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Communication

Identification of Potential Metabolic Markers for the Selection of a High-Yield Clone of *Quercus acutissima* in Clonal Seed Orchard

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Abstract: *Quercus acutissima* Carruth. is one of the most economically important deciduous tree species in Korea. The acorns of *Q. acutissima* are used for both food and medicinal purposes in Korea and China. In this study, we analyzed plant hormones and metabolite profiles to identify their correlation with the yield production of *Q. acutissima*. The contents of sucrose and inositol in the leaves of high-yield clones were significantly higher ($p < 0.05$) than those of low-yield clones. In addition, high-yield clones have a higher content of phosphoric acid, succinic acid, malic acid, and butane-1,3-diol in stems compared with low-yield clones. Among the identified metabolites, zeatin-9-glucoside showed highly significant negative correlations with tree height, crown volume, and acorn production. It is considered that these metabolites could be useful metabolic markers for the selection of a high yield clone of *Q. acutissima*.

Keywords: acorn; clonal seed orchard; high-yield clone; metabolites; metabolic marker; oak; hormones; quercus; sawtooth oak; zeatin-9-glucoside(Z9G)

1. Introduction

Quercus acutissima Carruth. is an Asian oak tree native to Korea, China, and Japan and it is a keystone species in these countries [1]. Especially in Korea, the *Q. acutissima* is one of the most economically and ecologically valuable deciduous tree species, its standing volume making up nearly 27% of the total tree inventory [2]. *Q. acutissima* (sawtooth oak) acorns are consumed in several Asian countries including Korea and China as traditional food and medicine resources [3]. In addition, the wood from this species is used for charcoal and architectural materials [4]. Particularly, the acorns make a crucial contribution to the regeneration of oak forest as well as being important wildlife food in the forest [5,6].

A seed orchard is a stand that consists of genetically improved trees. It is one of the important seed sources for seed production and forest plantation. In this regard, the Korean government launched the seed breeding project in the late 1950s [7,8]. Clonal seed orchards (CSOs) of *Q. acutissima* with grafted trees of superior phenotypes have been established and managed by the government to produce abundant seeds of a high genetic quality.

To maintain seed orchards and control seedling distribution successfully, the screening and selection of highly productive oak trees are very important. In recent years, various methods have been applied to estimate acorn production [9–12]. However, there have been no reports on the study of metabolic profiling to select superior and high yield clones. Most studies have focused on structural characteristics such as diameter at breast height (DBH), height and crown area (m^2).

Thus, an effective prediction technique for the selection of superior trees is needed for the grafted trees in CSOs. Recently, a large number of targeted or non-targeted metabolic profiles, through the use of various analytical methods (GC–MS, LC–MS, ^{1}H-NMR ...), have been generated [13]. Their results demonstrated that metabolite profiling and identification are significantly correlated with phenotype characteristics in several model plant species such as potato [14], wheat [15], maize [16], and poplar [17,18].

Therefore, the successful development of biomarkers for accurately screening and predicting superior clones with high yield production would have a significant impact on tree breeding programs. In this study, plant hormones and metabolic profiling were analyzed from leaves and stems between high- and low-yield clones of *Q. acutissima* to identify their correlation.

2. Materials and Methods

2.1. Plant Material and Sample Preparation

The studied *Q. acutissima* clonal seed orchard, established in 1999 with 389 grafted oak trees, has been managed for 19 years at a local orchard covering 1.2 ha in Hwaseong, Gyeonggi province, South Korea (37°16′15″ N, 126°55′30″ E). The collected scions were grafted onto the same species and the individual clones were planted randomly within the CSO. The elevation of the CSO is 92 to 103 m above sea level (masl). The soil from the CSO had a pH of 5.3, a total nitrogen content of 0.38%, a CEC of 6.07 cmolc/kg, an average available phosphorus content of 9.25 mg/kg, and an organic matter content of 3.11%.

In this study, the number of fruiting acorns per tree has been observed annually and morphologically differentiated; six high-yielding clones and six low-yielding clones of oak trees were investigated from the clonal seed orchard. New terminal shoots and leaves of the youngest branches were randomly collected from the selected trees during the flower bud differentiation period.

2.2. Metabolic Profiling and Hormone Analysis

The freeze-dried leaves and stem tissue samples were extracted using the two-phase methanol–chloroform method described by Robinson et al. [17]. Approximately 60 mg of frozen ground tissue was accurately weighed into a pre-chilled, 2 mL, lock-cap centrifuge tube. To this, 600 µL of HPLC-grade methanol (CH_3OH) (JT Baker, Phillipsburg, NJ, USA) was immediately added and the mixture was vortexed for 10 s to halt biological activity and minimize degradation; 40 µL of distilled deionized water and 20 µL of an internal standard (5 mg·mL^{-1} of phenyl-D-glucopyranoside in H_2O) were then added, and the sample was incubated for 15 min at 70 °C with constant agitation and centrifuged at 14,000× *g* for 5 min. The supernatant containing the extracted metabolites was retained. A quantity of $CHCl_3$ (800 µL; JT Baker, Phillipsburg, NJ, USA) was then added to the pellet, and the mixture was vortexed for 10 s to resuspend the pellet and then incubated for 5 min at 35 °C with constant agitation. The resultant supernatant recovered following a second 5 min centrifugation at 14,000× *g*, was pooled with the supernatant from the initial CH_3OH extraction. H_2O (600 µL) was added to the combined supernatant, and the mixture was vortexed for 10 s and then centrifuged for 15 min at 1350× *g* to permit the separation of polar (methanol–water) and nonpolar (methanol–chloroform) phases. This combination and separation of phases allowed metabolites extracted in one phase but with greater affinity for the other phase to repartition. A 1 mL aliquot of the polar (upper) phase was taken and either processed immediately or stored at −20 °C until further analysis. Only soluble metabolites were then analyzed by GC-MS (Finnigan Polaris Q GC/MS, Thermo Electron Corporation, Waltham, MA, USA), which was equipped with a Trace TR5-MS GC column (30 m × 250 µm × 0.25 µm; Thermo Fisher Scientific Inc., Waltham, MA, USA) according to Robinson et al. [18] and Ossipov et al. [19]. Cytokinins, abscisic acid and indole-3-acetic acid were extracted using a modified protocol described by Farrow and Emery [20]; then, they were analyzed with HPLC-ESI(+)-MS/MS. Quantification of hormones was accomplished using the stable isotope

dilution method with the addition of the labelled standards to each sample. The statistical analysis was performed with SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). For the principal component analysis (PCA), XLSTAT 18.06 (Addinsoft, Paris, France) was used.

3. Results and Discussion

Metabolites and phytohormones play an important role in the regulation of plant development and yield in trees. The interactions have also been observed and reported in a number of researches. From the correlation between plant hormones and growth development, it has been identified that plant hormones regulate the growth and development of plants [21]. Therefore, plant hormone analysis and metabolite profiling might provide a useful screening tool in breeding and propagation programs to identify superior genotypes at an early age [22,23].

In this study, a total of twelve morphologically superior (n = 6) and inferior (n = 6) clonal oak trees were selected on the basis of physical characteristics (Figure 1). The average values of their physical characteristics, including height, crown volume, and acorn bearing numbers in the superior group, were significantly higher than those of the inferior group.

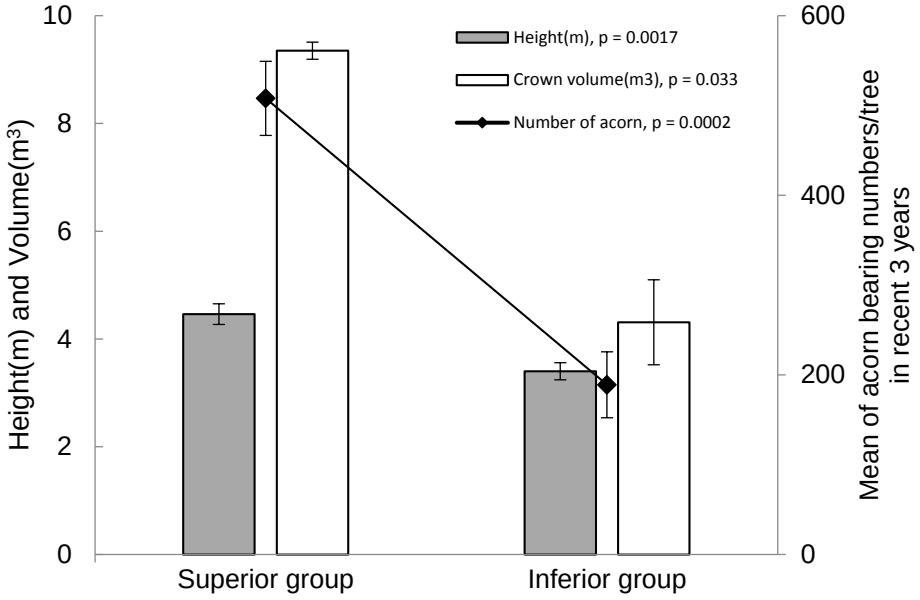

Figure 1. Growth characteristics of *Quercus acutissima* in the clonal seed orchard (n = 12; bars represent standard error of the mean).

Firstly, metabolite profiling experiments were performed on the groups to investigate the relationship between plant metabolites and morphological characteristics. A list of 20 metabolic compounds in leaves and stems was generated at probability of 0.05 and fold change >1.5 (Table 1). The concentration of some metabolic compounds was significantly associated with their growth characteristics. Most compounds were about two-fold higher in the superior group than in the inferior group. In the leaves, the concentration of maltose, sucrose, galactose and inositol was 2.98, 2.78, 2.73 and 2.91 times higher than the inferior group, respectively. In addition, Pearson's correlation analysis reveals that both isocitric acid and glucitol showed a high-value correlation coefficient with acorn bearing numbers and crown volume. However, phosphoric acid, succinic acid, malic acid, butane-1,3-diol, xylitol, and maltose had a significantly high correlation only with acorn bearing numbers and rythronic acid was only correlated with crown volume (Table 2). Although the metabolites seemed relevant to physical characteristics, further studies are needed to clarify their functions and effects in trees.

Table 1. Significant metabolites in the leaves and stems of *Quercus acutissima* ($n = 12$, $p \leq 0.05$).

Metabolites	Relative Contents (Mean)		Fold Changes (Superior/Inferior)	*t*-Test *p*-Value
	Superior Group	Inferior Group		
		<leaves>		
Ethylene glycol	74	34	2.18	0.045
Butane-1,3-diol	509	194	2.62	0.049
2(3H)-Furanone	62	23	2.70	0.042
Malic acid	3514	1520	2.31	0.048
Butane-1,4-diol	264	100	2.64	0.019
Rythronic acid-2	53	25	2.12	0.046
Ononitol	10,663	4997	2.13	0.048
Phenylpropanolamine	32,444	11,389	2.85	0.021
Quinic acid	22,284	9341	2.39	0.026
D-Ribose	1727	968	1.78	0.002
Galactose	205	75	2.73	0.044
Myo-inositol	172	90	1.91	0.037
Muco-inositol	282	97	2.91	0.017
Sucrose	39,070	14,068	2.78	0.025
Maltose	456	153	2.98	0.035
		<stems>		
Phosphoric acid	146	86	1.70	0.043
Succinic acid	10	5	2.02	0.000
Rythronic acid	42	30	1.40	0.021
Xylitol	15	10	1.51	0.033
Unknown 6	9	5	1.79	0.018

Table 2. Pearson correlations between growth characteristics (crown volume and acorn bearing numbers per tree) and metabolites in stems.

Metabolites	Acorn Bearing Numbers/Tree	Crown Volume
	Correlation Coefficient	Correlation Coefficient
Phosphoric acid	0.4301 *	0.2380
Succinic acid	0.5317 *	0.2252
Malic acid	0.5430 **	0.2821
Butane-1,3-diol	0.5951 **	0.3848
Xylitol	0.4308 *	0.3070
Isocitric acid	0.4738 *	0.6522 **
Glucitol	0.4591 *	0.4317 *
Maltose	0.4110 *	0.3657
Rythronic acid	0.2458	0.4498 *

* $p < 0.01$; ** $p < 0.001$.

In the plant hormone analysis, the concentrations of nine endogenous plant hormones including auxin (IAA) and abscisic acid (ABA), and endogenous cytokinins (CKs) including (9G)Z, (9R)Z, (7G)iP, iP, (9R)DZ, BA, and cis-Z, were quantified in 12 new terminal leaves and stem tissues collected from the superior and inferior groups (Table 3). There was no significant difference between groups in concentrations of IAA and ABA. Interestingly, only the zeatin 9-glucoside (9G)Z showed a significant negative correlation between its concentration and morphological characteristics including height ($r^2 = 0.704$, $p = 0.001$), crown volume ($r^2 = 0.490$, $p = 0.011$), and acorn bearing numbers ($r^2 = 0.478$, $p = 0.015$) (Figure 2). For the other five CKs, including (9R)Z, (7G)iP, iP, (9R)DZ and BA, there were no significant differences, but the concentrations in the superior group were about 1.4- to 3.0-fold higher than those of the inferior groups.

Table 3. Hormone contents in the new terminal stem tissues of *Quercus acutissima*.

Characteristic of Acorn Production	Hormone Contents (ng/g Dry Weight)									
	(9G)Z	(9R)Z	(7G)iP	iP	(9R)DZ	BA	cis-Z	IAA	ABA	IAA/ABA
Superior group	11.4 ± 1.6	108.8 ± 34.7	57.2 ± 25.5	150.4 ± 102.0	19.5 ± 6.1	6.8 ± 2.6	1.9 ± 0.6	280.5 ± 166.0	1,802.9 ± 666.3	0.19 ± 0.15
Inferior group	14.5 ± 2.0	107.2 ± 30.4	47.1 ± 22.5	94.3 ± 40.7	18.4 ± 5.6	6.2 ± 4.0	1.5 ± 0.6	299.2 ± 72.1	2,197.6 ± 576.2	0.15 ± 0.05
Sig.	**	-	-	-	-	-	-	-	-	-

(9G)Z: zeatin-9-glucoside; (9R)Z: trans zeatin riboside; (7G)iP: isopentenyladenine-7-glucoside; iP: isopentenyladenine; (9R)DZ: dihydrozeatin riboside; cis-Z: cis-zeatin; ABA: abscisic acid; IAA: indole-3-acetic acid; Significance (Sig.) given as the following: ** $p < 0.01$; - not significant.

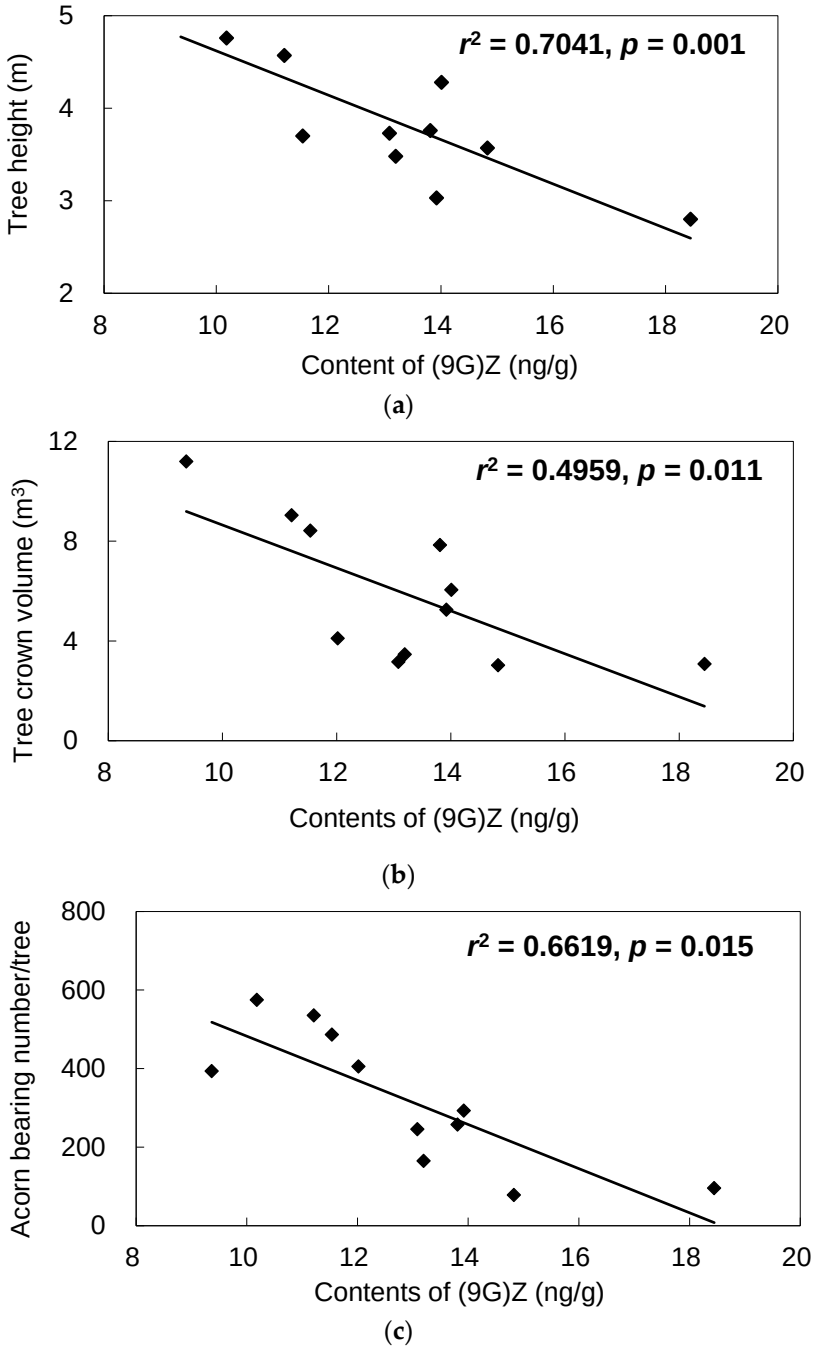

Figure 2. Pearson's correlations for the concentration of (9G)Z versus physical characteristics in the clonal seed orchard. (**a**) tree height; (**b**) tree crown volume; (**c**) acorn bearing number per tree.

Cytokinins play an important role in several aspects of plant growth, metabolism and development under normal growth conditions [24]. In particular, cytokinin is commonly converted to glucosyl conjugates and zeatin is the most active and ubiquitous form of the naturally occurring cytokinins [25]. Scott and colleagues [25] reported the first conclusive identification of an endogenous (9G)Z and demonstrated the occurrence of (9G)Z as the major endogenous cytokinin of *Vinca rosea* Linn. crown gall tissue. Martin et al. [26], also, reported similar results, i.e., that glycosyl conjugates of zeatin are found in many plant tissues and are considered important for storage and protection against degradative enzymes. Scott et al. (1980) documented that zeatin-0-glucoside (Z0G) was the main glucoside formed in soybean callus [27], lupin seedlings [28] and poplar leaves [29]. However, the (9G)Z in trees has not been well documented.

Principal component analysis (PCA) was conducted on 29 compounds to compare the metabolic concentrations of the groups with two principal components explaining 66.75% of the overall variance of the metabolite profiles, 52.38% and 14.37% for PC1 and PC2, respectively. The PCA scores revealed that the concentrations of metabolites and hormones of the *Q. acutissima* varied according to the groups (Figure 3). Interestingly, ABA and (9Z)G were similarly distributed among all the compounds and related with low-yielding clones. It is known that the plant hormone ABA regulates many key processes involved in plant development and it is also acclaimed as a stress modulator hormone. Therefore, it is possible that biotic and abiotic factors affect the concentration of (9Z)G and ABA. To verify the correlation, the stress-related compounds including malondialdehyde (MDA), proline, jasmonic acid, and salicylic acid should be tested in the field depending on the growth and development stages.

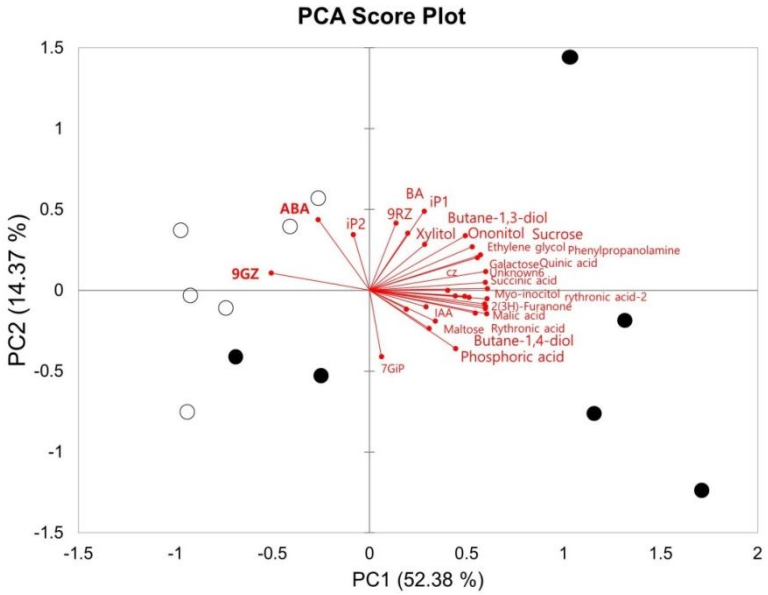

Figure 3. PCA (principal component analysis) of metabolites and hormones in superior (closed circles) and inferior (open circles) groups of *Quercus acutissima*.

4. Conclusions

Numerous efforts, such as metabolic profiling, have been made to develop markers for improving the yield and quality of crop species [30]. Our results show that several metabolites have strong correlations with phenotype characteristics. Notably, this is the first unambiguous identification of an endogenous (9G)Z in trees. We believe that our study provides valuable results to estimate acorn production. Therefore, (9G)Z could be a useful metabolic marker for the selection of a high yield clone of *Q. acutissima*.

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Author Contributions: Jun-Won Kang and Hyunseok Lee carried out the experiments and wrote the manuscript. Hyemin Lim contributed to the experiments and data analysis. Wi Young Lee directed the project and edited the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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