comprehensively evaluated to determine the suitability of the cultivars as materials for Yakuzen dishes.

3. Results and Discussion

Cultivars NK and SA (with money-pouch fruit shape) tended to have harder fruits, and long-fruit cultivars tended to be less sweet (Table 2). The aroma of the Italian cultivar, Ze was characterized as both "good" and "grassy". However, most cultivars were very similar to Se2 (the control cultivar in this study) in the sensory test.

Taste sensor analysis showed that the local cultivars tended to have a less unpleasant taste than Se2 (Table 3).

Table 3 - Evaluation of the taste of eggplant cultivars by taste sensor [z]

Fruit shape	Cultivar	Bitterness	Astringency	Acridity	Pungency
Small, Round	De [y]	-0.27 [x]	0.06	-1.74	-0.69
	Min	-0.38	0.04	-2.27	-0.96
Round	AM	-0.23	0.20	-1.14	-0.48
	KN	-0.11	0.21	-2.11	-0.94
	To	-0.11	0.13	-2.18	-1.18
	YM	-0.16	-0.02	-1.33	-1.30
Money pouch	NK	-0.26	0.27	-1.78	-0.64
	SA	-0.07	0.30	-2.30	-1.07
Oval	HM	0.22	0.13	-1.83	-0.99
	Se2 (Control)	0.00	0.00	0.00	0.00
	SK	-0.28	0.05	-1.51	-0.76
	WS	-0.06	0.23	-2.26	-1.17
	Ya	-0.09	0.08	-2.57	-1.30
Long	Chi	-0.33	0.12	-1.39	-0.61
	Hak	-0.28	-0.09	-2.55	-1.40
	Hho	-0.15	0.05	-1.61	-0.86
	HN	-0.01	0.15	-2.87	-1.45
	KC	-0.12	0.11	-2.24	-1.28
	KO	-0.43	0.07	-0.52	-0.15
	SS	-0.26	0.04	-2.03	-1.00
	SN	-0.47	-0.05	-2.27	-1.09
Big. Oval	Ze	-0.32	-0.02	-2.73	-1.23
Small. Round	MP	-0.34	0.19	-2.15	-0.75

[z] Taste sensor was prepared to express the value of Se2 was zero.

[y] Amino acid which is considered to be important for the functionality of eggplant in Yakuzen theory.

[x] Positive and negative value means the taste was stronger and weaker than Se2, respectively.

This result did not correspond to the results of the sensory test, and the difference may be due to the heating of samples before the sensory test but not before the taste sensor analysis. Nevertheless, we can conclude that the local cultivars were not unpalatable compared with the commonly used cultivar.

Min, YM and some other cultivars tended to have higher soluble solids contents, but there was no significant difference between cultivars (Table 4). "Mi" (the taste) of eggplant is "Kan" (sweet). In Yakuzen theory, Mi means not only the taste on the tongue but also specific functions in the human body (Tokui *et al.*, 2003). In this study, we could not discern differences of Kan characteristics from the results of sensory test and soluble solids contents among cultivars; therefore, the suitability of cultivars was evaluated on the basis of other characteristics.

Ash content was higher in cultivars De and Min (both of which have small, round fruit) and lower in cultivar WS, and tended to be lower in cultivars with oval fruit. "Sei" (the property) of eggplant is to cool the human body. Potassium accounts for most of the ash of eggplant fruit (USDA, 2013), and the function of this mineral is to release heat inducing diuresis. This function is closely related to the property of eggplant, and higher ash content may be related to greater suitability of cultivars for Yakuzen dishes.

Polyphenol content was also higher in De and Min. Eggplant contains polyphenols such as chlorogenic acid and nasunin, which are considered to have antioxidant activity, and to suppress lipid peroxidation, aging, various lifestyle diseases, and cancer (Kimura *et al.*, 1999; Noda *et al.*, 2000; Kitsuda *et al.*, 2005; Singh *et al.* 2009). Das *et al.* (2011) reported that grilled eggplant had a higher polyphenol content, though the cardioprotective ability was not different. The high polyphenol content in De and Min may increase the pharmacological value of these cultivars.

As mentioned above, Mi (the taste) also encompasses specific functions in the human body, and Mi of eggplant is Kan (sweet). Kan is considered to have functions such as supplying nutrition and energy, promoting relaxation, etc. Some amino acids are considered to have Kan functions. For example, glutamine is an energy source for digestion and plays an important role in the maintenance and improvement of immunity and the repair of organs (Ajinomoto Co. Inc., 2003 a, b), and this may correspond to a Kan function. Alanine has a sweet taste and supplies sugars to the body, and it is also considered to have a Kan function. The amino acids strongly related to the Kan of eggplant include alanine, citrulline, glycine, glutamine, proline and serine. Therefore, these amino acid contents were summed to give specific amino acid content (Table 4): it was highest in Se2 and SN and tended to be higher in the long-fruit cultivars and lower in the round-fruit cultivars. Total amino acid content also tended to be higher in the long-fruit cultivars, especially in SN. However, no other trends in amino acid content were observed. The higher content of specific and total amino acids in SN would indicate greater suitability of this cultivar for Yakuzen dishes.

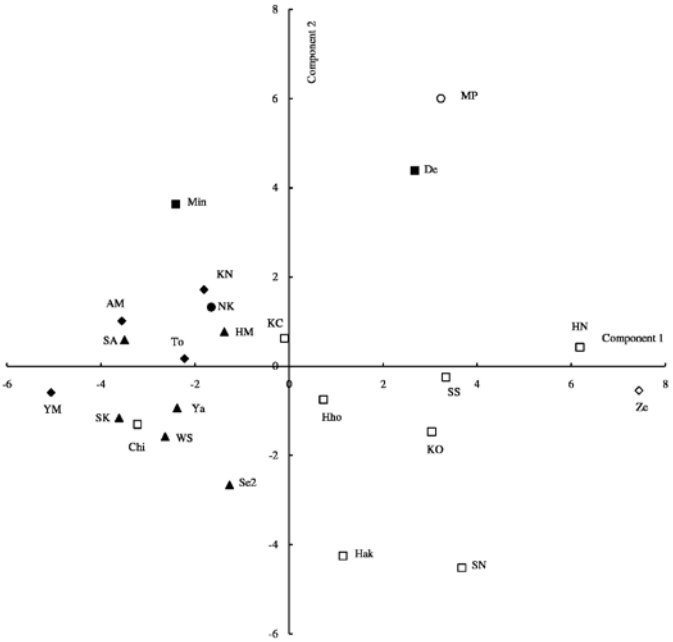

Fig. 1 Two dimensional scatter diagram of the principal component score of eggplant cultivars
■: small, round ◆: round ●: monry pouch ▲: oval □: long ◇: Italy ○: Thailand. Abbreviations as in Table 1.

From the PCA, 23 principal components (PCs) were derived and 14 PCs were considered to be meaningful (contribution rate>1). These 14 meaningful PCs accounted for 96.6% of the total rate (data not shown). Although the first two PCs accounted for only 41.3% of the total rate, a two-dimensional scatter diagram of factor loading was constructed. Specific and total amino acid contents were in the positive direction along the x-axis, and juiciness, sweetness, percent Brix and some unpleasant taste were in the negative direction. Ash, polyphenol content, and bitterness were in the positive direction along the y-axis, and softness was in the negative direction (data not shown). A two-dimensional scatter diagram constructed from the PC1 and PC2 scores of each cultivar enabled classification of the cultivars (Fig. 1). Many of the common F_1 cultivars of eggplant used for commercial production in recent years have been developed to improve ease of consumption and cultivation. Se2 is one of the common cultivars in Japan. It was characterized as juicy and sweet by the PCA and is considered easy to eat. The other cultivars with round or oval fruit were also characterized as juicy and sweet. On the other hand, most of the long-fruit cultivars were distinct from the round-fruit and oval-fruit

Table 4 - Soluble solid, ash, polyphenol and amino acid content of eggplant cultivars

Fruit shape	Cultivar	Soluble solids (% Brix)	Ash (g 100 g^{-1} FW)	Polyphenol [z] (mg 100 g^{-1} FW)	Specific amino acid [y] (mg 100 g^{-1} FW)	Total amino acid (mg 100 g^{-1} FW)
Small. Round	De [x]	5.1 a [w]	0.60 a [w]	673.4 a [w]	120.6	284.9
	Min	5.2 a	0.60 a	434.0 a	71.1	186.0
Round	AM	4.8 a	0.45 ab	169.0 b	37.2	151.2
	KN	5.4[v]	0.53 [v]	150.0[v]	48.8	203.1
	To	4.8 a	0.39 b	253.7 ab	52.5	196.6
	YM	5.3 a	0.50 ab	255.0 ab	36.3	144.3
Money pouch	NK	5.0 a	0.45 ab	172.9 b	63.1	197.8
	SA	4.6 a	0.50 ab	247.3 ab	39.5	167.3
Oval	HM	4.7 a	0.50 ab	300.6 ab	64.5	203.1
	Se2 (Control)	4.6 a	0.44 ab	129.1 b	145.6	302.0
	SK	4.3 a	0.39 b	158.4 b	48.0	170.6
	WS	5.1 a	0.37 b	181.9 b	52.8	199.1
	Ya	4.3 a	0.40 b	248.5 ab	58.1	185.4
Long	Chi	4.5 a	0.42 ab	188.1 b	34.1	161.2
	Hak	4.3 a	0.38 b	127.9 b	116.3	286.5
	Hho	4.8 a	0.52 ab	166.2 b	76.8	253.3
	HN	4.5 a	0.52 ab	144.1 b	83.6	288.6
	KC	4.4 a	0.48 ab	236.6 ab	50.8	211.0
	KO	5.1 a	0.45 ab	135.1 b	93.6	314.2
	SS	4.1 a	0.38 b	250.6 ab	106.1	281.8
	SN	4.5 a	0.41 ab	119.2 b	139.2	342.0
Big. Oval	Ze	3.8 a	0.46 ab	124.0 b	95.1	308.9
Small. Round	MP	5.1 a	0.56 ab	245.9 ab	63.9	196.7

[z] Polyphenol content was expressed as ethyl gallate equivalent.
[y] Amino acid strongly related to Kan of eggplant.
[x] Abbreviations as in Table 1.
[w] Different letter within the row indicates significant difference by Tukey's multiple range test at 5% level (n=5).
[v] Number of harvested fruit was not enough for statistical analysis.

cultivars. They were characterized as having higher specific amino acid contents and little unpleasant taste or sweetness. The small and round fruit cultivar De and the Thai species (MP) were considered to contain many functional ingredients, such as ash, polyphenols, and specific amino acids, and to have greater suitability for Yakuzen dishes.

4. Conclusions

Among the local Japanese cultivars used in this study, cv. De is highly suitable for Yakuzen dishes because it contains many ingredients associated with the properties and taste (Sei-Mi) of eggplant in Yakuzen theory. In addition, the Italian cultivar (Ze) and the Thai species were highly distinct. Different results may have been obtained had we grown the cultivars in another area or under different conditions. Nevertheless, we have demonstrated that some local eggplant cultivars have stronger Sei-Mi than current F_1 cultivars. These characteristics could add value to the local cultivars and lead to regional development.

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Physiological and psychological relaxing effects of visual stimulation with foliage plants in high school students

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Key words: heart rate variability, minors, house plant, stress reduction, visual stimuli.

Abstract: With lifestyles in modern society becoming increasingly stressful, there is growing interest in the physiological relaxing effects of the natural environment. Particular interest has been paid to the physiological effects of indoor plants, however no studies have revealed the effects of such visual stimulation on minors. In this study 85 (41 male and 44 female; 16.5±0.9 years; mean±SD) students were exposed, or not as control, to a typical foliage plant, dracaena (*Dracaena deremensis;* Lemon Lime), for 3 min. Physiological indices included heart rate variability (HRV) and pulse rate, using an accelerated plethysmography at the fingertip, were collected continuously during the experiments. The results indicated that the high frequency component (HF), a general index of parasympathetic nervous activity, was significantly higher; the low frequency component [LF/(LF+HF)], a general index of sympathetic nervous activity, was significantly lower; and the pulse rate was significantly lower. After exposure, or not, the subjects completed a questionnaire as psychological evaluation. A 13-point rating scale was used for following parameters: "comfortable-uncomfortable," "relaxed-awakening," and "natural-artificial." Results of the study showed that subjects felt more comfortable, relaxed and natural after visualizing the dracaena plants. Overall, the physiological and psychological relaxing effects of visual stimulation with foliage plants in high school students is confirmed.

1. Introduction

Recent studies have focused on the physiological relaxing effects of the natural environment (Park *et al*., 2009; 2012). It has been reported that staying in a forest environment enhances parasympathetic nervous activity (Park *et al*., 2012; Tsunetsugu *et al*., 2013), suppresses sympathetic nervous activity (Park *et al*., 2012; Tsunetsugu *et al*., 2013; Lee *et al*., 2014), decreases blood pressure and pulse rate (Park and Mattson, 2009; Park *et al*., 2012), and decreases cortisol concentration (Park *et al*., 2012). Studies by Li *et al*. (2007, 2008 a, b) demonstrated that staying in a forest environment for three days and two nights improved the immune function of office workers (Li *et al*., 2007), and this effect was sustained for approximately one month (Li *et al*., 2008 a, b). Another study reported that walking in an urban park enhances parasympathetic nervous activity and decreases heart rate (Song *et al*., 2013). In addition, spending time in rooftop gardens enhances parasympathetic nervous activity and suppresses sympathetic nervous activity in elderly people requiring care (Matsunaga *et al*., 2011).

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Evidence-based medicine has been attracting attention globally, with physiological data from field tests making a significant contribution. We expect that accumulating physiological data from field experiments will continue to demonstrate the preventive medical effects of nature therapy in the future (Lee *et al*., 2012).

In modern society, many individuals spend the majority of their time in intensely stressful states, and they have no time to make contact with nature outside of their immediate surroundings. High school students, who spend most of their everyday life at school, are typical examples. Previous studies have evaluated the psychological stress levels in high school students (Anda *et al*., 2000; Takakura and Sakihara, 2001). Moreover, many high school students have stressful relationships with friends or teachers (Miura and Kawada, 2008). In a document by the Japanese Ministry of Education, the percentage of students who progressed to universities or junior colleges in 2010 was 56.9%, which was 18.5% higher than the rate in 1975 (Statistics Bureau, Ministry of Internal Affairs and Communications, 2012), and the pressure from entrance examinations is extremely high among high school students (Equal Employment, Children and Families Bureau, Ministry of Health, Labour and Welfare, 2009).

Flowers and foliage plants are common natural surroundings that can be incorporated into the school and home. We previously conducted surveys to evaluate the physiological effects of visual stimulation with fresh rose flowers in high school students (Ikei *et al*., 2013), middle-aged and elderly medical staff (Komatsu *et al*., 2013), and office workers (Ikei *et al*., 2014). The results showed enhanced parasympathetic nervous activity (Ikei *et al*., 2013; Komatsu *et al*., 2013; Ikei *et al*., 2014), suppressed sympathetic nervous activity (Ikei *et al*., 2013), and decreased pulse rates (Komatsu *et al*., 2013) during visual stimulation.

It was reported in a previous study that natural views from hospital windows or the presence of indoor plants hasten the recovery of patients after surgery and decrease systolic blood pressure (Park and Mattson, 2009). These effects have also been studied in a classroom, demonstrating that the ambience of indoor space can be improved by including foliage plants (Doxey *et al*., 2009), as reflected by enhanced feelings of comfort among the students (Han, 2009). However, there have been no reports on the influence of visual stimulation with foliage plants on heart rate variability (HRV) and subjective feelings in minors. Therefore, this study was conducted to examine the effects of exposure to the foliage plant dracaena (Ministry of Agriculture, Forestry and Fisheries, 2008) on physiological and psychological variables (HRV, pulse rate, and subjective responses) in high school students.

2. Materials and Methods

The experiments were conducted in a classroom of the Chiba Prefectural Kashiwanoha Senior High School in October 2012. The room temperature was approximately 25.9°C, relative humidity approximately 52.6%, and illumination approximately 900 lux. Eighty-five high school students (41 male and 44 female; 16.5±0.9 years; mean±SD) participated in the experiment. The study was conducted with the approval of the Ethics Committee of the Center for Environment, Health and Field Sciences, Chiba University. All subjects provided written informed consent.

Three dracaena plants (*Dracaena deremensis*, Lemon Lime), 55-60 cm high, were placed at intervals of 8 cm on a desk in front of each subject (test situation). The distance from the subject's eyes to the plants was approximately 55 cm, and they were adjusted according to the height of the subjects. No exposure to foliage plants was used as the control condition. Before visual stimulation, the plants and the control were covered by a corrugated cardboard box (rest condition). Figure 1 shows the study protocol, figure 2 the rest condition, and figure 3 the visual stimuli (the dracaena

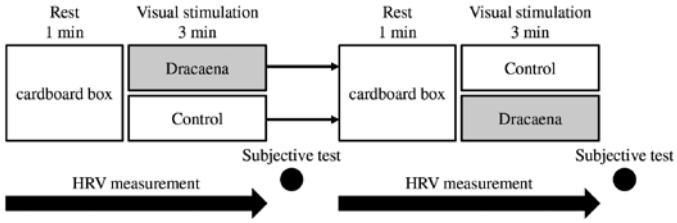

Fig. 1 - Study protocol for testing the physiological and psychological relaxing effects of visual stimulation with foliage plants in high school students.

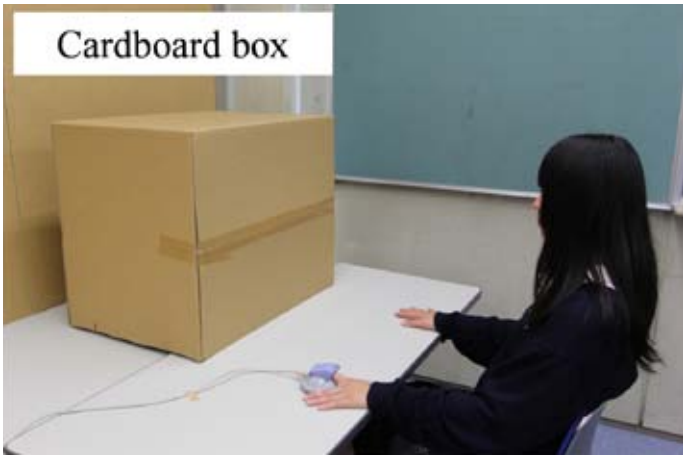

Fig. 2 - The rest condition.

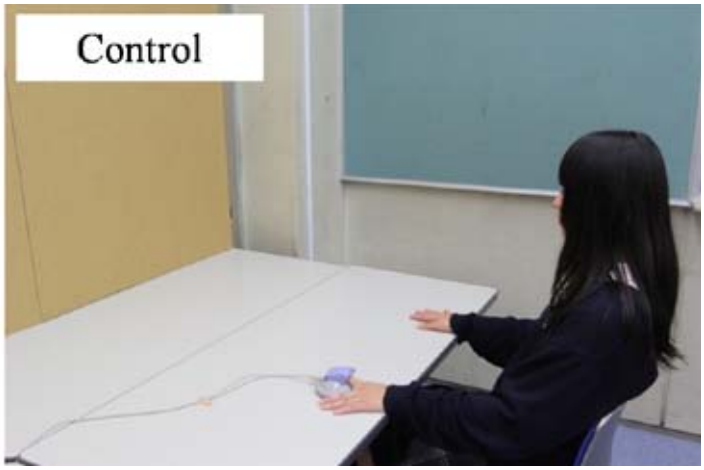

Fig. 3 -The visual stimulation condition.

plants or control). After viewing the cardboard box at rest in a sitting posture for 1 min (Fig. 2), the subject was exposed to the plants or control for 3 min (Fig. 3). After the experiments, each subject completed a questionnaire. The order of stimuli was counterbalanced among subjects.

HRV and pulse rate were measured as physiological indices. HRV was calculated by spectral analysis of the coefficient of variation of the a-a interval on an accelerated plethysmograph (APG; ARTETT, U-Medica Inc., Osaka, Japan). Previous studies have reported that the a-a interval on an APG and R-R interval on an electrocardiogram are strongly correlated (Takada and Okino, 2004; Takada *et al*., 2008). The sampling frequency was set at 1000 Hz. The maximum entropy method was used for frequency analysis, and variance of the low frequency (LF; 0.04-0.15 Hz) and high frequency (HF; 0.15-0.40 Hz) components were calculated. The LF/(LF+HF) ratio for R-R interval variability was also assessed. The HF component was used as an index of parasympathetic nervous activity and the LF/(LF+HF) ratio was used as an index of sympathetic nervous activity (Weise and Heydenreich, 1989; Cacioppo *et al*., 1994; Sawada *et al*., 1997). Generally, parasympathetic nervous activity is enhanced during relaxation and sympathetic nervous activity is enhanced at the time of awakening and stress (Ackerknecht, 1974). Therefore, the pulse rate was converted by dividing 60 by the a-a interval on APG. The HRV and pulse rate data were collected continuously during the 3-min experiments and averaged.

In addition, the subjects subjectively evaluated the emotional effects of the dracaena plants and control using the modified semantic differential (SD) method (Osgood *et al*., 1957), which uses three pairs of adjectives on 13 scales, including "comfortable-uncomfortable," "relaxed-awakening," and "natural-artificial."

The Statistical Package for Social Sciences software (v20.0, IBM Corp., Armonk, NY, USA) was used for all statistical analyses. A paired t-test was used to compare the physiological responses to visual stimulation with dracaena plants or control, followed by Holm correction of the changes in each 1-min average, while the Wilcoxon signed rank test was used to compare the psychological responses to visual stimulation with dracaena plants or control. For both conditions, one-sided tests were used because of the hypothesis that humans are relaxed by visual stimulation with foliage plants. Statistical differences were considered significant at $P<0.05$.

3. Results

Significant differences were found in the values of the HF component and the LF/(LF+HF) ratio of HRV between dracaena and the control.

Figure 4 shows the HF component of HRV, an estimate of parasympathetic nervous activity. HF between 0 and 1 min was 1210.7±120.3 (mean±SE) ms^2 during the test condition and 1032.9±95.2 ms^2 during the control condition, showing a significant increase of 17.2% ($P<0.05$) (Fig. 4A) during the test condition. Similarly, HF between 1 and 2 min was 1052.0±109.3 ms^2 during the test condition and 893.1±87.1 ms^2 during the control condition, showing a significant increase of 17.8% ($P<0.05$) (Fig. 4A) during the test condition. There was no significant difference in HF between 2 and 3 min. The overall HF during the 3-min experiment was 1083.9 ± 101.5 ms^2 in the test condition and 954.8±83.5 ms^2 in the control condition, showing a significant increase of 13.5% ($P<0.01$) (Fig. 4B) with the test condition, indicating that parasympathetic nervous activity was significantly higher during dracaena plant exposure.

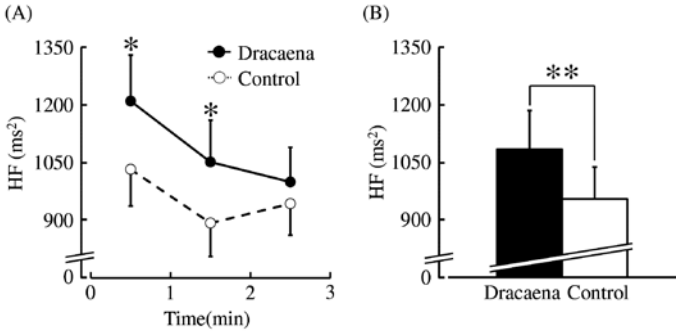

Fig. 4 - The 1-min averages and overall mean high frequency component (HF) of heart rate variability (HRV) during visual stimulation with dracaena plants and control.
(A) Changes in each 1-min average HF value over 3 min.
(B) Overall mean HF values.
n=85.
mean±SE.
*$P < 0.05$, **$P < 0.01$ as determined by the paired *t*-test, Holm correction.

The results of the LF/(LF+HF) ratio, a marker of sympathetic nervous activity, are shown in figure 5. For 1-min segment analysis, the LF/(LF+HF) ratio between 0 and 1 min was 0.47±0.02 during the test condition and 0.52±0.02 during the control condition, showing a significant decrease of 9.6% ($P<0.05$) (Fig. 5A) with the former.

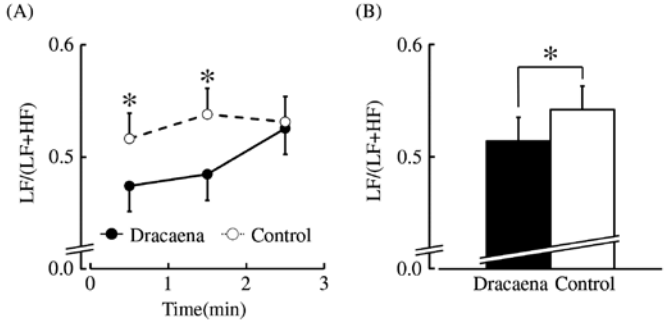

Fig. 5 - The 1-min averages and the overall mean LF/(LF+HF) ratio of heart rate variability. (HRV) during visual stimulation with dracaena plants and control.
(A) Changes in each 1-min average LF/(LF+HF) value over the 3 min.
(B) Overall mean LF/(LF+HF) values.
n=85.
mean±SE.
*$P<0.05$ as determined by the paired *t*-test, Holm correction.

Similarly, between 1 and 2 min, the ratio was 0.48±0.02 during the test condition and 0.54±0.02 during the control condition, showing a significant decrease of 11.1% (P<0.05) (Fig. 5A) during the test. No significant difference was observed between 2 and 3 min. For the entire 3-min duration, LF/(LF+HF) was 5.6% lower during the test condition than during the control condition (dracaena: 0.51±0.02, control: 0.54±0.02; P<0.05) (Fig. 5B), indicating that sympathetic nervous activity was significantly lower during dracaena plant exposure.

Clear differences in pulse rate were observed between dracaena and control exposure. The pulse rate between 0 and 1 min was 71.9±1.2 beats/min during the test condition and 72.7±1.2 beats/min during the control condition, showing a significant decrease of 1.1% (P<0.05) (Fig. 6A) during the test condition. Similarly, between 1 and 2 min, the pulse rate was 72.6±1.2 beats/min during the test condition and 73.3±1.2 beats/min during the control condition, showing a significant decrease of 1.0% (P<0.05) (Fig. 6A) during dracaena plant exposure. No significant difference was observed between 2 and 3 min. The mean pulse rate was 0.1% lower during the test condition than during the control condition (dracaena: 72.4±1.2 beats/min, control: 73.0±1.2 beats/min; P<0.05) (Fig. 6B).

The subjective evaluation data clearly showed the effect of the two different visual stimuli on the psychological states of participants. Participants felt significantly more comfortable (dracaena: "slightly comfortable"; control: "indifferent"; P<0.01) (Fig. 7, left), relaxed (dracaena: "slightly relaxed"; control: "indifferent"; P<0.01) (Fig. 7, center), and natural (dracaena: "slightly natural"; control: "indifferent"; P<0.01) (Fig. 7, right) in the dracaena condition than in the control condition after stimuli.

4. Discussion and Conclusions

An improved ambience resulting from the placement of foliage plants in a classroom may significantly enhance physiological relaxation and mental health in high school students, as shown in the present investigation.

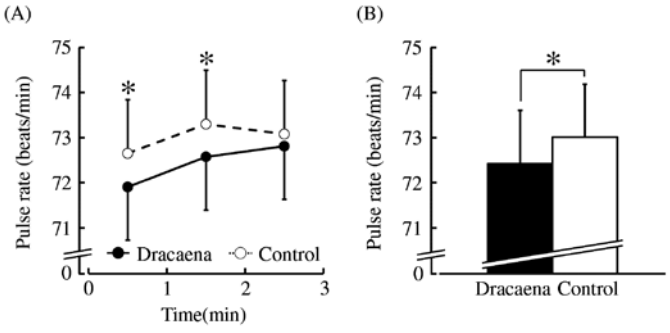

Fig. 6 - The 1-min averages and overall mean pulse rate during visual stimulation with dracaena plants and control.
(A) Changes in each 1-min average pulse rate over the 3 min.
(B) Overall mean pulse rates.
n=85.
mean±SE.
*P < 0.05 as determined by the paired t-test, Holm correction.

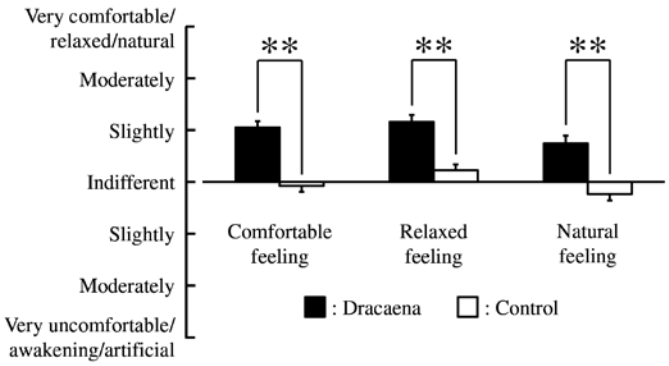

Fig. 7 - Changes in subjective evaluation for "comfortable–uncomfortable," "relaxed–awakening," and "natural–artificial" with dracaena plant exposure and control exposure.
n=85.
mean±SE.
**P < 0.01 as determined by the Wilcoxon signed-rank test.

Relatively brief (3 min) visualization of foliage plants resulted in significantly enhanced parasympathetic nervous activity (13.5%), suppressed sympathetic nervous activity (-5.6%), and decreased pulse rate (-0.8%), results which are consistent with previous studies involving the visualization of a forest scene (Park *et al*., 2010; Tsunetsugu *et al*., 2010; Park *et al*., 2011). Furthermore, our findings are consistent with those of our previous report on the calming effects of roses in high school students (Ikei *et al*., 2013), where similar physiological responses were found. Also in line with our findings, visual stimulation with fresh roses enhanced parasympathetic nervous activity and significantly decreased the heart rate in middle-aged and elderly medical staff (Komatsu *et al*., 2013), while it enhanced parasympathetic nervous activity in office workers (Ikei *et al*., 2014).

According to our analysis of the three questionnaires, the subjects in the present study felt more comfortable, relaxed, and natural after visualizing the dracaena plants. This result is consistent with that of our previous report on the calming effects of roses (Ikei *et al*., 2013; Komatsu *et al*., 2013; Ikei *et al*., 2014).

The results of this study support the hypothesis that placement of foliage plants in classrooms can induce a relaxing effect, improve physiological activity, and improve the psychological state in high school students. Because of the growing interest in mental health in modern times (Murray and Lopez, 1996), the psychological benefits of indoor plants are expected to play an important role in the promotion of mental health in the future.

However this study had limitations. First, we only evaluated HRV. Thus, the results cannot be interpreted in terms of a complete physiological evaluation. Other experimental indices such as brain activity and stress hormone levels should be assessed to determine the effects of visual stimulation with natural objects, such as foliage plants on human response. Second, only dracaena plants were used.

In future experiments, we will examine human responses to exposure to multiple types of foliage plants. We predict that the physiological data will support the physiological and psychological relaxing effects of foliage plants, which may subsequently lead to their increased use in educational establishments in attempts to decrease stress among students.

A brief visual stimulation of foliage plants shifted the sympathetic/parasympathetic balance and improved mood, suggesting a simple method to decrease stress and improve the health of high school students.

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