Plant Science 242 (2016) 260–269

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Water uptake in barley grain: Physiology; genetics and industrial applications

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ARTICLE INFO

Article history:
Received 3 June 2015
Received in revised form 8 August 2015
Accepted 13 August 2015
Available online 18 August 2015

Keywords:
Cell walls
Mutant library
QTL analysis
Starchy endosperm
Transgenic barley

ABSTRACT

Water uptake by mature barley grains initiates germination and is the first stage in the malting process. Here we have investigated the effects of starchy endosperm cell wall thickness on water uptake, together with the effects of varying amounts of the wall polysaccharide, (1,3;1,4)-β-glucan. In the latter case, we examined mutant barley lines from a mutant library and transgenic barley lines in which the (1,3;1,4)-β-glucan synthase gene, *HvCslF6*, was down-regulated by RNA interference. Neither cell wall thickness nor the levels of grain (1,3;1,4)-β-glucan were significantly correlated with water uptake but are likely to influence modification during malting. However, when a barley mapping population was phenotyped for rate of water uptake into grain, quantitative trait locus (QTL) analysis identified specific regions of chromosomes 4H, 5H and 7H that accounted for approximately 17%, 18% and 11%, respectively, of the phenotypic variation. These data indicate that variation in water uptake rates by elite malting cultivars of barley is genetically controlled and a number of candidate genes that might control the trait were identified under the QTL. The genomics data raise the possibility that the genetic variation in water uptake rates might be exploited by breeders for the benefit of the malting and brewing industries.

1. Introduction

Commercial malting processes are initiated by immersing the grain in water. This is known as steeping and the objective is to quickly raise the moisture content of the grain to about 40% so that the endosperm mobilisation step that follows will occur more quickly. During steeping, the water is drained once to allow re-aeration of the grain. This step is known as the air rest. The grain is subsequently re-immersed in water to complete the steeping treatment, which takes about 24 h overall. Germination of the moist barley grain continues under controlled conditions and mobilisation of the starchy endosperm is allowed to proceed for approximately four days. During this period enzymes secreted from the aleurone layer and the scutellar epithelium, together with some that pre-exist in starchy endosperm cells, collectively degrade cell walls, starch and storage protein. The malt is dried and transported to the brewery, where starch degradation products and other endosperm components are extracted and further hydrolysed, prior to the fermentation of released sugars to ethanol [1].

Currently around 4000 l of water is required to make 1 metric tonne (t) of malt. For example, in Australia the malting industry produces 790,000 t of malt per year, with corresponding water use of over 3000 Ml [2]. If the rate of water uptake into grain could be increased, the enhanced efficiency of the steeping process could result in significant time, energy and water savings in the malt house as well as improved malt quality.

The cell walls of the starchy endosperm of barley grain have been indirectly implicated in the rate of water uptake during steeping. These cell walls consist of approximately 70% w/w (1,3;1,4)-β-glucan, 20% w/w arabinoxylan and smaller amounts of cellulose, mannan and protein [3]. It is generally accepted that the (1,3;1,4)-β-glucans of cell walls are undesirable in the malting and brewing

Abbreviations: DarT, diversity arrays technology; DH, doubled haploid; GBS, genotyping-by-sequencing; Mx, mutant generation x; NIR, near infrared spectroscopy; QTL, quantitative trait locus; RNAi, RNA interference; SNP, single nucleotide polymorphism; SEM, scanning electron microscopy.

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http://dx.doi.org/10.1016/j.plantsci.2015.08.009
0168-9452/

processes [1] and the possibility arose that significant reductions in or the elimination of (1,3;1,4)-β-glucan in malting quality barleys might also lead to reductions in water usage with attendant benefits through faster grain modification in the malting process.

The genes that mediate (1,3;1,4)-β-glucan biosynthesis in barley have been identified as the cellulose synthase-like *CslF* and *CslH* genes [4,5]. The *CslF* genes, of which ten are present in barley [22], are the most important for (1,3;1,4)-β-glucan biosynthesis in barley grain, based on transcript abundance [6]. A sharp peak of *HvCslF9* transcripts is detected during endosperm cellularisation, 4–8 days after pollination; these transcripts disappear by about 15 days after pollination. More importantly, significantly higher levels of *HvCslF6* transcripts appear very early in grain development and persist until much later, more than 20 days after pollination [6]. Thus, the *HvCslF6* gene appears to be the most important for (1,3;1,4)-β-glucan biosynthesis in barley grain.

In previous work we modified levels of (1,3;1,4)-β-glucan in barley grain through manipulation of expression levels of the *HvCslF6* gene. Increases in grain (1,3;1,4)-β-glucan in transgenic barley lines of up to 50% have been achieved during attempts to increase the dietary fibre content of the grain [7]. However, in the present work the objective was to generate barley lines with decreased (1,3;1,4)-β-glucan levels. The identification of mutant barley lines with essentially no (1,3;1,4)-β-glucan in their grain [8] suggested that down-regulation of the *CslF6* gene would lead to lower levels of the (1,3;1,4)-β-glucan. Similarly, Aastrup and Munck [9] generated barley mutants in which the cell walls of the starchy endosperm were markedly thinner than wild type walls, and in which (1,3;1,4)-β-glucan levels were relatively low.

These reports indicated that it would be possible to use mutant libraries or RNA interference (RNAi) techniques to select or produce barley lines with low levels of (1,3;1,4)-β-glucan and thinner cell walls, with a view to investigating the effects of these physiological and chemical properties on the rate of water uptake into the grain. The results of these experiments are outlined in detail here. In addition, we phenotyped 200 doubled haploid lines generated from the F1 of two parent lines that showed relatively large differences in their rates of water uptake and identified regions of the genome that contribute to water uptake rates.

2. Materials and methods

2.1. Isolation of mutants

An ethyl methanesulfonate (EMS) mutant population generated by Patricia Warner from the barley cultivar Flagship at the Australian Centre for Plant Functional Genomics was generously provided by Peter Langridge. Approximately 2500 individual grains from the M3 generation were assayed using a half-grain Calcofluor assay developed for this purpose [10]. After four generations of self-fertilisation, more than 83 mutant M7 plants were generated from one original M3 line with grain containing reduced (1,3;1,4)-β-glucan content. Bulk (1,3;1,4)-β-glucan analyses were performed on flour from 10 grains and cell wall content was estimated in 50 individual half-grains from the same plant using the Calcofluor assay to detect the level of segregation for the low (1,3;1,4)-β-glucan phenotype and also to select single embryo-half grains containing reduced cell wall material for planting and generation of a pure line of low (1,3;1,4)-β-glucan barley.

2.2. Vector construction for transformation

dsRNAi gene silencing techniques were used to generate transgenic barley lines in which the *HvCslF6* gene was silenced. The 5′ untranslated region (UTR) of *HvCslF6* was selected as a suitable target for gene-specific dsRNAi silencing, due to the low level of sequence conservation in this region across the *HvCslF* family members. The fragment containing the 5′ UTR was isolated from cDNA populations generated from a mixture of barley tissues, including leaf, root, coleoptile and endosperm, in PCR reactions using the Elongase Taq polymerase, (Invitrogen, Carlsbad, CA, USA) and the primers CGGCCATGGCGCCAGCGGTGG and AGCGTGAAGGCGATCAGACGA. Fragments of the correct size were inserted into the pCR®8/GW/TOPO TA vector (Invitrogen) as recommended by the manufacturer. Plasmids containing cDNA inserts were subsequently sequenced on an ABI 3700 (Applied Biosystems Inc., Foster City, CA 94404, USA) at the Australian Genome Research Facility, Adelaide, Australia.

Correct inserts were transferred into pRB506, a modified version of the Gateway-compatible hairpin vector pTOOL1 (kindly provided by Peter Langridge) using an LR clonase reaction (Invitrogen) carried out according to the manufacturer's instructions. The pRB506 vector was constructed by modifying pTOOL1 through the removal of the double CaMV 35S promoter using a double HindIII and AscI restriction digest. The remaining vector backbone was purified using a Nucleospin® column (Macherey-Nagel GmbH and Co., Germany). The oat globulin promoter, *AsGlo*, was used as a replacement because this directs grain-specific expression and silencing [7]. The 1 kb *AsGlo* described by Vickers et al. [11] was amplified with Elongase from a plasmid kindly provided by Claudia Vickers, using the PCR primers OGFH5 (GACTAAGCTTCTGGAAAGTCATTTTGCCTCCTG) and OGRA3 (GGTTGGCGCGCCGAGATTGTAGAAGGTGGATTGG) and was subcloned into the pCR8®/GW/TOPO TA vector. The correct fragment was excised by double restriction digestion with HindIII and AscI and purified using a Nucleospin® column. The purified fragment was ligated into the pTOOL1 backbone and transformed into DB3.1 *Escherichia coli* cells. The RNAi construct targeting the *HvCslF6* gene by dsRNAi was transformed into Golden Promise and the malting quality breeding line WI4330.

Agrobacterium tumefaciens-mediated barley transformation

Transgenic barley was generated using the protocol of Tingay, et al. [12], as modified by Matthews, et al. [13] described in detail by Burton, et al. [7]. Immature embryos from the barley cultivar Golden Promise and the advanced breeding line WI4330 were aseptically excised from surface-sterilized grain and the embryonic axis was removed from the embryo. The remaining immature scutella were cultured on callus induction medium [14] prior to infection with the Agrobacterium cells. The initial transformations were performed in Golden Promise, because this variety is easily transformed and was used in the original study by Burton et al. [7]. However, Golden Promise is poorly adapted to Australian growing conditions, while the breeding line WI4330 is an experimental malting variety that is well adapted to Australian conditions and which we are able to transform at acceptable transformation frequencies.

Transgenic lines and the number of transgenic loci, were examined by Southern hybridization analysis (data not shown) and while they allowed the number of transgenic loci to be calculated, they did not provide information on the number of transgene copies at any particular locus. The presence of the transgene in selected T1 and T2 plants was confirmed by PCR using the REDExtract-N-Amp™ Plant PCR-Kit (Sigma–Aldrich, Australia). There were no discernible differences in grain morphology between the transgenic and control lines, or between their 1000 grain weight values.

All transgenic plants were grown under standard glasshouse conditions [15] and wild type, empty vector controls and null-segregants were grown alongside each batch of transgenic plants through all generations [7]. All analysis was performed on T2 grain. Grains (10) from each line were ground using a dental amalgam