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# NATURAL VARIATION OF WHEAT CUTICULAR WAXES IN RELATION TO DROUGHT TOLERANCE

Bread wheat, *Triticum aestivum*, represents about 30% of the world's cereal cultivation area and provides 20% of the calories for the human population. Wheat is cultivated over 220 million ha of soil worldwide, which is often under the influence of abiotic stresses such as limited water supply, high salinity and heat that significantly impair crop's yield. It is the growing consensus among the scientific community, that the need to compensate the potential yield losses associated with these challenges could be achieved through selection and adaptation of cultivars with improved genetic potential [1]. Understanding how plant copes with the environmental challenges has a vital significance for improvement of crop tolerance and yield. Plant cuticle, a continuous protective sheet that covers aerial surfaces of plant organs has evolved as an exterior extension of epidermal cell walls. The biochemical composition of cuticle is not only species/cultivar specific, but differs also between organs of the same species and is modulated by environmental conditions, defining the plant tolerance level to drought and excessive UV radiation. While there is a range of scientific indications about a connection of plant cuticle with plant stress tolerance, these data, obtained mostly on model plant *Arabidopsis* [2]. As a consequence, little is known about the biochemical details of cuticle composition and regulation in wheat and its relation to drought. The new knowledge on the biodiversity in cuticle composition of wheat varieties, the cuticle composition changes during the drought stress and particularly, the differences between tolerant and intolerant varieties, would be a great support for the wheat breeding programmes.

## Materials and methods

**Plant material.** Wheat plants for all experiments were grown from seeds available at the ACPFG collection in greenhouse under well-watered and drought conditions in large containers. Containers were equipped with an automatic watering system and four soil water tensiometers were installed at 0.1 and 0.3 m soil depths, and connected to a data logger for continuous monitoring of soil water tension. **Cuticular wax metabolomics.** For wax component analysis, 6.5 cm long flag leave blades from the base at 24 days after anthesis were used. Leaf samples were weighted before placing into liquid nitrogen, after which samples were stored at –80 freezer until wax extraction with chloroform or hexane. Following extraction, waxes were dried using stream of nitrogen and redissolved in a small amount of n-hexane prior to analysis using gas chromatography-mass spectrometry. Mass spectra of eluted waxes were identified using commercial mass spectra library NIST08 (http:/www.nist.gov) and the in-house Metabolomics Australia (Schholl of Botany, Melbourne) mass spectral library. **Scanning electron microscopy (SEM).** Flag leaf blades collected at 10 days after anthesis were examined using Philips XL30 Field Emission Scanning Electron Microscope, equipped with a Gatan CT1500 HF Cryotransfer Stage (Adelaide Microscopy). Samples attached to the holder were frozen in nitrogen slush, and transferred under vacuum to the preparation chamber where it was coated with platinum under low temperature of –110 °C. It was then loaded onto the microscope chamber (held at a temperature lower than –150 °C) and examined. **Gene expression analysis.** Wheat genes coding for 10 cuticle biosynthesis related transcription factors were amplified by PCR from cDNA of wheat cv RAC875 using homology cloning based on previously characterized genes in Arabidopsis, Medicago and maize. Following cloning and sequencing the confirmed wheat gene sequences were used to design specific primers for gene expression analysis by quantitative RT-PCR using a series of cDNA from different tissues of T. aestivum cv. Chinese Spring available at the ACPFG qPCR facility.

**Results and discussion**

Due to recent technological advances in plant genomics and metabolomics, cuticle emerged as a crucial barrier that control water lose and help plant to survive under drought and high UV radiation conditions [3]. Accumulation of epicuticular wax on plant surfaces often results in a bluish-white coloration termed glaucousness (fig. 1 A), which is the visible form of densely distributed epicuticular wax crystals. Glaucousness increases light reflectance and reduces leaf temperatures and transpiration, thereby enhancing leaf survival under water stress and improving water use efficiency [4]. As a classical genetic marker and agricultural trait, glaucousness has been intensively studied in association with drought/heat tolerance and yield in Australian wheat varieties [5, 6]. However, the precise value of this trait in biomass production and grain yield remains somewhat uncertain because of the complex biochemical/genetic nature of the phenotype. Based on solubility in organic solvents, the cuticle components can be divided into insoluble cutin and soluble cuticle waxes. Intracuticular wax is embedded in the underlying cutin framework and epicuticular wax is overlaid on the cutin matrix and intracuticular wax.

The waxes are typically a complex mixture of derivatives of very-long-chain (24–34 carbons) saturated fatty acids, such as alkanes, aldehydes, ketones, primary alcohols, and secondary alcohols [2]. Fig. 1 B schematically summarizes the gene networks and biochemical pathways responsible for biosynthesis of major cuticle wax species which have been deciphered through characterization of plant mutants, mostly in Arabidopsis, barley and maize.

In order to get insights into the nature of cuticle components variations in spring wheats in relation to drought tolerance, we initiated a project on targeted/quantitative analysis of cuticle lipophilic waxes and the genes related to wax biosynthesis. Primarily, five elite Australian wheat varieties: Kukri, Excalibur, RAC875, Gladius and Drysdale, which are under intensive genetic [5, 6] investigation related to drought tolerance at the ACPFG, have been included in the project. Earlier comparative metabolite of Kurki (nonglaucous, drought intolerant, high quality grain) vs Gladius, Excalibur and RAC875 (all are glaucose, drought tolerant cultivars) revealed significant differences in their stress reactions. Other wheat cultivars which have been included in the analysis are commercial varieties cultivated in different regions of Australia: Alsen, Baxter, Berkut, Chara, Pastor, Volkani, Westonia and Wialkat [8]. Altogether, twelve Australian wheat cultivars with different levels of tolerance to drought and heat were compared for cuticle structure properties (Light and Electron Microscopy) and wax composition (GC–MS). Metabolomics analyses of chloroform and hexane extracted waxes using GC–MS demonstrated significant quantitative differences between drought-sensitive and drought-tolerant cultivars in

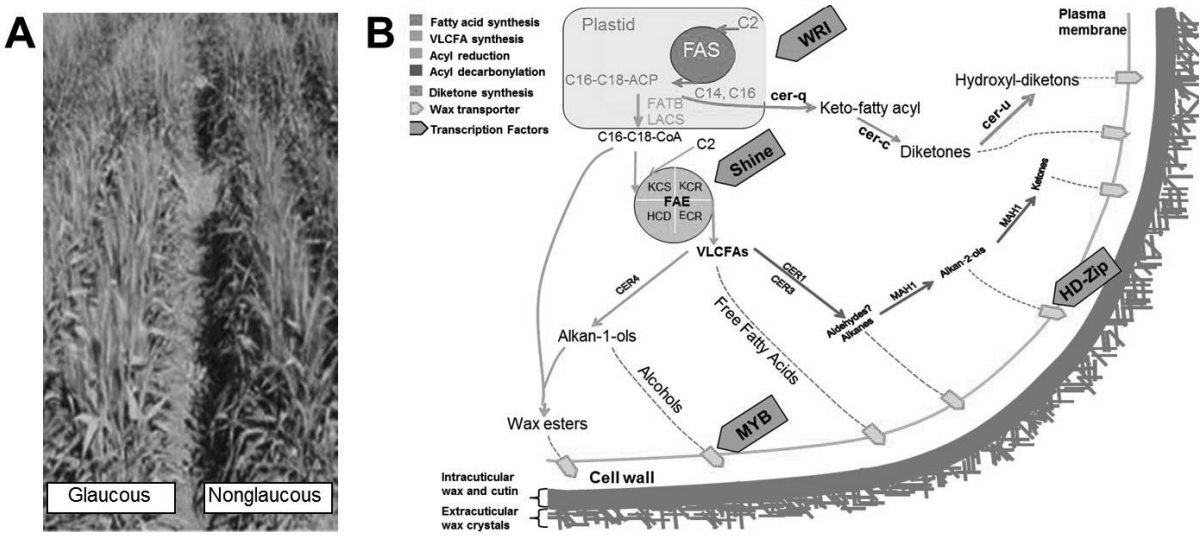


**Fig. 1.** Glaucous (waxy) and non-glaucous (waxless) wheat varieties (**A**), and schematic overview of pathways involved in cuticle wax biosynthesis (**B**). CER1–4: cuticular wax mutations; FAS: Fatty Acid Synthase complex; FAE — Fatty Acid Elongation complex; WRI, Shine, MYB, HD-Zip — Transcription Factors involved in biosynthesis pathways (reviewed in Borisjuk et al., 2014) [7]

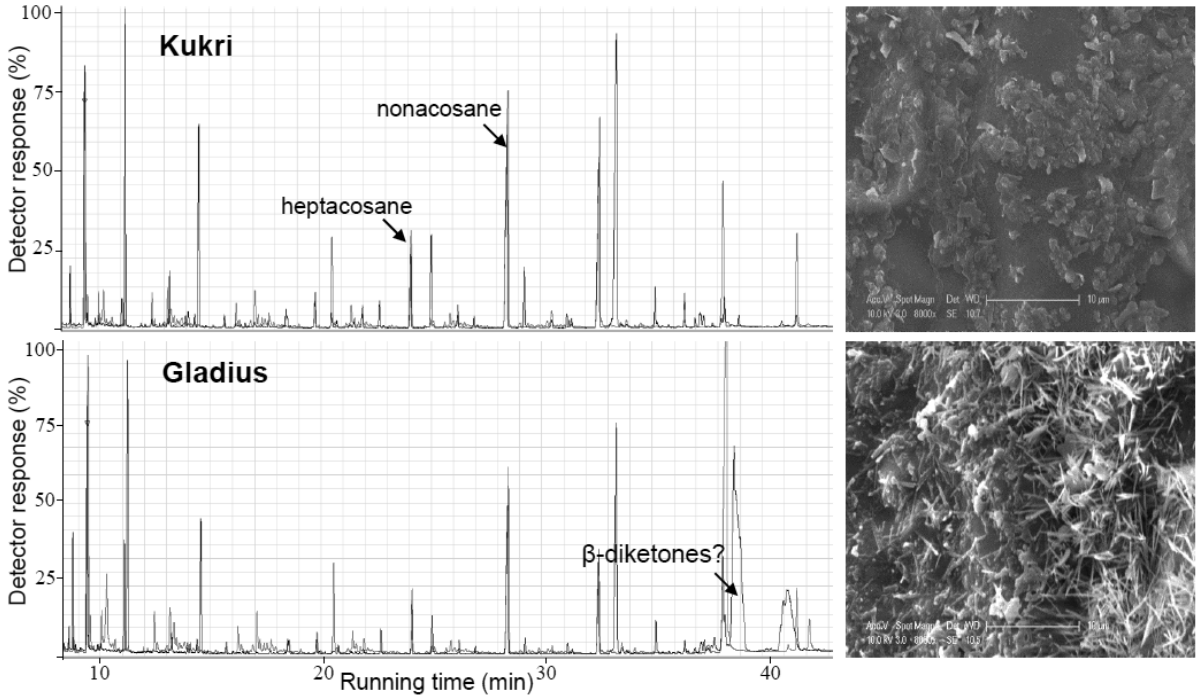


**Fig. 2.** GC–MS chromatograms of wax components and SEM image of flag leaf surface crystals in wheat cultivars Kukri (waxless, not tolerant; top) and Gladius (glaucous, drought tolerant; bottom)

several types of wax components. Table 1 represents the cumulative relative amounts (chloroform plus hexane extraction) of wax component in the analysed cultivars compared to cv. Kukri. While the majority of wax components, many of them still not properly identified, are relatively even represented in all analysed cultivars within two-folds range of variation, the unidentified components 7–9 show much higher representation in most cultivars (up to 186.9 fold difference for unknown 8 in cv Gladius) compared to Kukri. The chromatogram in fig. 2, where the peaks between 36 and 43 min represent unknown components 7–9 in the table 1, further details the cuticular wax components difference between Kukri and Gladius. While we were not able to precisely identify the 36–43 min components, there are good reasons to assume that those components belong to beta-diketones, which has been recently identified as key cuticle compounds contributing to wheat drought tolerance [9]. The revealed variation in wax component biochemistry is also translated in the drastic difference of the morphology of leaf surface wax crystal as revealed by SEM (fig. 2, right block).

The SEM has been useed to assess cuticular waxes in cv Gladius grown in greenhouse under well watered and drought conditions. As evident from fig. 3, the load of wax crystals on wheat leaves grown under drought is higher compared to well watered plants. The wax crystals under drought also are bigger and are differently shaped. The detailed comparative metabolomics analysis of waxes extracted from well-watered and drought treated plants is in progress.

Twelve Australian wheat cultivars with different levels of tolerance to drought and heat were compared for cuticle structure properties (SEM) and wax composition (GC–MS). Metabolomics analyses of chloroform and hexane extracted waxes using GC–MS demonstrated significant quantitative differences between drought-sensitive and drought-tolerant cultivars in several types of wax components. Acquired data are currently being used for identification of enzymes and upstream regulatory genes responsible for the biosynthesis of cuticular components.

**Conclusions**

Abiotic stresses such as drought, heat and high UV-irradiation adversely influence crop growth and productivity. While the nonglaucous phenotypes dominate among wild wheat ancestors, glaucousness positively correlates with yield in cultivated wheat varieties, especially under drought conditions. The trait depends on cuticle wax composition. Using GC–MS analysis we have identified wheat cuticle

*Table*

**Comparison of wax components in 12 selected Australian cultivars**

| Cultivar/ Compound | Kukri | Gladius | RAC 875 | Excalibur | Alsen | Baxter | Chara | Pastor | Volcani | Westonia | Wildkat | Drysdale |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| IS Nonadecane | 1 | 0.9 | 1.0 | 1.0 | 1.2 | 1.1 | 1.1 | 1.1 | 1.0 | 1.0 | 1.5 | 1.0 |
| Alkane-1 | 1 | 1.2 | 1.2 | 0.8 | 1.0 | 1.7 | 0.8 | 0.9 | 1.7 | 0.9 | 1.4 | 1.4 |
| Alkane-2 | 1 | 1.2 | 1.2 | 0.8 | 1.0 | 1.7 | 0.8 | 0.8 | 1.7 | 0.9 | 1.4 | 1.4 |
| Octanoic Ac* | 1 | 1.5 | 1.6 | 1.1 | 2.2 | 4.4 | 0.2 | 1.2 | 5.3 | 3.4 | 3.7 | 1.3 |
| Dodecanoic Ac | 1 | 0.6 | 0.7 | 0.9 | 0.4 | 0.9 | 0.7 | 0.4 | 1.1 | 0.7 | 0.7 | 1.4 |
| Alkane-3 | 1 | 1.2 | 1.2 | 0.8 | 1.0 | 1.7 | 0.9 | 0.9 | 1.5 | 0.8 | 1.3 | 1.5 |
| Aconitic Ac | 1 | 1.2 | 1.0 | 0.8 | 4.3 | 5.4 | 1.0 | 1.8 | 5.4 | 7.7 | 3.5 | 2.8 |
| Azelaic Ac | 1 | 1.4 | 1.6 | 1.2 | 1.0 | 2.2 | 0.3 | 0.9 | 2.8 | 1.7 | 1.8 | 1.0 |
| Alkane-5 | 1 | 1.2 | 1.2 | 0.8 | 1.2 | 1.6 | 1.3 | 1.1 | 1.4 | 1.2 | 1.6 | 1.5 |
| Alkane-6 | 1 | 1.0 | 1.2 | 1.0 | 0.8 | 1.0 | 0.1 | 0.7 | 1.3 | 0.8 | 0.9 | 1.6 |
| Unknown-1 | 1 | 1.2 | 0.7 | 0.3 | 1.1 | 1.4 | 0.6 | 1.1 | 1.3 | 3.3 | 1.2 | 1.0 |
| Linoleic acid | 1 | 1.9 | 0.6 | 0.3 | 5.1 | 7.9 | 0.2 | 2.9 | 5.6 | 12.1 | 5.8 | 2.8 |
| Unknown-2 | 1 | 0.9 | 0.9 | 0.6 | 3.2 | 1.0 | 0.5 | 2.2 | 1.0 | 2.8 | 1.2 | 0.9 |
| Octadecanoic Ac | 1 | 1.0 | 1.2 | 1.0 | 0.7 | 1.1 | 0.8 | 0.6 | 1.1 | 0.9 | 1.1 | 1.1 |
| Alkane-7 | 1 | 0.9 | 1.0 | 0.7 | 1.3 | 1.5 | 1.6 | 1.0 | 1.4 | 1.3 | 1.4 | 1.1 |
| Unknown-3 | 1 | 1.0 | 1.1 | 1.2 | 1.3 | 1.2 | 0.6 | 1.2 | 1.2 | 1.0 | 1.1 | 1.1 |
| Alkane8 | 1 | 0.9 | 1.2 | 1.3 | 1.8 | 2.6 | 1.7 | 1.4 | 1.7 | 1.3 | 1.6 | 1.5 |
| Docosanoic Ac | 1 | 1.1 | 1.8 | 0.9 | 1.4 | 1.5 | 0.7 | 1.4 | 1.5 | 1.4 | 1.6 | 1.5 |
| Heptacosane | 1 | 0.9 | 1.3 | 1.1 | 1.4 | 1.8 | 1.8 | 0.9 | 1.8 | 1.8 | 1.7 | 1.6 |
| Tetracosan-1-ol | 1 | 0.8 | 0.7 | 0.2 | 0.7 | 1.4 | 0.5 | 0.5 | 1.5 | 1.1 | 1.5 | 0.5 |
| Octacosane | 1 | 1.0 | 1.2 | 1.0 | 1.6 | 2.6 | 3.8 | 1.2 | 2.4 | 3.0 | 1.8 | 1.6 |
| Hexacosanol | 1 | 0.4 | 1.2 | 1.1 | 1.3 | 3.3 | 0.8 | 2.0 | 1.1 | 0.7 | 1.8 | 1.0 |
| Hexacosanoic Ac | 1 | 1.3 | 1.4 | 1.3 | 1.5 | 1.3 | 0.3 | 0.9 | 1.0 | 0.7 | 1.4 | 1.0 |
| Unknown-4 | 1 | 1.0 | 1.8 | 1.1 | 1.4 | 1.3 | 0.2 | 0.9 | 1.2 | 1.1 | 1.6 | 1.1 |
| Hentriacontane | 1 | 0.5 | 1.2 | 0.4 | 1.6 | 2.8 | 3.7 | 1.6 | 2.6 | 1.2 | 2.3 | 1.5 |
| Octacosanol | 1 | 1.1 | 1.3 | 1.0 | 1.0 | 1.0 | 0.6 | 0.7 | 1.0 | 0.7 | 1.5 | 1.0 |
| Octacosanoic Ac | 1 | 1.2 | 1.4 | 1.0 | 0.9 | 0.8 | 0.2 | 0.6 | 0.7 | 0.9 | 1.3 | 0.9 |
| Unknown-5 | 1 | 0.9 | 1.1 | 0.6 | 0.9 | 1.2 | 0.2 | 0.7 | 0.9 | 1.0 | 1.5 | 0.6 |
| Unknown- 6 | 1 | 1.7 | 0.9 | 1.4 | 1.1 | 1.7 | 0.1 | 1.2 | 1.2 | 0.6 | 1.3 | 1.0 |
| Dotriacontane | 1 | 0.6 | 1.2 | 0.5 | 1.4 | 3.6 | 2.7 | 1.5 | 1.0 | 1.1 | 2.5 | 1.7 |
| Triacontanol | 1 | 0.8 | 1.0 | 1.0 | 0.7 | 0.9 | 0.3 | 0.5 | 0.8 | 0.5 | 1.0 | 0.7 |
| Unknown- 7 | 1 | 33.9 | 8.8 | 1.4 | 2.2 | 0.8 | 6.1 | 33.4 | 0.2 | 1.5 | 1.4 | 12.1 |
| Unknown-8 | 1 | 186.9 | 26.2 | 2.7 | 3.7 | 0.7 | 39.1 | 146 | 0.0 | 1.4 | 0.7 | 49.2 |
| Unknown-9 | 1 | 176.8 | 28.5 | 2.5 | 1.4 | 0.6 | 44.2 | 40.1 | 0.0 | 1.1 | 0.1 | 13.6 |
| Tricontanoic Ac | 1 | 1.4 | 1.2 | 1.0 | 0.8 | 0.6 | 0.2 | 0.7 | 0.8 | 0.9 | 1.1 | 0.8 |

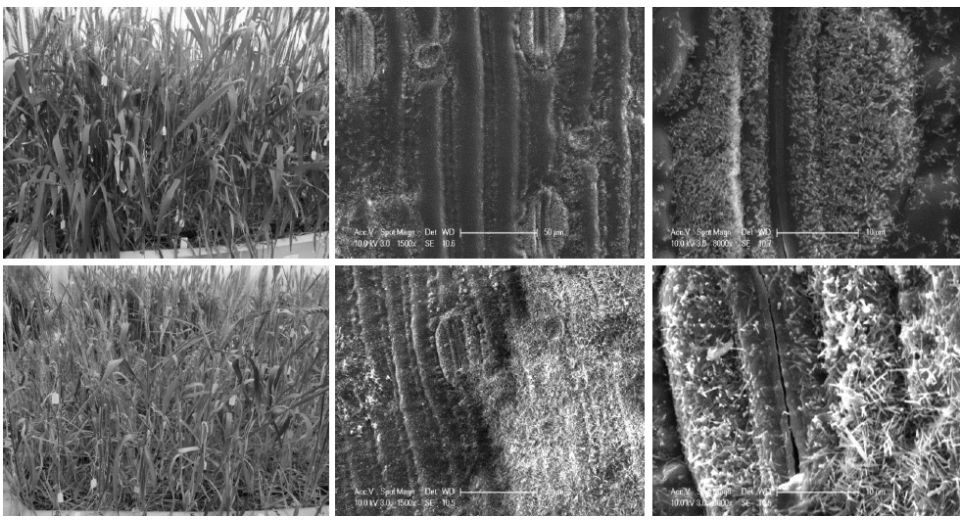

**Fig. 3.** Cryo-scanning electron micrographs of the abaxial side of the wheat flag leaf (cv. Gladius) grown under well watered (top) and drought conditions (bottom)

wax components that are present in drought resistant wheat cultivars and absent in the sensitive cultivar. Most likely these components represent beta-diketones which have been previously suggested to affect drought tolerance in wheat. The variation in wax components composition define shape of wax crystals on leaf surface as revealed by scanning electron microscopy. A detailed biochemical and molecular biology study of wheat cuticular wax regulation in relation to drought tolerance is in progress.

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**NATURAL VARIATION OF WHEAT CUTICLE WAXES IN RELATION TO STRESS TOLERANSE**

***Aims.*** The aim of our research is to investigate the role of wheat leaf cuticle in drought and heat protection by characterizing natural variability of cuticle components and revealing genetic and biochemical background of this variability in Australian wheat varieties. ***Methods.*** Cultivars with different levels of drought tolerance were analyzed for cuticle wax composition by gas chromatography (GC–MS) and for cuticle structure properties by scanning electron microscopy (SEM). ***Results.*** Metabolomics analyses demonstrated significant quantitative differences between drought-sensitive and drought-tolerant cultivars in several types of wax components. These data are complemented by differences in cuticle structure revealed by SEM. Ten wheat genes potentially involved in regulation of cuticle biosynthesis have been cloned and analysed for expression profiles in wheat by RT-qPCT. ***Conclusions.*** Acquired data are currently being used for identification of enzymes and genes responsible for drought-related cuticular components.

*Keywords*: wheat, drought tolerance, cuticle waxes, gene expression.