Combined GC- and UHPLC-HR-MS Based Metabolomics to Analyze Durable Anti-fungal Resistance Processes in Cereals

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Abstract: Introduction of durable resistance genes in crops is an important strategy to prevent yield loss caused by pathogens. The durable multi-pathogen resistance gene *Lr34* originating from wheat is widely used in breeding, and is functionally transferable to barley and rice. The molecular resistance mechanism of *Lr34*, encoding for an adenosine triphosphate-binding cassette transporter, is not known yet. To understand the molecular function and the defense response of durable disease resistance in cereals, the metabolic response of *Lr34* was investigated in, except for the *Lr34* gene, genetically identical lines of barley, rice and wheat. A broad range of compounds including primary, secondary and lipophilic metabolites were analyzed by a combination of gas (GC) and liquid chromatography (LC) mass spectrometry (MS) based methods. Data from metabolomics correlated well with transcriptomics data for plant defense responses such as the formation of anti-fungal hordatines or the components of the glyoxylate cycle. Induction of the glyoxylate cycle found in transgenic *Lr34* rice grown in the greenhouse was confirmed in field-grown natural *Lr34* wheat. Constitutively active plant defense responses were observed in the different cereals.

Keywords: *Lr34/Yr18/Sr57/Pm38* · Mass spectrometry · Plant defense · Plant metabolomics

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1. Introduction

An environmentally sustainable, effective method to control fungal infections in plants is the use of resistance genes. The wheat resistance gene *Lr34* (also known as *Yr18*, *Sr57* or *Pm38*) is an important source of disease resistance against leaf, stripe and stem rust in wheat breeding due to its durability with respect to pathogen adaptation. *Lr34* causes broad-spectrum resistance against the three wheat rusts and powdery mildew.[1] Keller and co-workers found that *Lr34* encodes for a full-size ATP-binding cassette (ABC) transporter protein of the ABCG subfamily, LR34.[2] The substrate(s) translocated by LR34 and also its location remain unknown.

Lr34 functionality can be transferred to other cereal species like barley, rice and maize, where the gene increased resistance against the adapted pathogens barley leaf rust, barley powdery mildew,[3] rice blast[4] and common maize rust.[5] In contrast to wheat, where *Lr34* is only expressed at adult plant stage, barley and rice already showed *Lr34* resistance at seedling stage. Therefore, transgenic barley and rice plants offered a valuable tool to study the molecular differences in pathogen resistance caused by *Lr34*. The *Lr34* resistance gene activity is associated with a senescence-like leaf-tip necrosis phenotype.[2]

In order to use plant defense against pathogens, it is crucial to understand biotic stress responses.[6] Metabolites play vital roles in plant–pathogen interactions, and reflect the molecular phenotype. Metabolomics, with its challenging aim to identify the entire metabolite content of an organism or tissue, is a valuable tool to better understand biological processes and mechanisms, *e.g.* in plant biotic interactions.[7] The workflow generally involves the analysis of the metabolic profiles, the identification of statistical differences in metabolites that are up- or downregulated upon treatment, and next the structure identification of the metabolites. Considerable challenges are the high chemical diversity associated with contrasting physicochemical properties, the wide dynamic range of metabolites, and the rapid biotransformations.[6] They are addressed by combining multiple partially complementary analytical approaches. Metabolite identification still poses a major bottleneck in metabolomics, given the high structural diversity, the limited availability of reference com-

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pounds and the restricted phyto-metabolites in mass spectral databases.

In order to gain a deeper insight into the metabolic pathways and responses related to *Lr34*, metabolomic profiling of *Lr34* was performed as part of a multi-disciplinary approach to unravel the molecular function of durable disease resistance in cereals. Here, we present a comprehensive characterization of metabolic differences caused by *Lr34* in the cereal species barley, rice and wheat. In order to find robust differences, transgenic barley and rice were grown under three well-defined conditions including hydroponic solution, mock-infection without pathogen, and infection with fungal pathogens. Finally, metabolic profiles of field-grown *Lr34* wheat were analyzed to evaluate *Lr34* function under agricultural conditions. A combination of UHPLC- and GC-MS based technologies were applied to cover a broad variety of plant metabolites including essential primary metabolites, lipids and non-essential secondary metabolites.

2. Experimental Procedures

Barley (*Hordeum vulgare* cv Golden Promise) and rice (*Oryza sativa japonica* cv Nipponbare) seedlings were grown under three conditions; hydroponics, mock-infected without pathogen and pathogen infected with barley leaf rust (*Puccinia graminis* sp hordei) or rice blast (*Magnaporthe grisea*), respectively. Barley and rice genotypes with low and high expression levels of *Lr34res* were investigated, resulting in low and high number of transcripts and respective transporter proteins. As controls, sister lines with identical genetic background lacking *Lr34res* were used. Flag leaves of mature field-grown winter wheat (*Triticum aestivum* L. cv Arina) of both non-infected and wheat leaf rust (*Puccinia triticina*) infected plants were investigated. For all samples, whole leaves were harvested and homogenized. In order to enlarge the metabolic coverage, the plant powder was extracted by a mixture of methanol, methyl-tert-butylether (MTBE) and water, allowing the simultaneous extraction of primary and secondary metabolites as well as lipids from the same samples.[8] Lipids were analyzed in the apolar organic fraction,[9] whereas secondary metabolites were detected by LC-MS and primary metabolites by GC-MS[10] in separate aliquots of the polar phase. MS data were preprocessed with *TagFinder*[11] or *Genedata Refiner MS* (Genedata, *http://www.genedata.com*) to result in two-dimensional matrices comprising metabolite identities/ feature information and intensities of all replicate samples. To account for variances that arose from sample preparation and metabolite analysis, data were normalized to the intensity of the internal standards and to fresh weight of the plant material, filtered with feature intensities in blank samples, occurrence, robustness and abundance. For principle component analysis (PCA), data were log2 transformed. The univariate statistical analysis was performed by analysis of variance (ANOVA) including multiple testing corrections and further evaluation by a post-hoc Tukey's test. The significantly different metabolites with *p*-values below 0.05 were considered for comparison between independent experiments.

Secondary metabolite identification by UHPLC-HR-tandem MS (MS/MS) was performed on a *QExactive* Orbitrap (*Thermo Fischer Scientific*, Waltham, MA, US) based on calculated molecular formula, MS/MS spectra comparison to databases and literature references. Detailed experimental procedures are documented in ref.[12].

3. Results and Discussion

To gain a deeper insight into the metabolites and metabolic pathways related to *Lr34* resistance, a comprehensive metabolomic profiling of barley, rice and wheat was performed. In order to find and characterize significant differences caused by the resistance gene, all experiments were designed to compare the metabolomic profiles of *Lr34* plants with control plants. Known primary metabolites and lipids were assigned based on MS spectra from libraries[8,13] and used to evaluate differences between control and *Lr34* plants. For secondary metabolites, preprocessed signals from UHPLC-HR-MS, so-called features characterized by mass-to-charge ratio (*m/z*), retention time and intensity, were evaluated by statistical analysis to find features that significantly increased or decreased between control and *Lr34* plants and were subsequently investigated for metabolite identification.

3.1 *Primary Metabolites and Lipids*

In extracts of transgenic barley and rice, eighty-four primary metabolites including amino acid derivatives, sugars, polyols and organic acids as well as 156 lipids including phospho-, sphingo-, galactolipids and triacylglycerols were found. Data from metabolic profiling were explored by multivariate analysis, namely principal component analysis (PCA). Two representative PCA score plots of primary metabolites found in mock-infected barley and rice are shown in Fig. 1. The strongest group separation was obtained for high-expressing barley (orange triangles), which was clearly separated from the high-expressing control group (orange circles) and the low-expressing groups (blue).

Significantly different metabolites between control and *Lr34* plants were evaluated by univariate analysis (ANOVA), and differences that were found in replicate experiments were compared between all three growth conditions (Fig. 2). The resulting overlapping metabolites were considered as robust metabolic changes. Subsequent results were restricted to high *Lr34*-expressing barley, as no robust differences were observed in low expression lines or in rice.

Nine primary metabolites and sixteen lipids were changed in barley plants with high *Lr34* expression levels grown under all three conditions. Amongst the robust metabolic differences, decreased dehydroascorbate concentration was related to plant defense regulation in terms of reactive oxygen species scavenging. Fructose and glucose were induced to higher levels in *Lr34* barley, hinting towards reallocation of energy or association with senescence.[14] Increased concentrations
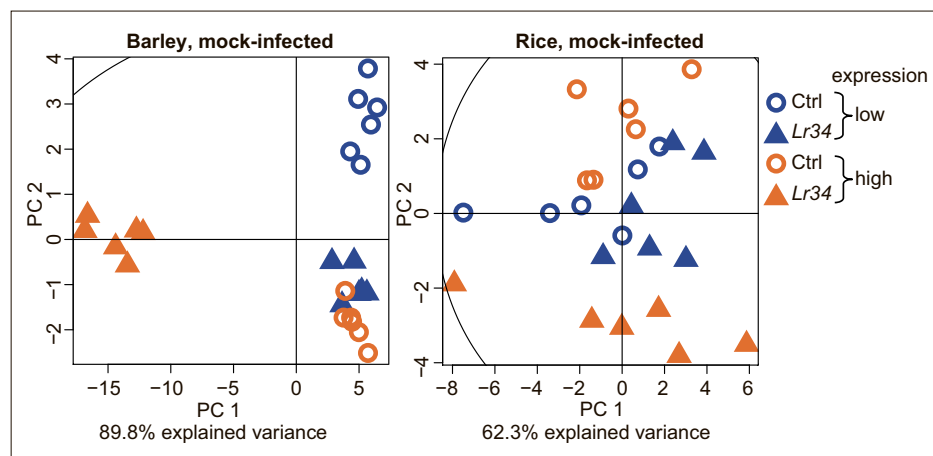

Fig. 1. Representative PCA score plots of identified primary metabolites in barley and rice grown under mock-infected conditions showing the first and second principal components (PC1, PC2). Largest group separation observed for high *Lr34*-expressing barley (left plot, orange triangles).
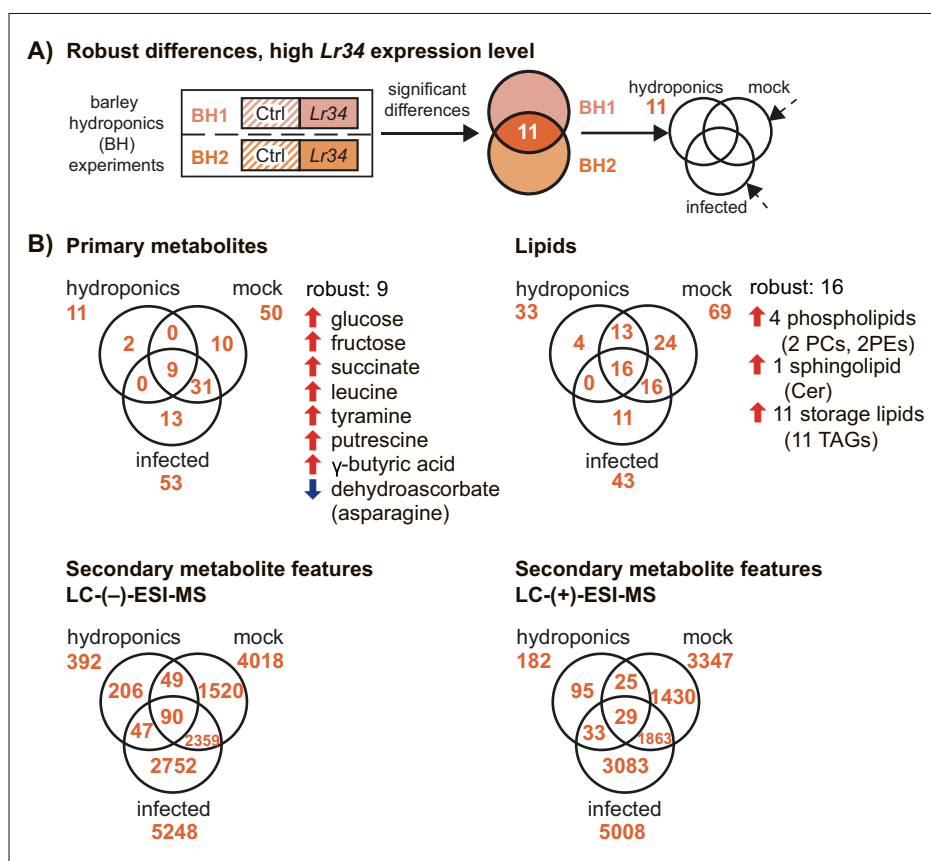
Fig. 2. Evaluation of robust significant differences in high *Lr34*-expressing barley; A) Significant differences between control and *Lr34* were evaluated by statistical analysis, and robust differences of both replicate experiments were considered for comparison of growth conditions. B) Robust significant differences of different metabolite classes.

glucoside ($C_{23}H_{38}O_{20}$) in barley leaves.[19] Flavonoid concentrations are known to fluctuate in response to plant stress,[14] or the observed metabolites might undergo an unknown biotransformation. Genes of the phenylalanine pathway and flavonoid biosynthesis were induced in *Lr34* barley, but the identified flavones were decreased.

Another group of barley-specific antifungal secondary metabolites called hordatines were increased in previously investigated *Lr34* barley plants without pathogen infection, correlating to the increased gene expression of agmatine-coumaroyl transferase[18] which is a key enzyme in hordatine biosynthesis. Besides the increase of hordatines, several defense responses were active in transgenic *Lr34* barley even in the absence of any infection.[18] The constitutive defense compounds being constantly synthesized by the plant suggest that *Lr34* contributes to constitutive, basal plant resistance in barley and might explain the negative impact of *Lr34* on the vigor of barley plants as trade-off in terms of biomass.

It is noteworthy that the majority of differentially regulated secondary metabolite features could not be identified. Plant secondary metabolites have a large structural diversity, the MS/MS spectra are difficult to reproduce and interpret, and only a few are available as reference compounds.[6]

of putrescine and the polyamine degradation product γ-butyric acid might be involved in plant stress tolerance.[15] The sixteen commonly changed lipids involved each two phosphatidyl cholins (PCs) and phosphatidyl ethanolamines (PEs), one ceramide (Cer) and eleven triacylglycerols (TAGs). TAGs are formed as intermediates upon membrane breakdown during stress or senescence, and elevated sphingolipids such as ceramides regulate cell death and pathogen-induced hypersensitive responses.[16] The observed lipid differences consequently indicate connections with plant defense responses and with the strong leaf-tip necrosis phenotype in barley.

Correlation of metabolomics and transcriptomics data were obtained for the glyoxylate cycle, a pathway that is induced in general stress response in rice (Fig. 3). Isocitrate concentrations were down-, and succinate concentrations upregulated in high-expressing transgenic *Lr34* rice grown under hydroponic conditions, and also gene expression of isocitrate lyase, an enzyme in the glyoxylate cycle, was increased in *Lr34* rice.[4] The only robust changes of primary metabolites found in non-infected and infected wheat plants grown in the field were isocitrate and succinate that were increased and decreased, respectively, and a tendency of increase

was also reported for gene expression of isocitrate lyase in *Lr34* wheat.[17] Thus, the findings from the transgenic model-plants rice were confirmed in natural, field-grown wheat. In contrast, transgenic *Lr34* barley showed a different response and isocitrate lyase gene expression was downregulated.[18]

3.2 Secondary Metabolites

Using the same strategy described above, a higher number of robustly significantly changed secondary metabolite features was found for high *Lr34*-expressing barley grown under the different conditions. Based on HR-MS and in-source fragments, a LC-(−)-electrospray ionization (ESI)-MS feature at *m/z* 315.0721 and retention time 3.65 min was putatively assigned as gentisic acid *O*-glucoside, a glycosylated oxidation product of the plant hormone salicylic acid. A series of decreased features in high *Lr34*-expressing barley and rice grown under mock- and pathogen infected conditions were annotated as *C*-glycosylated flavones based on flavones previously described in literature. An example is shown in Fig. 4 for a LC-(−)-ESI-MS feature at *m/z* 771.1992 and retention time 5.21 min. The MS/MS spectrum showed high agreement with fragments found for isoorientin-7-2"-di-*O*-

4. Conclusions

The extensive metabolomic investigation of *Lr34* in barley, rice and wheat characterized a variety of metabolites contributing to *Lr34* resistance and thus helped to understand durable anti-fungal resistance processes in cereals. This study shows successful examples of integrating metabolomics and transcriptomics data, *e.g.* for the glyoxylate cycle or for hordatine phytoalexins. Overall, a strong, constitutive re-programming of metabolism by *Lr34* in transgenic barley and rice was observed. In wheat, the results from the glyoxylate cycle were confirmed under most natural field-conditions. Multiple plant defense responses were constitutively active in *Lr34* resistant plants, but the substrate of the LR34 transporter could not be identified.

[1] S. G. Krattinger, E. S. Lagudah, T. Wicker, J. M. Risk, A. R. Ashton, L. L. Selter, T. Matsumoto, B. Keller, *Plant J.* **2011**, *65*, 392.

[2] S. G. Krattinger, E. S. Lagudah, W. Spielmeyer, R. P. Singh, J. Huerta-Espino, H. McFadden, E. Bossolini, L. L. Selter, B. Keller, *Science* **2009**, *323*, 1360.

[3] J. M. Risk, L. L. Selter, H. Chauhan, S. G. Krattinger, J. Kumlehn, G. Hensel, L. A. Viccars, T. M. Richardson, G. Buesing, A. Troller, E. S. Lagudah, B. Keller, *Plant Biotechnol. J.* **2013**, *11*, 847.

[4] S. G. Krattinger, J. Sucher, L. L. Selter, H. Chauhan, B. Zhou, M. Tang, N. M. Upadhyaya, D. Mieulet, E. Guiderdoni, D. Weidenbach, U. Schaffrath, E. S. Lagudah, B. Keller, *Plant Biotechnol. J.* **2015**, *14*, 1261.

[5] J. Sucher, R. Boni, P. Yang, P. Rogowsky, H. Buchner, C. Kastner, J. Kumlehn, S. G. Krattinger, B. Keller, *Plant Biotechnol. J.* **2016**, DOI: 10.1111/pbi.12647.

[6] H. Tenenboim, Y. Brotman, *Trends Plant Sci.* **2016**, *21*, 781.

[7] L. W. Sumner, Z. Lei, B. J. Nikolau, K. Saito, *Nat. Prod. Rep.* **2015**, *32*, 212.

[8] P. Giavalisco, Y. Li, A. Matthes, A. Eckhardt, H.-M. Hubberten, H. Hesse, S. Segu, J. Hummel, K. Kohl, L. Willmitzer, *Plant J.* **2011**, *68*, 364.

[9] B. D. Pant, A. Burgos, P. Pant, A. Cuadros-Inostroza, L. Willmitzer, W. R. Scheible, *J. Exp. Bot.* **2015**, *66*, 1907.

[10] J. Lisec, N. Schauer, J. Kopka, L. Willmitzer, A. R. Fernie, *Nat. Protoc.* **2006**, *1*, 387.

[11] A. Luedemann, K. Strassburg, A. Erban, J. Kopka, *Bioinformatics* **2008**, *24*, 732.

[12] R. Bucher, Ph.D. Thesis, University of Zurich, 'Mass-spectrometry Based Metabolomic Approaches to Characterize Plant Pathogen Resistance in Cereals and the Flavonols Modifying Development in *Arabidosis thaliana*', Zurich, **2017**.

[13] a) J. Kopka, N. Schauer, S. Krueger, C. Birkemeyer, B. Usadel, E. Bergmuller, P. Dormann, W. Weckwerth, Y. Gibon, M. Stitt, L. Willmitzer, A. R. Fernie, D. Steinhauser, *Bioinformatics* **2005**, *21*, 1635; b) A. Burgos, J. Szymanski, B. Seiwert, T. Degenkolbe, M. A. Hannah, P. Giavalisco, L. Willmitzer, *Plant J.* **2011**, *66*, 656.

[14] M. Watanabe, S. Balazadeh, T. Tohge, A. Erban, P. Giavalisco, J. Kopka, B. Mueller-Roeber, A. R. Fernie, R. Hoefgen, *Plant Physiol.* **2013**, *162*, 1290.

[15] P. T. Do, T. Degenkolbe, A. Erban, A. G. Heyer, J. Kopka, K. I. Kohl, D. K. Hincha, E. Zuther, *PLoS One* **2013**, *8*, e60325.

[16] Y. Okazaki, K. Saito, *Plant J.* **2014**, *79*, 584.

[17] S. H. Hulbert, J. Bai, J. P. Fellers, M. G. Pacheco, R. L. Bowden, *Phytopathology* **2007**, *97*, 1083.

[18] H. Chauhan, R. Boni, R. Bucher, B. Kuhn, G. Buchmann, J. Sucher, L. L. Selter, G. Hensel, J. Kumlehn, L. Bigler, G. Glauser, T. Wicker, S. G. Krattinger, B. Keller, *Plant J.* **2015**, *84*, 202.

[19] A. Piasecka, A. Sawikowska, P. Krajewski, P. Kachlicki, *J. Mass Spectrom.* **2015**, *50*, 513.
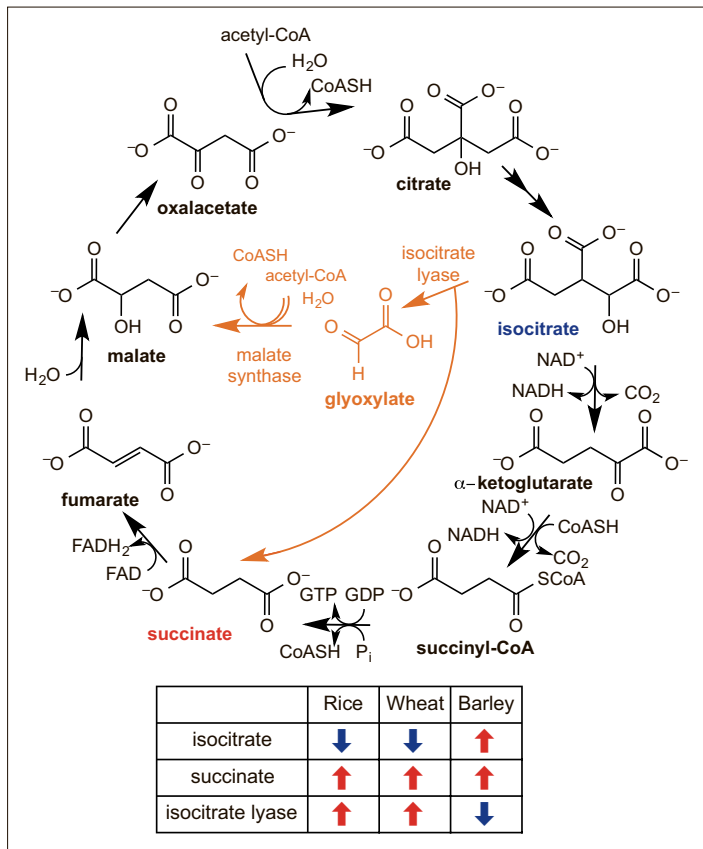
Fig. 3. Pathway interpretation of the glyoxylate cycle (orange) and citric acid cycle (black). In *Lr34* plants of rice (hydroponic growth condition, high expression) and wheat (non-infected and infected), the metabolite concentrations of isocitrate were down- and succinate upregulated. The mRNA of isocitrate lyase was increased in *Lr34* containing plants of rice[4] and a tendency of increase was also reported for wheat.[17] In barley, the metabolic response was different and isocitrate lyase gene expression downregulated.[18]
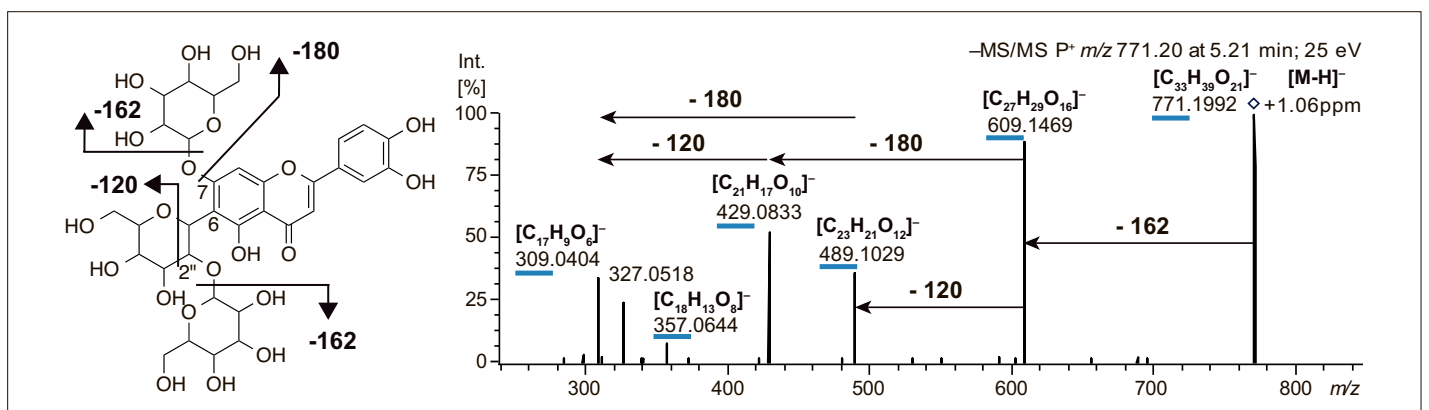
	Rice	Wheat	Barley
isocitrate	↓	↓	↑
succinate	↑	↑	↑
isocitrate lyase	↑	↑	↓

Fig. 4. LC-(–)-ESI-MS/MS of a *C*-glycosylated flavone [M-H]⁻ *m/z* 771.1992 at 5.21 min putatively assigned as isoorientin-7-2"-di-*O*-glucoside; fragments reported in literature are underlined.[19]