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SUMMARY OF DOCTORAL THESIS

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Title: Exploring metabolome changes in wheat (*Triticum aestivum*) under heat stress using Fourier transform infrared spectroscopy
（フーリエ変換赤外分光法を用いた高温ストレス下におけるコムギのメタボローム変動の探索）

Wheat (*Triticum aestivum* L.) is one of the most important crops globally. It contributes with rice, maize, and soybean to two-thirds of calories required for world population. Wheat is very sensitive to heat stress. An increase of 1ºC temperature is estimated to reduce wheat yield by 6.0%. Therefore, understanding wheat response to heat stress is crucial for facilitating the development of new heat tolerant varieties.

In this study, metabolomic approach was chosen because it is arguably more closely related to the phenotypes than other "omics" data. Metabolomics is one of the omics tools used to analyze the molecular responses of plants, and has been utilized to study metabolic responses in plants under various stresses and genotypes differentiation. There are various tools to study plants metabolome. Among them Fourier transform infrared spectroscopy (FTIR) spectroscopy is unique in that it provides an opportunity to study biological samples *in vivo* in a non-destructive manner, is compatible with remote sensing in the field, and allows the analysis of complex biomacromolecules such as cell wall components. To our knowledge FTIR was not applied before to examine heat stress effects in wheat metabolome.

Therefore, sequences of studies were carried out starting by establishing a protocol to detect FTIR capability to characterize chemical changes of wheat metabolome under heat stress (chapter1) utilizing a genotype Norin 61 (N61). Subsequently, the established protocol was applied to wheat genotypes possessing different heat tolerance capabilities (chapter 2). Three genotypes were used in this study: Chinese Spring (CS) has been identified as a heat-sensitive genotype. Imam is a heat-tolerant cultivar widely grown in Sudan, which is regarded as the world's hottest wheat growing environment. N61 showed heat tolerance in hot regions in Sudan in field studies.

In the present studies, plants were grown in normal condition in control chamber in 18◦C for the night temperature for 10 h and the daily temperature of 22ºc. Heat stress was applied when the plants reached the three-leaf stage and the length of the third leaf exceeded that of the second leaf. Under the heat stress condition, the seedlings were transferred to a heat chamber with a daily maximum temperature of 42◦C. The obtained FTIR spectra from the leaves did not show visually-prominent discriminating peaks between heat stress and control conditions. Therefore, coupling the FTIR analysis with chemometric analysis was indispensable.

In the first chapter, visual inspection of FTIR spectra and their principal component analysis showed partially overlapping features between heat-stressed and control leaves in N61 genotype. In contrast, supervised machine learning through linear discriminant analysis (LDA) of the spectra demonstrated clear discrimination of heat-stressed leaves from the controls. Analysis of LDA loading suggested that several wavenumbers in the fingerprinting region (400–1800 cm^{-1}) contributed significantly to their discrimination. Six novel spectrum-based biomarkers, designated as Fm482, Fm576, Fm1251, Fm1465, Fm1502, and Fm1729, were developed using these

discriminative wavenumbers, which enabled successful diagnosis of heat-stressed leaves.

In chapter 2, the metabolome responses of heat-tolerant genotypes, Imam and N61, and susceptible genotype CS were comparatively analyzed using FTIR in combination with chemometric data mining techniques. Similar to the chapter 1, principal component analysis of the FTIR data showed partially overlapping spectral feature between the three genotypes. However, the six FTIR-based markers developed in the study presented in chapter 1, together with LDA data detected contrasting metabolome behaviors between the three genotypes, demonstrating the capacity of FTIR-chemometrics approach in differentiating genotypes, environment, and their combination thereof.

The FTIR-chemometrics described above showed a wide range of metabolome changes in wheat leaves under heat stress; some of them were commonly observed in three wheat genotypes, while others were genotype-specific. The former example includes the markers Fm482 and Fm1502, which were reduced in all genotypes, indicating similar chemical response between these genotypes. Wavenumber 482 cm^{-1}, a target wavenumber for the marker Fm482, was positioned outside the" fingerprinting region" and was related to a methoxy group (472/475 cm^{-1}) and S–S stretching (450–550 cm^{-1}). The latter annotation may be related to a previously reported heat-induced protein disulfide isomerase, which promotes covalent cross-linking of sulfhydryl groups of cysteine residues, leading to stabilization of the structure of cellular proteins under heat stress. The Fm1502 marker is potentially annotated to lignin, suggesting that physicochemical modifications in cell wall compositions may occur under heat stress in these wheat genotypes.

This study identified several FTIR markers that showed differential behaviors between genotypes. The Fm1465 marker, which may be associated with suberin/cutin, lipids, and/or cell wall polysaccharides, elevated under heat stress in CS and N61, but reduced in the Imam genotype. The Fm576 marker increased under heat stress in CS, but decreased in N61, and was statistically unchanged in Imam. No sufficient information on the assignment of the wavenumber 576 cm^{-1} to chemical structures are available, except for carbon halogen stretching (400–800 cm^{-1}), P=S stretching (500–850 cm^{-1}), and P–Cl stretching (300–600 cm^{-1}). These observations suggested that biochemical responses to heat stress may be largely different between these genotypes.

It noteworthy that, the markers Fm1251 and Fm1729 showed different responses between heat-tolerant and -susceptible genotypes. The Fm1251 marker, which is related to hemicellulose and/or pectin, decreased under heat stress in the heat-tolerant Imam and N61 genotypes, while it increased in the heat-sensitive CS genotype. Those signs may indicate that chemical modification in the extracellular matrix, which potentially functions as a controller for cell wall porosity and heat conductance, are contrastingly different between heat-tolerant and susceptible genotypes. The Fm1729 marker, which is located in the carbonyl ester region (1720–1760 cm^{-1}) and/or its oxidized derivatives, was increased under heat stress in heat-tolerant Imam and N61 genotypes, whereas the value was unchanged in the heat-susceptible CS genotype. This spectral region provides information on the polar interfacial regions of pectin or membrane lipids. Thus, the markers Fm1251 and Fm1729 may potentially serve as tools for distinguishing heat-tolerant and susceptible wheat genotypes.

Overall, in the present study, an FTIR-based fingerprint technique was applied to characterize the metabolome response of wheat leaves to heat stress. Application of chemometrics techniques to the FTIR spectral data, especially the LDA technique, revealed specific spectral regions that may reflect metabolome changes in wheat leaves under heat stress. Several spectral biomarkers were developed that correctly reflected the heat-stress status of the leaves. Application of the developed markers to wheat genotypes with different heat tolerance abilities showed common and differential metabolomic response among genotypes. Among these biomarkers; Fm1251 and Fm1729 markers potentially discriminate heat-tolerant and -susceptible genotypes, suggesting that these markers may serve as a selection tool for heat-tolerant genotypes. Overall, the present study suggests the potential of FTIR spectroscopy, coupled with chemometrics analysis, for studying the heat-stress response and tolerance mechanisms in wheat