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Relationship of Transformation Efficiency and Metabolites Induced in Korean Soybean Cotyledons Treated with Sonication

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ABSTRACT The interaction between *Agrobacterium* and soybean has been studied at the transcriptome level but not at the metabolic level. However, it is necessary to investigate the difference in metabolites between susceptible and non-susceptible cultivars for high efficiency transformation. We investigated the difference in metabolites from sonicated soybean cotyledons of Korean cultivars and Bert cultivar. To identify difference in metabolites, sonicated extracts were analysed by Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR/MS). The soybean cultivars were classified by susceptibility using green fluorescent protein expression. We found a difference in metabolites between the high susceptible and low susceptible cultivars. The FT-ICR/MS experimental m/z data of different metabolites were compared with theoretical m/z in KNApSAcK database. The candidate list was made using KNApSAcK and focused on phenolic compounds. These candidate metabolites are speculated to influence factors in the interaction. This list of candidates may be useful to investigate the interaction between *Agrobacterium* and plants to increase transformation efficiency.

Keywords : soybean, metabolites, interaction, *Agrobacterium*, FT-ICR/MS

Soybean (Glycine max L.) is an important crop because of its high oil and protein contents in seeds. Soybean is the predominant protein source in animal feed and cooking oil (Schmutz *et al*. 2010). Soybean is responsible for more than $5 billion in annual export value to the U.S. economy (Gunstone, 2001).

Considerable success has been obtained in *Agrobacterium*-mediated transformation of soybean (Trick & Finer, 1998; Santarém *et al*. 1998; Olhoft & Somers, 2001; Olhoft *et al*. 2003), after the first transgenic soybean plant was obtained (Hinchee *et al*. 1988). In an effort to solve problems with host-specific *Agrobacterium* strains and low transformation efficiency researchers have included thiol compounds in the co-cultivation medium (Trick and Finer 1998; Olhoft and Somers 2001; Negishi 2007). Various transformation methods has been developed using powerful tools such as green fluorescent protein (GFP) and sonication. The use of GFP has provided a tool for fast and simple monitoring. Because visualisation of GFP does not kill cells, fluorescence can be used to study gene expression in vivo (Chalfie *et al*. 1994) or to monitor viral or bacterial infection events over time (Baulcombe *et al*. 1995; Gage *et al*. 1996; Oparka *et al*. 1997). Sonication-assisted *Agrobacterium*-mediated transformation (SAAT) is an efficient *Agrobacterium*-based transformation technology (Trick and Finer, 1997). This method consists of subjecting the target plant tissue to brief periods of ultrasound while it is immersed in an *Agrobacterium* suspension. The wounding due to sonication creates entry points for the bacteria and may stimulate the production of signalling molecules involved in the T-DNA transfer process (Meurer at el., 1998; Gelvin 2000). However, successful transformation has been accomplished in only a few mature soybean cultivars (Yan *et al*. 2000; Donaldson & Simmonds, 2000; Olhoft *et al*. 2003).

Improvements in *Agrobacterium* transformation depend on several factors including plant genotype, explant vigour, *Agrobacterium* strain, and co-cultivation. Some studies have shown an interaction between strains of *Agrobacterium* tumefaciens and soybean cultivars (Delzer *et al*. 1990; Ditt *et al*. 2001; Veena *et al*. 2003). Acetosyringone is a plant phenolic compound naturally secreted by wounded plant

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cells that acts as an inducer of *Agrobacterium* virulence genes (Stachel *et al*. 1985; Tang 2003). Phenolic compounds have benzene rings with some hydroxyl groups and are produced by plants mainly to protect against stress. In the majority of cases, the phenolic compounds kill many microorganisms, and some pathogens can use these metabolites to their own advantage. *Agrobacterium* has evolved mechanisms to counteract and utilise plant defences for their own advantage (Matilla *et al*. 2007; Hartmann *et al*. 2008). These metabolites are related to the interaction between *Agrobacterium* and host plant cells (Long 1996; Parker *et al*. 1997).

Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR/MS) is a analytic method with extremely high resolution and sensitivity (Marshall 1985). FT-ICR/MS allows direct analyses of samples without chromatographic steps and structural reactions, and high-throughput metabolic profiling studies can be conducted by assigning only one or a few molecular formulas to each single m/z value in the spectra. Several applications using FT-ICR/MS for plant metabolomics have been published (Aharoni *et al*. 2002; Hirai *et al*. 2004; Nakamura *et al*. 2007; Ohta *et al*. 2007). Algorithms have been developed to generate reliable liquid chromatography-mass spectrometry and FT-ICR/MS data to identify and measure the abundance of molecules. In the peak-picking algorithm, the highest intensity data points are used without an m/z centroid (Clauser *et al*. 1999). The frequency and mass difference between the data points is given to describe the error due to this simple peak detection (Titulaer *et al*. 2006) and the average noise signal intensity, the baseline correction, is calculated according to a method developed by Horn *et al*. Many programs has been developed and used such as “mMass”, which is a cross-platform environment to precisely analyse individual mass spectra (Strohalm *et al*. 2010).

These programs make analysis of FT-ICR/MS data and metabolic profiling easy. In particular, development of data processing (Dr. DMASS) and a metabolite-species relationship database (KNApSAcK) enables the identification of candidate metabolites (Oikawa *et al*. 2006). This system was developed to obtain information on metabolites, secondary metabolites, and their corresponding species. The database has a tool that can be used for analysing FT-ICR/MS data. The database enables searches using FT-ICR/MS data to identify metabolite-species relationships with detailed metabolite information.

The objective of this study was to examine the interaction between *Agrobacterium* and soybean cotyledons. GFP expression on cotyledons and the genotype-specific metabolites induced from sonicated cotyledons were evaluated using Korean soybean cultivars and the U.S. cultivar ‘Bert’. This is the first report to study the interaction between *Agrobacterium* and soybean at the metabolic level.

MATERIALS & METHODS

Preparation of explants

The three Korean soybean cultivars, ‘Taekwang’, ‘Iksannamul’, ‘Pungsannamul’, and the US cultivar ‘Bert’ were used. Seeds were obtained from the Rural Development Administration, Republic of Korea in 2010. Soybean seeds were washed with 70% ethanol with shaking for 1 min. The seeds were kept in a desiccator for 16 hr under chlorine gas for sterilisation, which was produced with 100 ml of bleach and 5 ml of 12 N HCl. After sterilisation, the seeds were transferred to germination media containing B5 salts and vitamins (Gamborg *et al*. 1968), 30 mg/l sucrose, pH 5.7. All plants were grown under a 16 hr photoperiod at 25℃ After 4-5 days, the seed coat and hypocotyl were removed, and the cotyledon was separated. The cotyledonary node, containing the cotyledon, was used for transformation experiments.

Binary vectors and *Agrobacterium* strains

The *Agrobacterium* tumefaciens strains used for transformation was EHA105. The strains contained the pCAMBIA 1303 binary vector (CAMBIA, Canberra, Australia) including the mGFP gene. A single colony was isolated and cultured in 5 ml liquid YEB media containing 50 mg/l rifampicin and 50 mg/l kanamycin overnight with 200 rpm shaking at 25℃ A 25 μl aliquot of culture media was transferred to 500 ml of YEP medium with antibiotics overnight at 25℃ with shaking until optical density (OD) reached a value of 0.4-0.5 in 600 nm. The resulting culture media was centrifuged at 4000 rpm for 10 min to collect the bacterial pellet. The pellets were resuspended and

adjusted to an OD_{600} of 0.8-1.0 with co-cultivation medium. The co-cultivation medium was composed of B5 salts and vitamins, 44 μM BAP, 1 μM IBA, 100 μM acetosyringone, and 30 mg/l sucrose, pH 5.5. The bacterial suspension was prepared immediately prior to use.

Inoculation and co-cultivation of *Agrobacterium*

The transformation protocol was based on the SAAT protocol of Townsend and Thomas (1993). The sonication is useful to wounding equally, the wounding induced metabolites related to interaction. The transformation experimental units were composed of 10 explants placed in 30 ml of *Agrobacterium* suspension and sonicated for 600 seconds. After the sonication, cotyledons were cultured for an additional 30 min at 150 rpm with shaking. A 200 μl aliquot of *Agrobacterium* suspension was transferred to 30 ml liquid YEB media to measure the relationship between sonicated cotyledons and *Agrobacterium* growth,. The O.D. value of *Agrobacterium* suspension was measured at 600 nm after 14 and 16 hr. An *Agrobacterium* suspension without soybean cotyledons was measured as a control. After sonication, each tube of explants was rinsed with double distilled water and placed in solid co-cultivation media (as above with agarose added) and incubated at 25℃ in the dark for 72 hr. After co-cultivation, cotyledons were rinsed with liquid post co-cultivation medium containing B5 salts and vitamins, 5 μM BAP, 1 μM IBA, and 30 g/l sucrose (pH 5.7), 250 mg cefoxtaxium, and 100 mg ticarcillin. The cotyledons were subsequently cultured on solid post co-cultivation medium.

Visualization of GFP expression

The soybean cotyledons on a plate were assayed for GFP gene expression. All cotyledons were assayed at 10 days after co-cultivation. GFP expression was observed by a LED 450 nm blue light source with a Kodak Wratten filter #15. The GFP foci per cotyledon (Foci/Cot) and percentage of response (Resp/Cot) were measured on the abaxial surface of the cotyledon (Meurer *et al*. 1998). The Foci/Cot was the number of GFP expressing foci on each cotyledon that responded with more than one spot of GFP expression, and the percentage of the response was the number of cotyledons with more than one spot of GFP expression among total explants.

Analysis of FT-ICR/MS

FT-ICR/MS was used to compare the substances secreted from soybean cotyledons. Cotyledons were sonicated in water without *Agrobacterium* for an accurate analysis. Metabolites were analysed using an FT-ICR/MS instrument (Korea Basic Science Institute, Osong, Chungbuk, Korea) with a 15T-FT-ICR, set on electrospray ionisation positive mode. The samples were injected directly by syringe pump at a flow rate of 2 μl min^{-1}. The samples were diluted 1:10 with 100% acetonitrile. Twenty spectral scans were performed for each sample. Experimental values for the m/z data were corrected to their theoretical values to calibrate all spectral data. A metabolite-species relationship database (KNApSAcK) was used to predict the candidate metabolites (Oikawa A *et al*. 2006; Shinbo Y *et al*. 2006).

Normalization

The FT-ICR/MS data were controlled by the mMASS, version 5.0 program. For normalisation, peaks were separated from noise if they had a signal to noise intensity (S/N) > 3. The spectra were smoothed using the moving average method and deisotoped. Precursor error tolerance was set to 100 ppm, and mass fragment error tolerance was set to 0.5 DA. Normalised peaks were compared with the tolerance set to 0.005 Da.

Data were analysed by analysis of variance, and the means were compared using Duncan's multiple range test. A $p < 0.05$ was considered significant.

RESULTS & DISCUSSION

GFP expression on cotyledons

To identify relation between transformation efficiency and metabolites, we used the GFP expression. GFP expression on cotyledons was evaluated at 10 days after co-cultivation with *Agrobacterium* (Table 1, Fig. 1). The spots of GFP expression from EHA105 were counted on the abaxial side of the cotyledon. The Foci/CotN and Resp/CotN were used to evaluate GFP expression. The percentage response was 26.3, 20.0, 16.3, and 18.0% in 'Bert', 'Taekwang', 'Iksannamul' and 'Pungsannamul', respectively. The 'Bert' Foci/CotN

Table 1. GFP expression on cotyledons of Bert, Taekwnag, Iksannamul, Pungsannamul at 10 days after co-cultivation with EHA105.

Variety	Foci/CotN[1]	Response/CotN[2]
Bert	9.8±1.7[a3]	26.3
Taekwang	7.8±1.4[ab]	20.0
Iksannamul	3.0±0.4[c]	16.3
Pungsannamul	4.8±0.7[bc]	18.0

[1]Foci/CotN is the number of GFP spots on each cotyledon responsed with more than one GFP spot.
[2]Response/CotN is the number of cotyledon with more than one GFP spot among total explants.
[3]Different letters within column indicate significant differences at $p<0.05$ (DMRT).

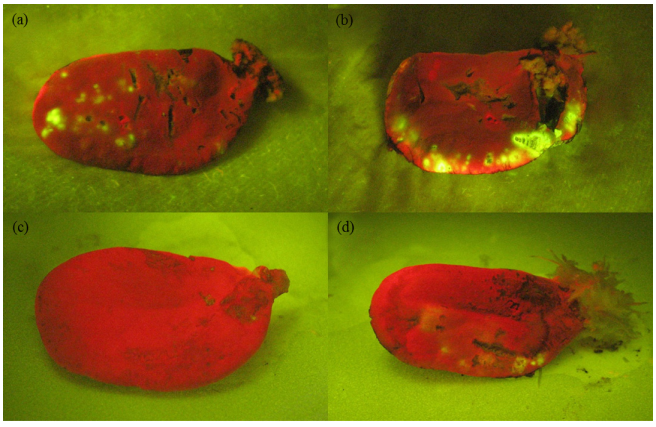

Fig. 1. Photographs showing GFP expression in cotyledon of soybean cultivars. (a) Bert, (b) Taekwang, (c) Iksannamul, (d) Pungsannamul.

was significantly different, and 'Taekwang' showed the highest Foci/CotN. As a whole, 'Bert' and 'Taekwang' were showed higher score in Foci/CotN and Resp/ContN than 'Iksannamul' and 'Pungsannamul'. These results strongly suggest a relationship between the variants and *Agrobacterium*.

These results are suggesting that genotype-specific metabolites affect susceptibility to *Agrobacterium*. Many studies have reported that some metabolites, such as phenolic compounds, affect the interaction between *Agrobacterium* and plant cells (Owens and Smigocki, 1988; Tzfira and Citovsk, 2002; Zaltsman *et al*., 2010). The 'Bert' and 'Taekwang' cultivars were more susceptible than 'Iksannamul' and 'Pungsannamul' from the GFP expression results.

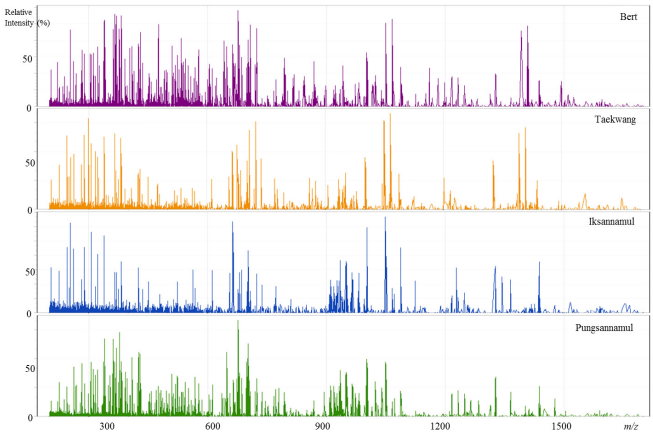

Fig. 2. Positive mode ESI FT-ICR mass spectrum of whole library.

Difference in metabolites

Several studies have shown that metabolites such as phenolic compounds affect bacterial growth and interaction (Cushnie & Lamb, 2005; Ferrazzano *et al*., 2009; Taguri *et al*., 2006). As known, 'Bert' was used for soybean transformation as a good explant (Olhoft *et al*., 2003; Zeng *et al*., 2004). But Korean soybean cultivars have low transformation efficiency compared with other cultivars, also successful transformation in Korean soybean had not yet reported. To identify the reason of low transformation efficiency, the metabolites from sonicated cotyledons were analysis using FT-ICR/MS (Fig. 2). The results showed that some peak of Bert is different from peak of Korean cultivars. However, Taekwang have some similar peaks of Bert's peak. It is expected that Taekwang's susceptibility is similar to Bert.

Candidate metabolites

The FT-ICR/MS spectra were analysed using mMASS software. After normalisation, the observed peaks were compared with a tolerance of 0.005 Da. We focused on the overlapping peaks between 'Bert' and 'Taekwang', which are highly susceptible cultivars, and between 'Iksannamul' and 'Pungsannamul', which are low susceptibility cultivars. We suggest that the metabolites of the highly susceptible cultivars are related to the interaction between *Agrobacterium* and soybean cells. The peaks at masses of 707.222, 723.196, 1065.312, and 1407.428 *m/z* had intensities above the S/N threshold in the 'Bert' and 'Taekwang' cultivars.

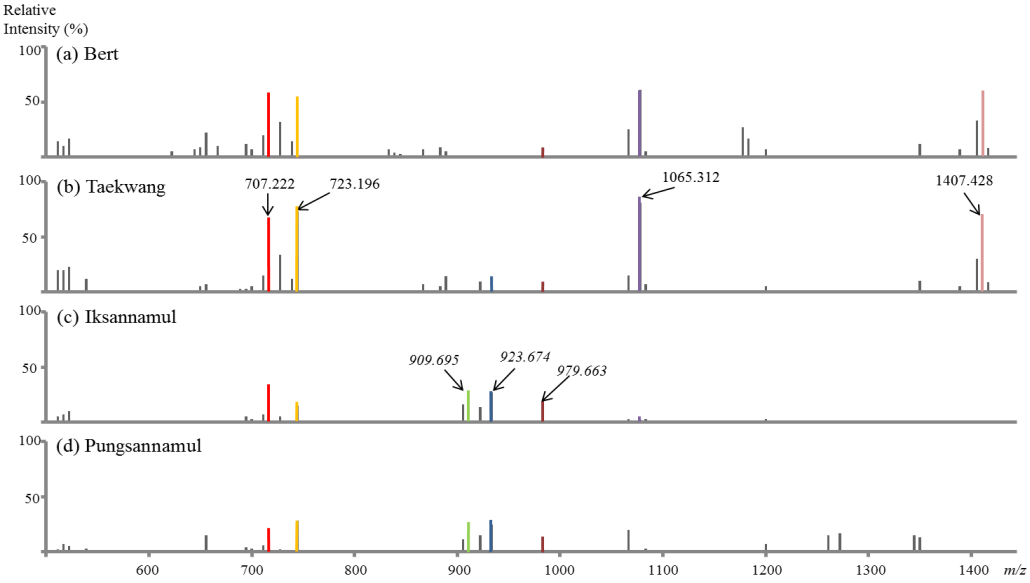

Fig. 3. The specific peaks of each cultivar selected by FT-ICR mass spectrum.

Table 2. The candidates at each peaks. The list were selected by comparing between theorical m/z and experimental m/z using KNApSAcK database.

Experimental *m/z*[1]	Adducts[2]	Theorical *m/z*[3]	Difference[4]	Molecular Formula	Metabolites	Organism	Bert
707.222	H^-	708.190	0.039	$C_{32}H_{36}O_{18}$	Kaempferide 3-Rhamnoside-7-(6"-Succinylglucose)	Allium	58.343
		708.204	0.026	$C_{33}H_{36}N_6O_8S_2$	Leptosin M1	Leptoshaeria	
		708.227	0.009	$C_{33}H_{40}O_{17}$	Glomeratose C	Polygala	
		708.263	0.034	$C_{34}H_{44}O_{16}$	4'-Cinnamoylmussatioside	Mussatia	
		708.205	0.024	$C_{36}H_{36}O_{15}$	Dicerandrol B	Phomopsis	
		708.252	0.023	$C_{36}H_{45}BRN_4O_6$	Jaspamide	Jaspis	
		708.242	0.013	$C_{37}H_{40}O_{14}$	Firmianone C	Firmiana	
	H^+	706.211	0.004	$C_{33}H_{38}O_{17}$	Sinocrassoside A5	Sinocrassula	
		706.120	0.018	$C_{40}H_{34}N_4O_{16}$	Antibiotic Pa 42702B	Flexibacter	
	Na^+	684.263	0.03	$C_{32}H_{44}O_{16}$	Yadanzioside E	Brucea	
		684.205	0.027	$C_{34}H_{36}O_{15}$	Agnucastoside C	Vitex Agnus-	
723.196	H^-	724.221	0.018	$C_{33}H_{40}O_{18}$	Kaempferol 3-Rhamnosyl-(1->4)-Rhamnoside-7-	Cassinopsis	54.638
					5,7,4"-Trihydroxyis O-Flavone 7-O-Alpha-L-	Sophora	
			0.008	$C_{35}H_{36}N_2O_{15}$	Pradimicin Fb	Actinomadur	
		724.200	0.003	$C_{36}H_{36}O_{16}$	Kaempferol 3-(2"-P-Coumaryl-Rhamnoside)-7-	Cheilanthes	
					3-O-Alpha-L-Rhamnopyranosylcatechin-(4Alpha-	Quercus	
					Catechin-(4Alpha->8)-Catechin-3-O-	Quercus	
					Kaempferol 3-(3"-P-Coumaryl-Rhamnoside)-7-	Cheilanthes	
	H^+	722.206	0.017	$C_{33}H_{38}O_{18}$	6'''-(3-Hydroxy-3-Methylglutaroyl)Isoviolanthin	Glycyrrhiza	
		722.185	0.004	$C_{36}H_{34}O_{16}$	5-O-Beta-D-Glucosylluteoliflavan-(40>8)-	Sorghum	
		722.200	0.011	$C_{40}H_{34}O_{13}$	Formononetic 7-O-(2",6"-Di-O-(E-P-	Pterocarpus	
	Na^+	700.221	0.015	$C_{31}H_{40}O_{18}$	Welloside	Veronica	
923.674	Na^+	900.690	0.005	$C_{51}H_{96}O_{12}$	Sch60065	Acremonium	0
1065.312	N/A[5]	1065.288	0.024	$C_{51}H_{53}O_{25}$	Cyanidin 3-[2-(6-P-Coumaryglucosyl)-6-P-	Ipomoea	61.006
					Pelargonidin 3-O-[2-O-(6-Ć-Caffeoyl-Beta-D-	Raphanus	
					Pelargonidin 3-(2-(6-Caffeylglucosyl)-6-P-	Raphanus	
	H^+	1064.301	0.004	$C_{48}H_{56}O_{27}$	Kaempferol 3-Neohesperidoside-7-2"-[-	Allium	
					Capilliposide I	Lysimachia	
					Chakaflavonoside A	Camellia	
1407.428	NH^+	1389.372	0.022	$C_{66}H_{69}O_{33}$	Delphinidin 3-Glucoside-7,3',5'-Tri(6-O-P-	Dianella	57.404
					Cyanidin 3-O-[2-O-(6-O-E-Coumaroyl-Beta-D-	Ipomoea	

Peaks at masses of 907.679, 909.695, 923.674, 931.679, and 979.663 *m/z* were shown in 'Iksannamul' and 'Pungsannamul' (Fig. 3). Some peaks overlapped in all cultivars, nevertheless, there were some differences in intensity.

The masses of the peaks were searched using KNApSAcK, which is a mass spectra dataset. The KNApSAcK data were analysed focusing on ion adducts $[M+K]^+$, $[M+Na]^+$, $[M+NH4]^+$, $[M+H]^+$, and $[M-H]^-$ (Tautenhahn *et al*., 2007; Iijima *et al*., 2008). The experimental FT-ICR/MS *m/z* data were compared with the theoretical *m/z* in the KNApSAcK database. The candidates were sorted by errors < 0.05 *m/z* between the experimental *m/z* and the theoretical *m/z*. The common metabolites of the highly susceptible cultivars had a few errors from the theoretical *m/z*. The *m/z* of 723.196, 1065.312, and 1407.428 showed that some phenolic compounds may have influenced the interaction between *Agrobacterium* and soybeans (Table 2).

Organisms in the KNApSAcK database are used help to speculate on the origin of metabolites such as the species, genus, family, or kingdom. Therefore, we focused on the phenolic metabolites in the plant kingdom. Phenolic compounds play a role activating bacterial nodulation (*nod)* and virulence (*vir*) gene networks as well as quorum signalling (Brencic *et al*., 2004; Bhattacharya *et al*. 2010). Eleven candidates were searched at 707.222 *m/z*. The candidates included phenolic compounds such as kaempferol and cinnamaldehydes. The families of kaempferol and hydroxycinnamides are associated with *vir* gene induction (Zerback *et al*., 1989; Berthelot *et al*., 1998; Joubert *et al*., 2004). Some candidate metabolites had no relationship with the interaction between *Agrobacterium* and soybeans. Dicerandrol B from fungi, jaspamide from the animal kingdom and the antibiotic pa 42702B from bacteria were not associated with the soybean metabolites. Many candidates, which were related to phenolic compounds, were shown at 723.196 *m/z*. All metabolites at 723.196 *m/z* were from plants, including many phenolic compounds such as kaempferol, flavonone, catechins, and coumaroyl hydrates. Kaempferol and flavonone are metabolites that induce the *vir* gene in *Agrobacterium* (Latha and Mahadevan, 1997; Jiang, 2003). Catechins and coumarins are metabolites used as recognition receptors in *Agrobacterium* (Spencer and Towers, 1988; John *et al*., 2009). After normalisation, two peaks were shown at 1065.288 *m/z* and 1064.301 *m/z* at 1065.312 *m/z*. The 1065.288 *m/z* peak was potentially cyanidin or pelargonidin, which are anthocyanidins. Anthocyanidins are flavononols, which determine floral colour, but they are not associated with the interaction between *Agrobacterium* and soybean. However, 1064.301 *m/z* with H^+ adducts included metabolites related to the interaction (Koes *et al*., 1994; Holton and Cornish, 1995). Kaempferol is induced by the *vir* gene of *Agrobacterium*, and flavonoids and flavonols induce the *nod* gene in *Rhizobium* (Cohen *et al*., 2001; Stougaard, 2000). The 1407.428 *m/z* peak contained two candidates coumaroyl and cyanidin. Coumaroyl and cynidin are anthocyanins related to flower colour (Holton and Cornish, 1995). Some common metabolites in the highly susceptible cultivars were candidates associated with the interaction between *Agrobacterium* and soybean. Especially, 707.222, 723.196, and 1065.312 *m/z* were involved in phenolic compounds such as keamperol, catechin, cinnamaldehydes that play an important role in the interaction between *Agrobacterium* and soybean cells (Nester. 1995; Bhattacharya *et al*. 2010). In contrast, the common metabolites of the low susceptibility cultivars had large errors > 0.05 except 923.674 *m/z*. SCH 60065 was the only candidate at 923.674 *m/z*. SCH 60065 is an antibiotic metabolite that has a role as a receptor inhibitor in microbes (Hegde *et al*. 1997). No other peaks were detected in the low susceptibility cultivars with < 0.05 error. This may have been due to a lack of studies on secondary metabolites; thus, these peaks are unresolved. Differences in metabolites were observed between the high and low susceptible cultivars and some candidate metabolites had an effect on the interaction between *Agrobacterium* and soybean cells.

We found the difference of metabolites between each cultivar. The metabolites were identified by sonication and FT-ICR/MS may have caused the difference in susceptibility to *Agrobacterium*. 'Bert' and 'Taekwang' were classified as highly susceptible cultivars based on GFP expression. To clarify reason of different transformation efficiency, the experimental FT-ICR/MS *m/z* data were compared with the theoretical *m/z* in the KNApSAcK database and we found some candidate metabolites. The candidates included some phenolic compounds that influence the interaction between

Agrobacterium and soybean cells. The candidate metabolites of the highly susceptible cultivars may influence interaction and transformation efficiency. The metabolite data of the high and low susceptibility cultivars may be useful to investigate the interaction between *Agrobacterium* and plants and increase transformation efficiency.

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