DFG-Project report

1. Allgemeine Angaben:

DFG Geschäftszeichen: WE1641/19-1

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Thema: Genetic and metabolic control of grain size and weight in wheat.

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Publikationsliste:

a) Wu, B., Andersch, F., Weschke, W., Weber, H., Becker, JS. (2013) Diverse accumulation and distribution of nutrient elements in developing wheat grain studied by laser ablation inductively coupled plasma mass spectrometry imaging. METALLOMICS 5, 1276-1284.

Saalbach, I., Mora-Ramirez, I., Weichert, N., Andersch, F., Guild, G., Wieser, H., Koehler, P., Stangoulis, J., Kumlehn, J., Weschke, W., Weber, H. (2014) Increased grain yield and micronutrient concentration in transgenic winter wheat by ectopic expression of a barley sucrose transporter. JOURNAL OF CEREAL SCIENCE 60, 75-81.

b), c), not applicable

2. Arbeits- und Ergebnisbericht

2.1 Objectives

The general aim of this project was to analyse mechanisms determining grain size and grain weight in wheat, traits of high economic relevance. It was planned to focus on two winter wheat models: a) the homozygous W6-introgression line (W-IL) and the respective basis line Prinz (Roeder *et al.*, 2008), b) the homozygous transgenic winter wheat line HOSUT overexpressing the HvSUT1 gene from barley carrying a single insert of the sucrose transporter gene HvSUT1 and the respective wild type Certo (Saalbach *et al.*, 2014; Weschke *et al.*, 2000). Results from preliminary work support the assumption that increased grain size in the W-IL line can be due to differences in the maternal tissues pericarp/integuments/nucellus. Evidence for that is the observed effect not only on grain development but also on development of vegetative organs (increased stem and rachis length in W-IL). On the other hand, the HOSUT-grains differ in grain thickness but also in grain length, which indicates major alterations during the filling phase. One important aim of this project was to confirm these hypotheses by performing:

1) A histological characterisation associated with the specification of the yield-determining traits grain length and width with emphasis on both maternal and filial seed organs.

2) A comprehensive phenotypic analysis of two contrasting wheat models displaying increased grain length and grain width.

3) An analysis of the underlying tissue-specific physiological, molecular and metabolic network of grain development of the models including transcript, metabolite and hormone profiling.

The overall aim was to get a deeper understanding of the complex network underlying specification of grain size and weight and to highlight the significance of maternal and filial effects on thousand grain weight in wheat. Furthermore, we expected to identify new genes relevant to the traits grain length and grain width.

2.2 Entwicklung der durchgeführten Arbeiten (Development of the proposed work)

The main problem was that initially the project was outlined to be executed by two PhD students and one technician. Originally it was planned to divide the foreseen experiments in such a way that one candidate would concentrate on the histological and molecular biological studies (WP1, WP4) whereas the other candidate should concentrate mainly on transcript and metabolite analysis (WP2-3). However, only the position for one PhD student was granted and the foreseen schedule was therefore no longer possible. Franka Andersch was employed on the granted position for three years. Therefore, due to restricted time and personnel resources several parts of the project could not be performed as planned. Concerning the histological analysis (WP1) both models. HOSUT/Certo and W-IL/Prinz were analysed. However, only the model HOSUT/Certo could be subjected to the planned transcriptome analysis. In addition, also the planned metabolic and hormone profiling (WP3, 4, 5) could not be performed so far, for reasons outlined above. A further delay in the planned schedule arose due to the insufficient annotation of the 60K wheat array. Since a satisfiable annotation for wheat was not available we cooperated with Klaus Mayer/Helmholtz Center München. So far, the final evaluation of the transcript data is still underway.

2.3 Histological and physiological analysis of seed model development, histological analysis, seed composition analysis (WP 1)

A barley cDNA encoding HvSUT1 (Weschke *et al.*, 2000) under the control of the barley Hordein B1 promoter and the Hordein B1 terminator (Fig. 1a), was expressed in the winter wheat variety Certo via *Agrobacterium* mediated transformation. The marker gene, initially required for the transformation procedure, was removed from the lines by segregation. Three independent transgenic lines were characterised with respect to yield-related parameters in small greenhouses, in soil under normal stands density in micro-plots (0.5 m^2, 200 plants), (Fig. 1b). Cultivation of three independent lines, grown in three consecutive years, in total 28 plots grain revealed a large yield increase by around 20 % compared to Certo, which was largely due to an increased thousand grain weight but also to a higher ear number per plant. However, grain number per ear was lower. Whereas the percentage of nitrogen was not significantly different the nitrogen yield per plot was around 20 % higher due to the higher grain yield (Fig. 1c).

Yield-related parameters and growth characteristics

Frequency distribution of mature grain weight, length and width (Fig. 1c, d) were determined for HOSUT24 and Certo. The distribution of these parameters does not follow a Gaussian distribution and frequency classes of lower values are overrepresented. While the general distribution between HOSUT24 and Certo grains is similar, the means and medians for grain weight, length and width are shifted to higher values for HOSUT24 when compared to Certo.

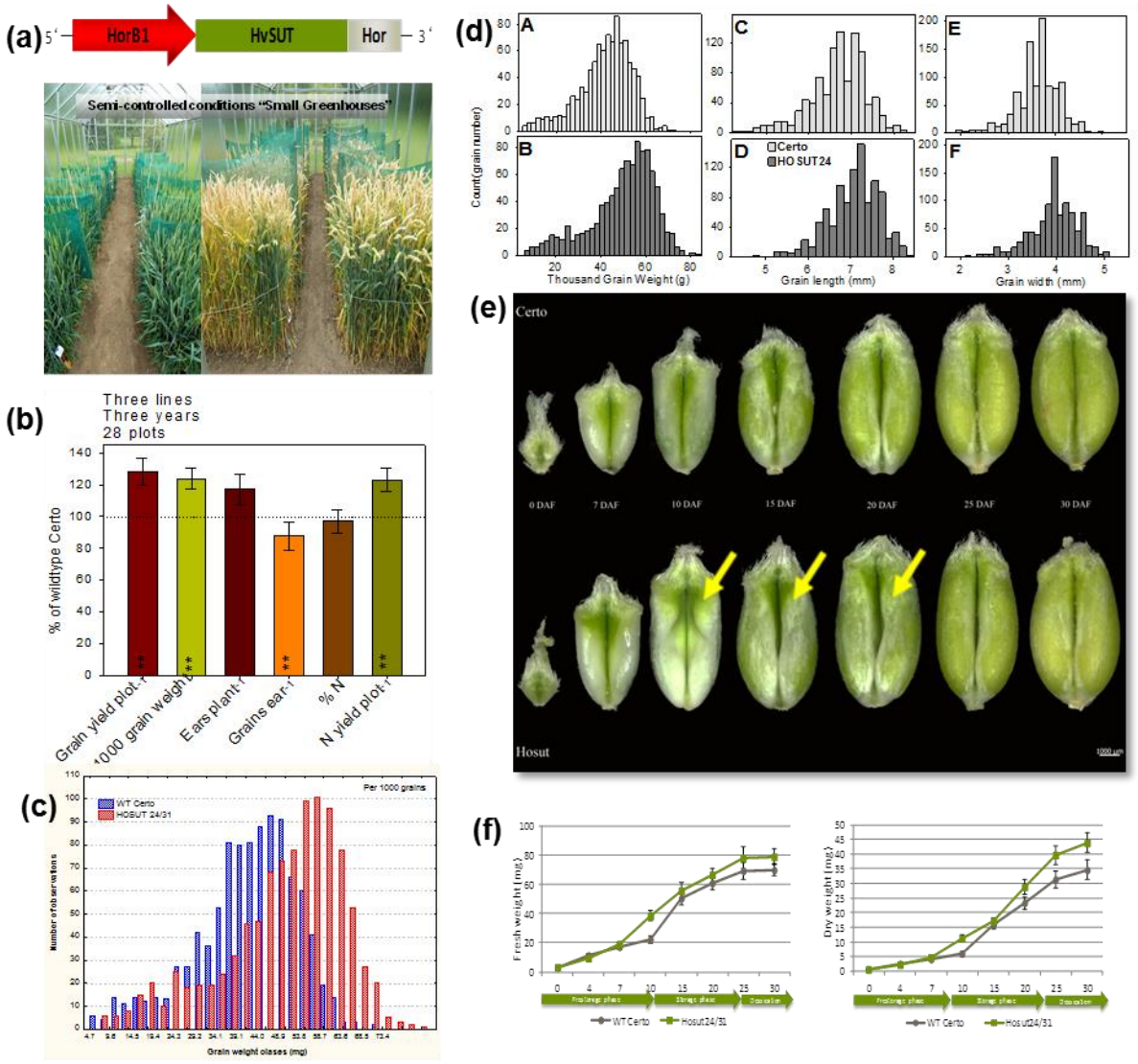


Fig. 1, Yield-related parameters and grain growth characteristics of HOSUT-lines; (a), construct used for plant transformation, (b), yield-related parameters, (c), distribution of grain weight classes for HOSUT24 and Certo, (d), shape of mature grains of HOSUT24 and Certo, (e), caryopses shape of HOSUT and Certo during development, (f), fresh/dry weight accumulation of grains from HOSUT24 and Certo.

Fig. 1e shows the shape of developing caryopses of HOSUT24 and Certo and reveals potential differences in the size of the lobes of the endosperm, which seem to be larger for HOSUT caryopses, beginning at 10 DAF (arrows). Fresh/dry weight accumulation is shown in Fig. 1f, for grains from HOSUT24 and Certo, and reveals higher values for the HOSUT lines staring at 10 DAF, the beginning of the storage phase.

Developing caryopses of HOSUT and Certo at 4, 7, 10 and 20 DAF were wax-embedded and sectioned in the transversal direction (Fig. 2a). A comparison revealed very minor changes at 4 and 7 DAF whereas at 10 DAF the lobes of the pericarp are more extended and stretched. This was also evident at 20 DAF. These results indicate a stimulated endosperm development in HOSUT caryopses, especially of the lobes, compared to Certo. The cross-sections were used to measure the areas of total caryopses, pericarp and endosperm (Fig. 2b) at 4, 7, 10 and 30 DAF. Caryopses cross-section area is not different at 4 and 7 DAF but is increased by around 20 % at 10 and 20 DAF for HOSUT. The pericarp areas are higher at 4 and 7 DAF for HOSUT but are lower at 10 and 20 DAF. Endosperm areas are lower for HOSUT at 4 and 7 DAF but are higher at 10 and 20 DAF. Caryopses length, width and thickness were determined along

development (Fig. 2c). The results show that caryopses length is higher in HOSUT at all stages from 7 to 20 DAF. Width is higher at 7, 10 and 30 DAF but is not different between 10 and 25 DAF. Caryopses thickness in HOSUT is increased at all stages between 7 and 30 DAF.

In summary, these results indicate an altered development of HOSUT caryopses compared to Certo. Obviously, endosperm growth in HOSUT is delayed at the early prestorage phase but is increased during the grain filling stage. Stimulated growth potentially applies to the endosperm and the especially lobes of the endosperm. This is also confirmed by the fact that caryopses thickness rather than width is higher in the HOSUT caryopses.

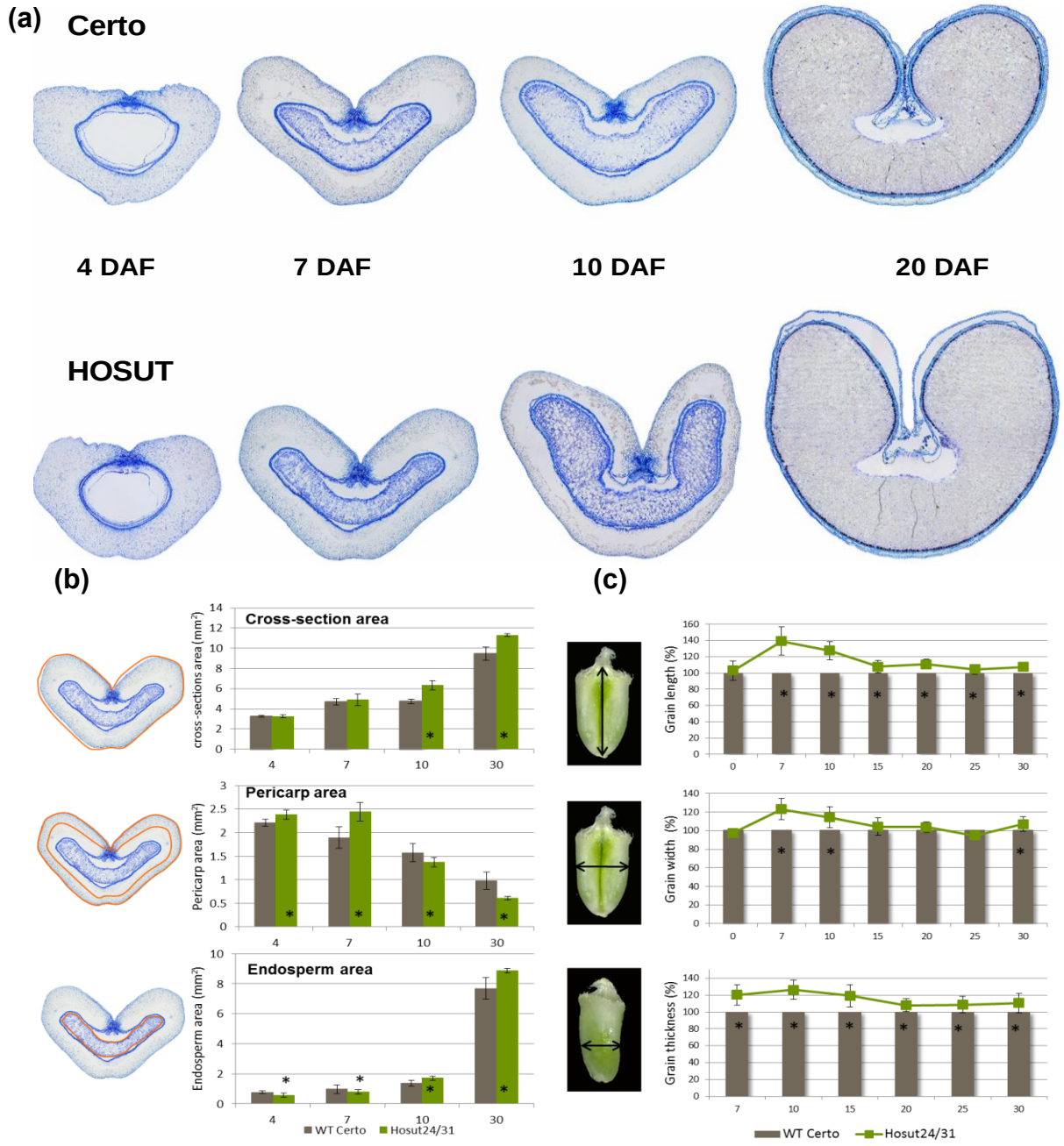
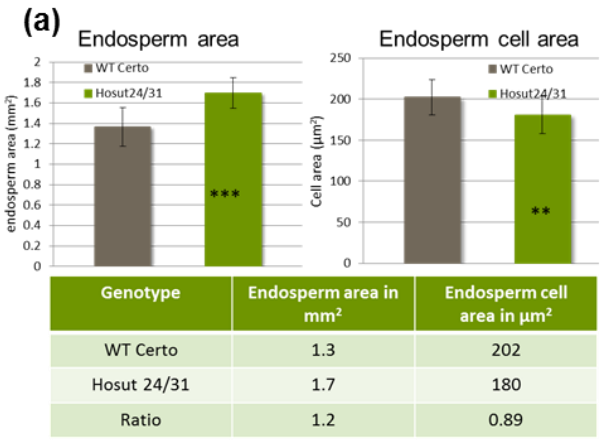


Fig. 2, Growth characteristics of developing caryopses of line HOSUT24 and Certo; (a), cross-sections of caryopses in the transversal direction, (b), areas of total caryopses, of pericarp and of endosperm, (c), caryopses length, width and thickness, determined along development. For (b) and (c), n = 10, *, significant differences after T-Test, $P < 0.05$.

Endosperm area was determined along with endosperm cell area in equal regions of developing endosperm of the line HOSUT24 and Certo at 20 DAF (Fig. 3a). Endosperm area in HOSUT is significantly higher by around 20 % compared to Certo whereas the endosperm cell area is significantly lower in HOSUT by around 10 %. This indicated that the HOSUT endosperm at 20 DAF contains smaller but a higher number of cells.

Cell number and endosperm cycle value was analysed using fluorescence-based flow cytometry. Cell number was directly measured by fluorescence staining and by counting of cell nuclei preparations of the endosperm in cooperation with Jörg Fuchs, IPK Gatersleben. The profiles of cell elongation during endosperm development can be estimated by monitoring endopolyploidisation, which was measured together with that of cell number using the flow cytometer (Fig. 3b). The results show that in HOSUT endosperm the cell number is higher from around 12 DAF (only significant at 20 and 25 DAF). Endopolyploidisation, measured as endosperm cycle values, is significantly lower for HOSUT in almost all stages and especially between 10 and 20 DAF. These results indicate that the increased endosperm size in HOSUT probably results from an increased endosperm cell number. Moreover, endopolyploidisation, which is a direct measure for cell elongation and the degree of differentiation, is lower in the HOSUT endosperm. Together the results suggest that the larger endosperm in the developing HOSUT caryopses contains a higher number of smaller cells.



Genotype	Endosperm area in mm²	Endosperm cell area in µm²
WT Certo	1.3	202
Hosut 24/31	1.7	180
Ratio	1.2	0.89

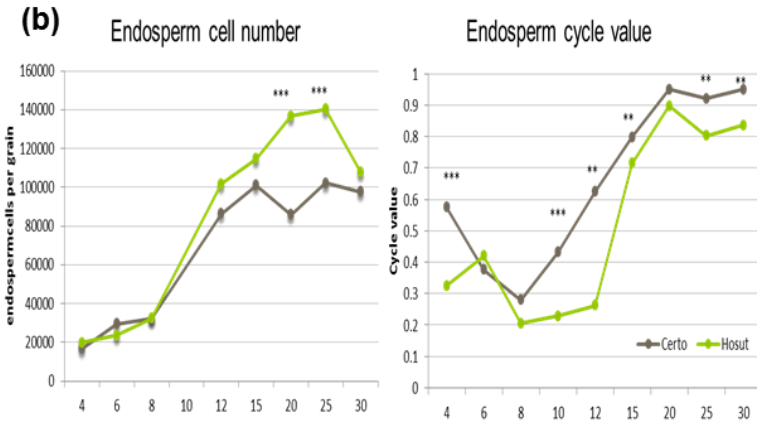


Fig. 3, Endosperm growth characteristics of line HOSUT24 and Certo; (a) endosperm area and endosperm cell area measured in equivalent regions of 20 DAF caryopses, (b), cell number and endosperm cycle values analysed using fluorescence-based flow cytometry, n = 10, significant differences after T-Test, **, P < 0.01, ***, P < 0.001.

Grain and endosperm growth characteristics of line-IL and Prinz

Fig. 4 a shows mature grains of line W-IL carrying a genomic fragment of the synthetic wheat M6 in the genetic background of the winter wheat variety Prinz. The introgression is related to a quantitative trait locus (QTL) of grain size and the QTL interval was genetically delimited by fine mapping (Roeder *et al.*, 2008). Fig. 4b shows an analysis of mature grain shape parameters of line W-IL compared to Prinz revelaling significantly increased thousand grain weight, grain area, grain length and grain width.

Developing caryopses of line W-IL and Prinz at 4, 7, 10 and 20 DAF were wax-embedded and sectioned in the transverse direction (Fig. 4c). The cross-sections were used to measure the areas of total caryopses, pericarp and endosperm (Fig. 4d). Caryopses cross-section area is higher at 4 and 30 DAF for the line W-IL compared tp Prinz. The pericarp areas are higher for the line W-IL at all stages. Endosperm areas are lower for line W-IL at 7 DAF but are higher at 30 DAF. Caryopses length, width and thickness were determined along development (Fig. 4e). The results show that caryopses length and thickness are higher

in line W-IL at nearly all stages from 4 to 30 DAF whereas width is higher in the line W-IL only at 7, 10 and 20 DAF but not different between 0 and 4 and at 25 DAF.

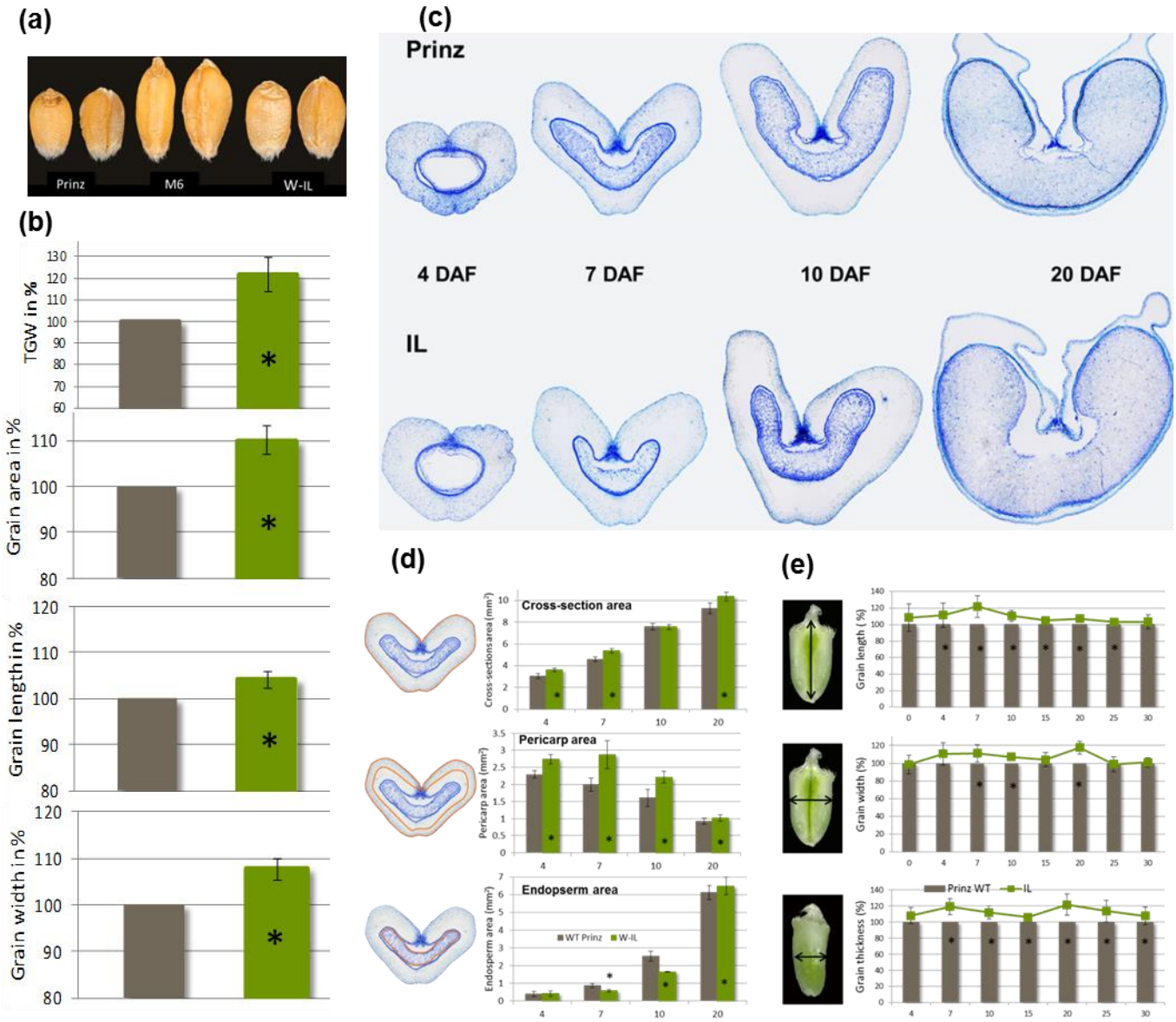
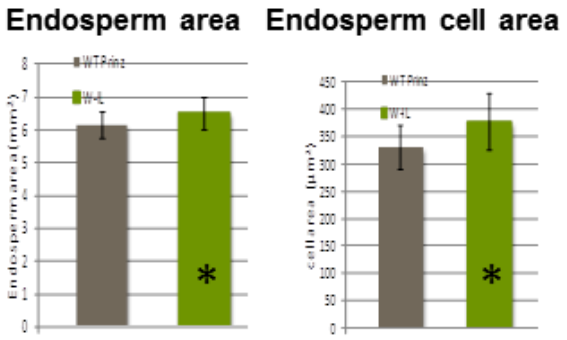


Fig. 4, Growth and grain shape characteristics of developing caryopses of line W-IL and Prinz; (a), intact mature grains, (b), mature grain shape parameters of line W-IL compared to Prinz, (c) cross-sections of caryopses in the transversal direction, (d), areas of total caryopses, of pericarp and of endosperm, (e), Caryopses length, width and thickness, determined along development; n = 10, significant differences after T-Test, *, P < 0.05.

Endosperm area was determined along with endosperm cell area in equal regions of developing endosperm of the line W-IL and Prinz at 20 DAF (Fig. 5). Endosperm area and endosperm cell area in line W-IL are significantly higher compared to Prinz. This indicated that the IL-endosperm at 20 DAF contains larger cells, which could be the reason for the larger endosperm size. The finding for the line W-IL is clearly different from the results from the HOSUT24/Certo, where the increased endosperm is probably due to a higher number of cells.



Genotype	Endosperm area in mm^2	Endosperm cell area in μm^2
WT Prinz	6.1	328
W-IL	6.5	375
Ratio	1.05	1.14

Fig. 5, Endosperm growth characteristics of line-IL and Prinz, endosperm area and endosperm cell area measured in equivalent regions of 20 DAF caryopses, n = 10, significant differences after T-Test, *, $P < 0.05$.

2.4 Transcript profiling (WP 2)

This part of the project should start after evidence was available for altered development/histology of particular seed tissues. Decisions about these particular tissues to be subjected for transcript profiling should be made from results from the histological analysis, in the case of HOSUT24 see Figs. 2 and 3. It was concluded that the inscreased grain size of HOSUT is mainly due to a higher cell number in the endosperm, especially the lobes of the endosperm. On the other hand, line W-IL probably reveals differences in the pericarp development (see Fig. 4), which could be the reason for the increased grain weight.

Micro-dissected tissue from the endosperm was chosen for a comparative transcript profiling between HOSUT24 and Certo grains and four stages of development were analysed, 4, 7, 10 and 15 DAF. Transcript profiling was performed using the custom-made 60k AGILENT wheat microchip. Array hybridisation was done in-house using the IPK facilities and the software package GeneSpring (Agilent technologies) and the methodology described earlier (Kohl *et al.*, 2015; Thiel *et al.*, 2012). Fig. 6a summarizes the general workflow of the transcript profiling procedure whereas Fig. 6b shows the filtering of differentially expressed genes.

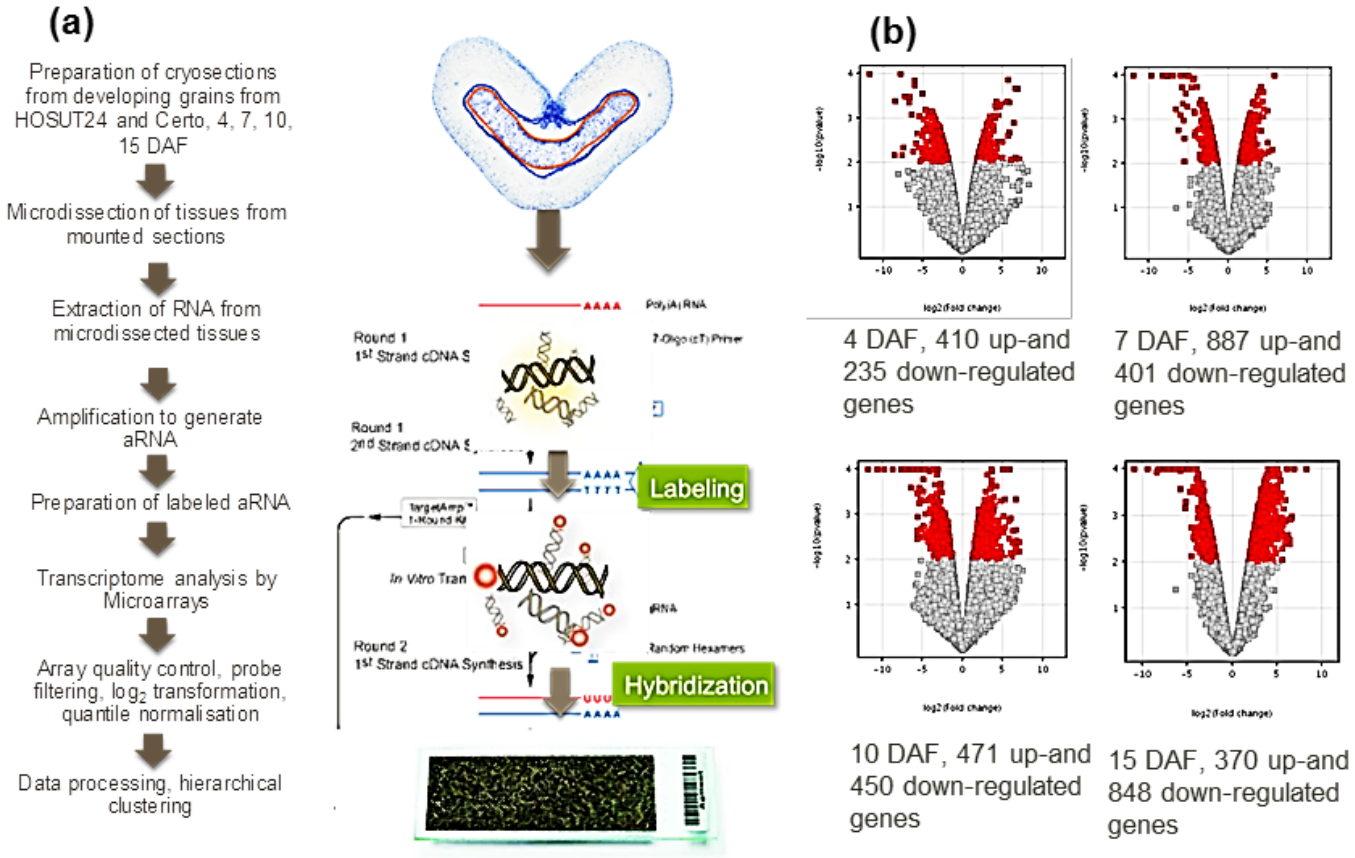


Fig. 6, Workflow and results from transcript profiling; (a), workflow of transcript profiling, (b), filtering of differentially expressed genes.

Differentially expressed genes were divided into functional categories, highlighting the biological processes, which are up- or down-regulated in the HOSUT24 endosperm compared to Certo (Fig. 7).

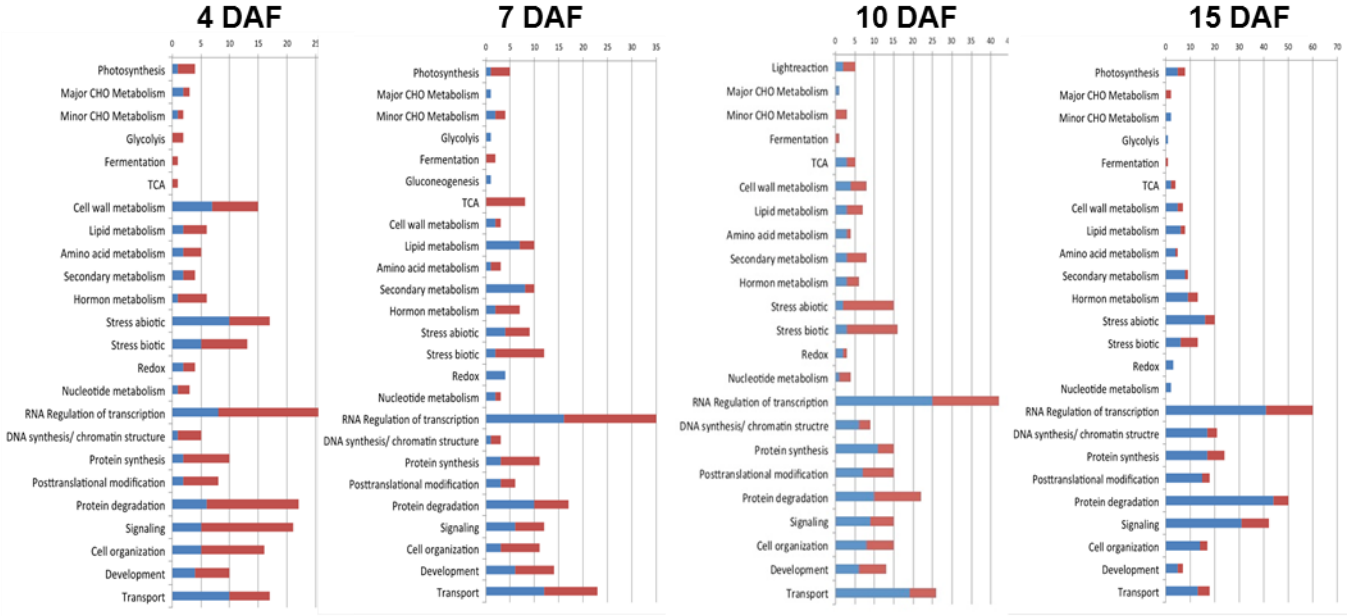


Fig. 7, Functional categories of differentially expressed genes in endosperms of HOSUT24 versus Certo, red, up-regulated in HOSUT, blue, down-regulated in HOSUT by at least the factor of two and significant differences in at least one stage with three biological replicates.

Categories of differentiall expressed genes

Data evaluation of differentially expressed genes as shown in Fig. 7 revealed that candidates are over-represented in the HOSUT endosperm at 4 DAF, which are related to early growth and development. These categories comprise RNA regulation of transcription, DNA synthesis/chromatin structure, protein synthesis/degradation, signaling, cell organisation, and cell wall metabolism. Under-represented genes at 4 DAF include the categories stress and transport. Such a distribution suggests a stimulated growth by cell division within the early endosperm of HOSUT24 compared to Certo.

At 7 DAF, there is still some over-representation of up-regulated genes in the categories early growth and development represented by the groups RNA regulation of transcription, DNA/Protein synthesis whereas other over-represented genes fall in the categories abiotic stress and TCA cycle. At 10 DAF, up-regulated genes in HOSUT are found in the groups biotic/abiotic stress, minor CHO metabolism and protein degradation. At 15 DAF, down-regulated genes are dominating in almost all categories, especially in those groups, which include genes, which are up-regulated in HOSUT24 at 4 DAF and which are related to early growth and development (see above). In summary, the distribution suggests that early cell growth processes probably are activated in the HOSUT endosperm compared to Certo.

Cell division activity and auxin/gibberellin signalling are transcriptionally up-regulated in the early HOSUT endosperm

Genes involved in cell cycle regulation are up-regulated in HOSUT endosperm at 4 and 7 DAF (Fig. 8a). This includes several types of cyclin genes, cyclin-dependent kinases and other genes associated to mitotic activity. On the other hand, a cyclin-dependent kinase inhibitor is strongly down-regulated in the HOSUT24 endosperm at 4 DAF as well as a cell-cycle switch protein, CCS 52A, which controls the switch from the mitotic cycle to endoreduplication cycle.

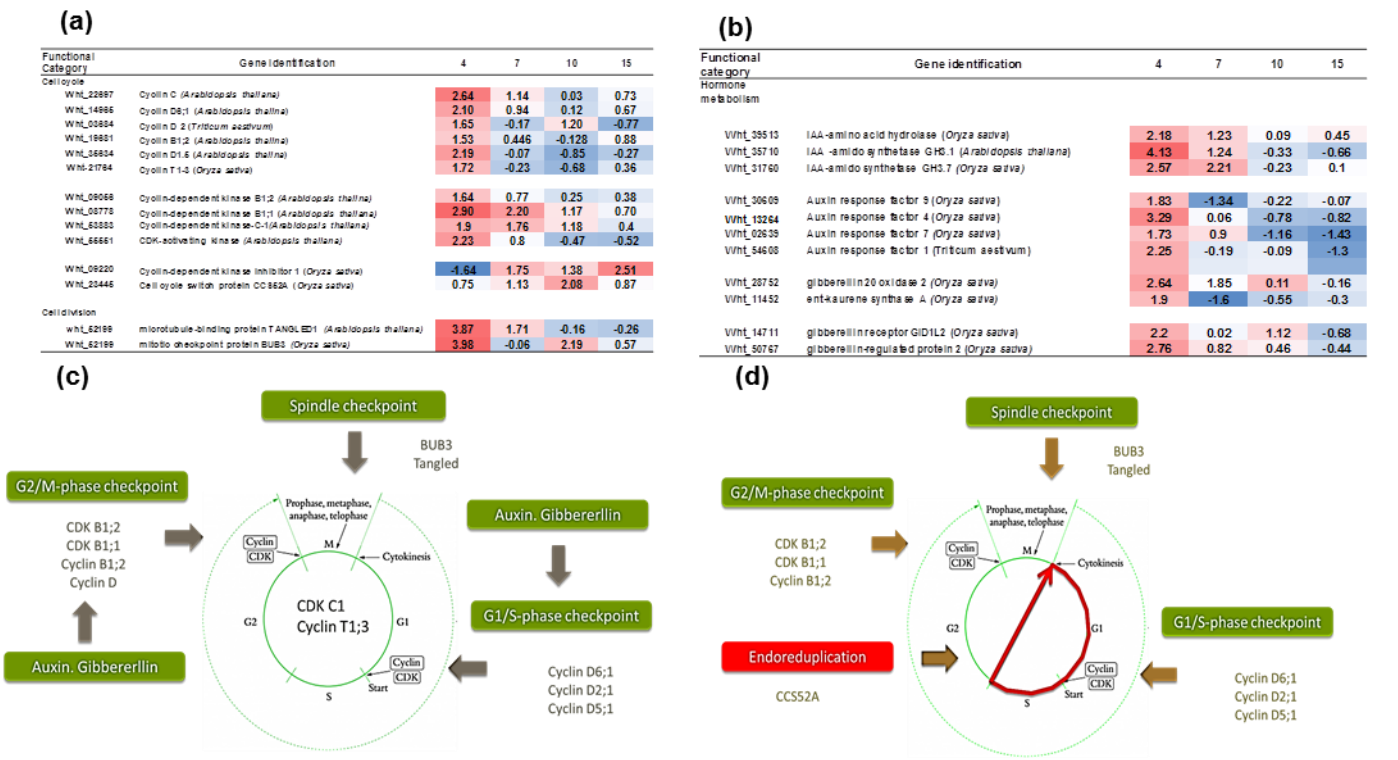


Fig. 8, Transcriptional changes in the HOSUT24 endosperm; (a), up-regulated genes involved in the mitotic cycle, (b), up-regulated genes involved in auxin and GA function, (c), possible model for the stimulation of mitotic activity in HOSUT, (d), possible model for the switch from mitotic cycle to endoreduplication in the HOSUT endosperm.

Together with mitotic activity there is transcriptional up-regulation of genes related to auxin functions at 4 and 7 DAF such as IAA-amido synthetases and several auxin response factors. In addition, gibberellin (GA) functions are also up-regulated at 4 DAF including *ent*-kaurene synthase, GA20-oxidase and GA receptor. Possibly, the concerted action of auxins and GAs could promote the cell division of HOSUT24 endosperm cells by up-regulating cell cycle regulation genes. A possible model for the stimulation of mitotic activity in HOSUT is given in Fig. 8c.

It could be hypothesised that over-expressing a sucrose transporter increase sucrose uptake capacity (Weichert *et al.*, 2010) and leads to stimulation of mitotic activity in the endosperm. Sugar effects on mitotic activity are well known and partially could be mediated by sucrose activation of cyclin D-type gene expression (Riou-Khamlichi *et al.*, 2000). Three members of D-cyclins are transcriptionally up-regulated in the earyl HOSUT endosperm (Fig. 8a).

Endocycles operate with the same machinery as the mitotic cycles except that the M-phase is omitted by inactivation of mitotic cyclin-dependent kinase (CDK) activities (De Veylder *et al.*, 2011). In this context, CCS52A apparently controls cell division and onset of endoreduplication. CCS52A is down-regulated at 4 DAF in the HOSUT endosperm and up-regulated at 10 DAF (Fig. 8a). In addition, a cyclin-dependent kinase inhibitor is roughly co-expressed with CCS52A. This suggests that in the HOSUT endosperm the switch from mitotic activity to endoreduplication occurs later compared to Certo, which is in agreement to the lower endosperm cycle level (Fig. 3b). The finding that the HOSUT24 endosperm contains smaller cells (Fig. 3a) also confirms this hypothesis. A possible model for the switch from mitotic cycle to endoreduplication in the HOSUT endosperm is shown in Fig. 8d.

Summary (Zusammenfassung)

This project aims to analyse mechanisms determining grain size and grain weight of two winter wheat models, the introgression line W-IL and the transgenic line HOSUT, over-expressing a sucrose transporter from barley. Both models display increased seed size compared to their basis lines Prinz and Certo, respectively. Increased grain size in the W-IL line probably derived from the maternal tissues pericarp/integuments/nucellus whereas the HOSUT-grains could be altered in endosperm development. These hypotheses should be confirmed in this project by applying histological characterisation, comprehensive phenotypic analysis and investigation of the molecular and metabolic network. Due to restricted time and resources, the foreseen analysis was predominantly restricted to the HOSUT/Certo model. Histological analysis shows altered development of HOSUT caryopses compared to the base line Certo. It was concluded that HOSUT endosperm growth is lower at early prestorage phases but is increased during grain filling stage. The stimulated growth potentially applies to endosperm and especially to the lobes of the endosperm. This is confirmed by the fact that caryopses thickness rather than width is higher in the HOSUT caryopses. The increased endosperm size in HOSUT probably results from an increased endosperm cell number. Moreover, endopolyploidisation, which is a direct measure for cell elongation and the degree of differentiation, is lower in the HOSUT endosperm. Together, this suggests that the larger endosperm in the developing HOSUT caryopses contains a higher number of smaller cells. For the W-IL line it was shown that its endosperm contains larger cells compared to Prinz, which is probably the reason for the larger endosperm size. Thus, the findings for the line W-IL are clearly different from the results from the HOSUT24/Certo, where the increased endosperm is probably due to a higher number of cells. Micro-dissected endosperm tissue was chosen for a comparative transcript profiling between HOSUT and Certo grains using 60k AGILENT wheat microchip. Preliminary data evaluation of differentially expressed genes reveals over-represented categories in the early HOSUT endosperm related to growth and development. Especially those genes are up-regulated, which are involved in cell cycle regulation suggesting stimulated cell division activity in the HOSUT endosperm. Transcript profiling further points to the concerted action of auxin and gibberellin functions in order to promote cell division within the HOSUT endosperm cells by up-regulating cell cycle regulation genes. Overall, it is concluded that over-

expressing the sucrose transporter increases sucrose uptake capacity in HOSUT endosperm and leads to stimulation of mitotic activity in the endosperm. Such sugar effects on mitotic activity are well known and could partially be mediated by sucrose activation of cyclin D-type gene expression. Confirmatively, three members of D-cyclins are transcriptionally up-regulated in the early HOSUT endosperm.

Primary Sources

Secondary Sources

Uncategorized References

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