Plant Physiology and Biochemistry 133 (2018) 142–148

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

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

Research article

Lignin synthesis and accumulation in barley cultivars differing in their resistance to lodging

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ARTICLE INFO

Keywords:
Lignin
Hordeum vulgare
Internode
Cinnamoyl-CoA reductase
Cinnamyl-alcohol dehydrogenase
Phenylalanine ammonia-lyase
Lodging resistance

ABSTRACT

Since lignin greatly affects stem strength, which is an important agronomical trait, understanding the relationship between lodging resistance and lignin synthesis is important in barley breeding and selection processes. The aim of the study was to reveal the connection between physiological aspects of lignin synthesis and genetic background of barley cultivars with different lodging phenotype. Three barley cultivars Astor, Scarlett and Jaran were compared by measuring lignin, cellulose and total soluble phenolics content, phenylalanine ammonia-lyase activity (PAL) and expression of cinnamoyl-CoA reductase (*CCR*) and cinnamyl-alcohol dehydrogenase (*CAD*) in three lower internodes at flowering and grain filling stage. To assess their genetic background simple sequence repeats (SSR) markers, connected to lodging resistance and plant height, were analyzed. Compared to lodging susceptible cultivars Scarlett and Jaran, a lodging resistant cultivar Astor revealed different dynamics of lignin synthesis and deposition, showing higher PAL activity and total soluble phenolics content as well as higher expression of *CCR* and *CAD* genes in the second internode at grain filling stage. Analysis of SSR markers associated with quantitative trait loci (QTL) for lodging resistance revealed that Astor discriminates from Scarlett and Jaran by marker Bmag337 connected with elongation of the second internode. Lignification process is under a strong influence of genotype and environmental factors which determine lignin synthesis dynamics and deposition of lignin in the cell walls of barley.

1. Introduction

Lignin synthesis presents one of the largest energy consuming processes for the plant (Amthor, 2003). Deposition of lignin in the cell wall provides mechanical strength and supports tissues, enables water transport and also proved to be important in defense against pathogens and many parasites (Bhuiyan et al., 2009; Jayamohan and Kumudini, 2011). Lignin is synthesized through a process of polymerization of monolignols: *p*-coumaryl, coniferyl and sinapyl alcohol differing in their degree of methoxylation (Boudet, 2000). Monolignols arise as products of three biosynthetic pathways: shikimate, phenylpropanoid and specific monolignol. The shikimate pathway ends with the production of phenylalanine, the starting point for the synthesis of cinnamic acid by the enzyme phenylalanine ammonia-lyase (PAL, EC 4.3.1.5). PAL catalyzes the deamination of phenylalanine which signifies the beginning of phenylpropanoid biosynthetic pathway. Through several steps of hydroxylation by cinnamate-4-hydroxylase (C4H), *p*-coumarate-3- hydroxylase (C3H), *O*-methyltransferase (OMT), ferulate-5-hydroxylase (F5H), 4-coumarate CoA: ligase (4CL) and methylation of 3-hydroxyl group by caffeoyl-CoA *O*-methyltransferase (CCoAOMT), cinnamic acid is modified to the feruloyl-CoA (Vanholme et al., 2012).

A specific monolignol pathway represents the last stage of modifications i.e., reduction of monolignols. The first enzyme in this pathway is a cinnamoyl-CoA reductase (CCR, EC 1.2.1.44) specific for this biosynthetic pathway, and considered to be essential in the regulation of phenylpropanoid metabolites entrance (Barceló, 1997). The enzyme catalyzes the conversion of cinnamoyl-CoA esters to

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https://doi.org/10.1016/j.plaphy.2018.10.036
Received 27 August 2018; Received in revised form 24 October 2018; Accepted 30 October 2018
Available online 31 October 2018

cinnamaldehydes. The next and final stage of lignin synthesis is the conversion of cinnamaldehydes into cinnamyl alcohols or monolignols, catalyzed by the enzyme cinnamyl-alcohol dehydrogenase (CAD, EC 1.1.1.195). Monolignols then undergo an oxidation process by peroxidase and/or laccase and finally polymerize into the lignin polymer through a radical coupling (Vanholme et al., 2012). An important agronomic trait in barley (*Hordeum vulgare* L.) is plant height, which determines crop production and plays an important role in achieving the desired yield. It is a complex trait controlled by both Mendelian genes and quantitative genes (Ren et al., 2014). Selection and breeding processes of dwarf and semi-dwarf cultivars resulted in a creation of cultivars with enhanced stem strength and improved lodging resistance which increased the grain yield (Ren et al., 2010).

Simple sequence repeats (SSR or microsatellite) showed to be useful in breeding processes, facilitating the construction of high-density maps used for identification of molecular makers linked with genes and/or quantitative trait loci (QTLs) for different important agronomic traits such as yield, plant height, resistance to lodging and stress. Available sequence data are used for diversity assessment of barley breeding germplasm collections by using marker-assisted selection (MAS) which facilitates the process of breeding and improvement of agronomically important traits (Varshney et al., 2006). Molecular markers that are strongly associated with QTLs could be used for selection of traits, as well as lines which carry valuable alleles (Ren et al., 2014; Von Korff et al., 2006).

It was shown in previous studies (Jones et al., 2001; Pedersen et al., 2005; Ma, 2009) that lignin synthesis and accumulation correlate to stem strength. Moreover, the basal part of the barley stem has an important role in stem lodging resistance (Berry et al., 2006). Therefore, in order to further elucidate stem lodging resistance/susceptibility in three barley cultivars, we explored the dynamics of lignin accumulation and expression of lignin biosynthesis genes *CCR* and *CAD* as well as SSR markers associated with the QTLs controlling stem height and resistance to lodging.

2. Materials and methods

2.1. Plant material and sampling

Two Croatian spring barley cultivars – Astor and Jaran and one German – Scarlett, with different stem lodging phenotypes, Astor being lodging resistant while Jaran and Scarlett lodging susceptible, were grown at experimental fields of Agricultural Institute Osijek (45°32′N, 18°45′E, eutric cambisol pH in 1M KCl: 7.10, humus 3.0%, P_2O_5 = 0.27 mg g^{-1}, K_2O = 0.259 mg g^{-1}). Each barley cultivar was planted in four repetitions (plots) in a random block design at experimental fields. Area of the basic plot was 7.56 m^2 with sowing density of 450 grains per m^2. Samples were collected from May to June in 2011 at flowering and grain filling stages according to Zadoks scale (Zadoks et al., 1974). From each plot approximately 100–120 plants were collected. Plants were pooled together and from such combined sample all analyses were performed in three to five replicates. Furthermore, first three internodes, starting from the bottom of the plant, were collected, excised and leaf sheaths were removed. Internodes of each sample type were combined. All samples were frozen immediately in liquid nitrogen and stored at −80 °C for further analyses. For each analyses approximately 50 plants were used and number of technical replicates (*n*) is written in figure legends.

Agronomic traits (grain yield, hectoliter and lodging resistance) and phenotypic characteristic (stem height) of all 48 spring barley cultivars were evaluated by the certified breeders at Agricultural Institute of Osijek in accordance with Value for Cultivation and Use (VCU) guide published by The International Union for the Protection of New Varieties of Plants international organization (UPOV, 1994) results are presented in Supplementary material (Table S1). Cultivar Astor is two-rowed barley with dwarf phenotype, firm and elastic stem with average height 79,8 cm, semiprostratum tillering type (Martinčić et al., 1991). Cultivar Jaran is also two-rowed spring barley, semierectum to intermedium tillering type with loose, nutans type with easily detached awns from grain (Kovačević and Lalić, 1997) with stem height of 89,5 cm. Scarlett is a German two-rowed spring barley cultivar, with dwarf phenotype similar to cultivar Astor (stem height 77.2 cm) with medium to large kernels (Braugerstengemeinschaft e.V., 2008).

2.2. Determination of total lignin content

Plant tissue was milled using 0.5 cm mesh (Retsch, Germany) and dried for 48 h at 65 °C. Tissue was extracted four times with 80% ethanol at 80 °C for 30 min. Finally, the pellet was washed with 100% acetone and dried overnight at 90 °C. One hundred milligrams of dried plant material was used for determination of Klason lignin (Kirk and Obst, 1988) and acid-soluble lignin (Dence, 1992). Results were expressed as total lignin content (sum of Klason and acid-soluble lignin) per mg of dry weight.

2.3. Crystalline cellulose content determination

Crystalline cellulose content was determined according to Foster et al. (2010). Briefly, 1 mL of Updegraff reagent (Updegraff, 1969) was added to the approximately 70 mg of previously dried and extracted tissue. Samples were heated at 100 °C for 30 min, centrifuged and the pellet was washed with water and acetone and air dried overnight. The pellet was hydrolyzed with 72% sulfuric acid. Crystalline cellulose content was determined using the colorimetric anthrone assay. For standard curve preparation, glucose and anthrone reagent (2 mg mL^{-1} sulfuric acid) were used. Crystalline cellulose content was expressed as glucose equivalent.

2.4. Measurement of phenylalanine ammonia-lyase activity

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.24) activity was determined according to Bauer et al. (2009) with slight modifications as follows: three to four hundred milligrams of plant tissue was ground in liquid nitrogen and homogenized in extraction buffer. PAL activity was measured in crude protein extracts. The reaction mixture contained 100–500 μL of protein extract, 0.5 M borate buffer (pH 8.8) and 0.01 M L-phenylalanine in a total volume of 2 mL. All samples were incubated at 40 °C and change in absorbance, as a result of *trans*-cinnamic acid production, was recorded every 20 min during 3 h at 290 nm. The results were expressed as pkat *trans*-cinnamic acid per milligram of protein (pkat mg^{-1} protein).

2.5. Total soluble phenolics content

Fifty milligrams of plant tissue was ground in liquid nitrogen. Total soluble phenolics were extracted with 2.5 mL of 96% ethanol for 48 h at −20 °C (Randhir and Shetty, 2005). Reaction mixture contained 0.1 mL of ethanol extract, 0.7 mL distilled H_2O, 0.05 mL Folin–Ciocalteu reagent and 0.15 mL sodium carbonate solution (200 gL^{-1}). Samples were incubated for 60 min at 37 °C and absorbance was measured spectrophotometrically at 765 nm using tannic acid (TA) as a standard. Total soluble phenolics content was expressed as tannic acid equivalents per g of fresh weight.

2.6. Isolation of total RNA and reverse transcription

For total RNA extraction 300–400 mg of ground plant material was homogenized in 2 mL of TRIzol reagent (Ambion), in three independent replicates for each sample. cDNA synthesis was carried out using 1 μg of total RNA previously treated with DNase I (Invitrogen) according to the manufacturer's instructions. The reaction mixture contained 2,5 μM Oligo $d(T)_{16}$ primer, 5 mM $MgCl_2$, 200 nM dNTPs, 1U μL^{-1} RNase

inhibitor and 2,5 U μL^{-1} MuLV reverse transcriptase (Applied Biosystems) in a total volume of 20 µL. The mixture was incubated at 25 °C for 10 min, at 42 °C for 25 min and at 99 °C for 5 min. Synthesized cDNA was stored at −20 °C until further use.

2.7. Relative gene expression analyses by qRT-PCR

Gene-specific primers for barley cinnamoyl-CoA reductase (accession no. AY1496707; *HvCCR*- F 5′-GAAGCAGCCTTACAAGATGTCC-3'; *HvCCR*- R 5′- TCGTACAACGACGTC-TACACC-3′), cinnamyl alcohol dehydrogenase (accession no. AK359640; *CAD*-F 5′-CGTA-CAGAGAGT CGTTCACAGG-3'; *CAD*-R 5′-TCACCACGAACTTCTGATCG-3′) and glyceraldehyde 3-phosphate dehydrogenase (accession no. M36650, *GAPDH*-F 5′-ATCAT-TCCAAGCAGCACTGG-3'; *GAPDH*-R 5′-TCATTCCA AGCAGCACTGG-3′), as a reference gene, were designed with Primer3Plus (http://primer3.ut.ee). The qRT-PCR assay was performed by using PowerSYBR green PCR master mix (Applied Biosystems), 5 µL of cDNA and 250 nm primers on ABI 7300 Real-Time PCR System (Applied Biosystems, California, USA) with following protocol: initial denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s annealing at 60 °C for 1 min, followed by a melt-curve analysis. *GAPDH* was used as a reference to normalize the amounts of cDNA between samples and for comparison of data from different PCR runs. The relative gene expression was calculated according to Pfaffl (2001) using previously determined efficiency calculated for every set of primers from 10-fold dilution series of cDNA. Expression of *CCR* and *CAD* genes was analyzed in triplicate and the mean values were used for calculations. The experiment was repeated three times using three technical replicates. The data from all experiments was pooled in a single analysis and represented as a mean value (Fig. 3).

2.8. Analysis of potential QTL placements

In order to assess genetic variability between them and to compare their genetic backgrounds three barley cultivars were screened by SSR markers (n = 20) for QTL traits with special reference to markers associated with stem height (Bmag223, Bmag120 and EBmac679) and lodging resistance (Bmag337, Bmag7 and HVM54). Plants were grown from three seeds (for every sample/cultivar) sown into the plastic container filled with loam substrate (40% sand, 40% silt, 20% clay; pH 6.5). At three leaf stage of vegetative growth Z13 (Zadoks et al., 1974) leaves were sampled from three to four plants. Genomic DNA was isolated by cetyltrimethyl ammonium bromide (CTAB) method (Doyle, 1990) from leaf tissue previously lyophilized and grounded with steel beads to powder. DNA concentration was determined spectrophotometrically. SSR markers were amplified by multiplex PCR (Table S2) in Veriti Thermal Cycler (Applied Biosystems). The PCR amplification procedure was performed as follows: initial denaturation step at 94 °C for 4 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50°- 65 °C for 30 s, elongation at 72 °C for 60 s and with a final elongation at 72 °C for 5 min in a total volume of 10 µL containing 20 ng of genomic DNA, 1xPCR buffer, 0.5 U AmpliTaq DNA polymerase (Applied Biosystems), 0.3 µM of each primer, 200 µM dNTPs and 1.5 mM $MgCl_2$. Primers and annealing temperatures are provided in Supplemental Table S2. PCR products were analyzed with the genetic analyzer (ABI 3130 - size standard used: GeneScan 500LIZ, Applied Biosystems) and its running module *"Fragment Analysis 36 POP7"*. Data were retrieved via GeneMapper 4.0 (Applied Biosystems) software with the use of *"Local Southern"* algorithm for determination and analysis of PCR fragments.

2.9. Data analysis

The statistical differences between internodes of three cultivars were analyzed using ANOVA and Fisher's LSD test (least significant difference) for each growth stage separately. For analysis of agronomic traits and stem height between 48 spring barley cultivars Duncan's multiple range test was used. P values ≤ 0.05 were considered significant. Analyses were performed with Statistica 7 (StatSoft, USA). Data presented in this study are the means ± standard deviation.

The analysis of genetic variability and polymorphisms of microsatellite markers was based on the number of alleles (N_a), genetic variability (H_e) and allelic polymorphic information content (PIC). All calculations were performed using PowerMarker (Liu, 2002). The expected heterozygosity (H_e) was calculated according to Nei (1973).

Using the equation:

$$He = 1 - \sum_{i-1}^{l} p_i^2$$

while PIC (Botstein et al., 1980) was calculated as follows:

$$PIC = 1 - \sum_{i-1}^{l} p_i^2 - 2 \sum_{i=j+1}^{l} \sum_{j=1}^{l-1} p_i^2 p_j^2$$

where p_i and p_j are the frequencies of alleles i and j, and l represents the total number of alleles.

The sizes of PCR products were used to calculate the proportion of shared allele distance (P_{SA}) (Bowcock et al., 1994) as follows: $P_{SA} = \frac{\sum_{l=1}^{L} s}{2L}$ where s denotes the number of shared alleles and L the number of loci. Based on this, genetic distance (D_{PSAM}) was calculated as $D_{PSAM} = 1\text{-}P_{SA}$. The genetic distance matrix was used to reveal relations based on the unweighted pair group method with arithmetic averages (UPGMA) analysis of similarity matrices and PAST 3.0 (Hammer et al., 2001) was used to generate a UPGMA dendrogram.

3. Results

3.1. Lignin and cellulose content in the internodes

Total lignin content varied between internodes and between growth stages in cultivar-dependent manner, with some general exceptions. At flowering stage, significantly higher lignin content in the first internode compared to the second and third, was measured only in cultivar Astor (Fig. 1A). However, compared to Astor, this content was higher in cultivars Jaran and Scarlett at flowering. In Scarlett the lignin content gradually decreased from the first to the third internode with a significant difference between the first and third internode. No significant difference in lignin content was detected between internodes in cultivar Jaran (Fig. 1A). Thus, at grain filling stage in all three cultivars the basal internode, which is also the oldest, contained more lignin compared to the second and the third internodes. The second and the third internodes of each cultivar did not differ significantly in lignin content (Fig. 1B). When compared, the overall lignin content of internodes of cultivar Scarlett at flowering and Jaran at grain filling stage reached a statistically significant maximum indicating developmentally specific change. At two growth stages, when comparing all three internodes within the same cultivar as well as the same growth stage, cellulose content did not change (Fig. 1).

3.2. Total soluble phenolics content and PAL activity

Total soluble phenolics content was measured in three spring barley cultivars at flowering and grain filling stage. Results showed that at both stages cultivars Scarlett and Jaran had significantly higher phenolics content in all three internodes compared to cultivar Astor, (Fig. 2A and B). In Scarlett and Jaran, the total soluble phenolics content was highest in the first internode and it remained constant or gradually decreased from the second to the third internode. A similar pattern was found in Astor only at grain filling stage (Fig. 2B). A significant increase in total soluble phenolics content was detected between the flowering and grain filling stage of cultivar Scarlett. No such

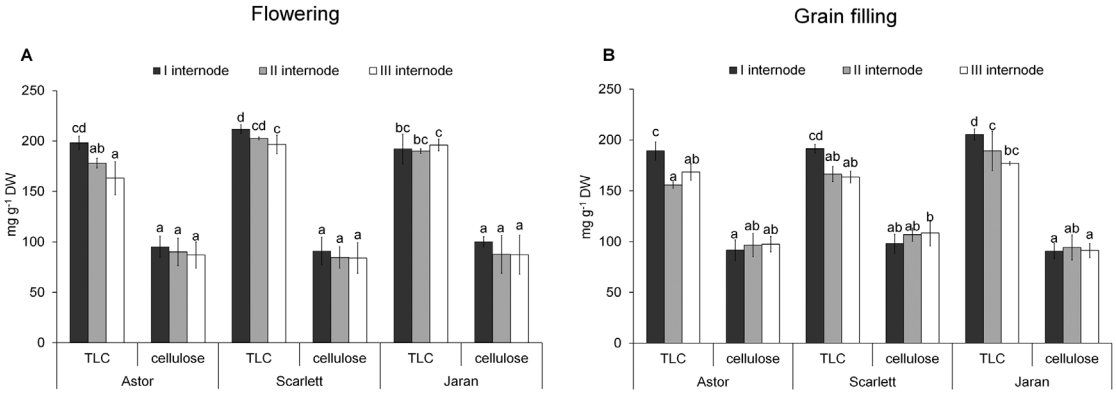

Fig. 1. Total lignin and crystalline cellulose content in the internodes of three barley cultivars (Astor, Scarlett and Jaran) at flowering (A) and grain filling (B) stage. Vertical bars represent mean values ± standard deviation ($n = 3$). Different letter represents significance at $P \leq 0.05$ for each growth stage separately.

difference between developmental stages was observed in cultivar Jaran (Fig. 2A and B; Table S3).

In general, PAL activity decreased from flowering to grain filling stage in all three cultivars (Fig. 2C and D). However, the highest PAL activity was measured in cultivar 'Astor' at both growth stages (Fig. 2C and D). At grain filling stage in Astor PAL activity significantly decreased in the first internode while in two younger internodes did not change. In Scarlett, PAL activity was lower at grain filling stage in all three internodes with the most dramatic decrease in the third internode. In cultivar Jaran PAL activity at grain filling equally decreased in all three internodes compared to the flowering stage (Fig. 2C and D; Table S3).

3.3. Expression pattern of CCR and CAD genes involved in specific monolignol synthesis pathway

Expression of genes encoding cinnamoyl-CoA reductase (*CCR*) and cinnamyl alcohol dehydrogenase (*CAD*) involved in specific monolignol biosynthesis pathway was analyzed using qRT-PCR (Fig. 3). In general, both transcripts were more abundant at flowering stage in all three cultivars (Fig. 3A, C). At flowering stage in cultivars Scarlett and Jaran, expression of *CCR* (Fig. 3A) and *CAD* (Fig. 3C) genes was lowest in the first internode and gradually increased in the second and third internode. The significantly higher expression of *CCR* gene was detected in the third internode of cultivar Jaran (Fig. 3A). The exception to this trend was found in cultivar Astor (Fig. 3C) where expression of *CAD* gene was highest (fivefold) in the first internode. The expression of *CCR* and *CAD* genes at grain filling stage differed between cultivars and internodes (Fig. 3B, D). In lodging resistant cultivar Astor higher expression of both *CCR* and *CAD* was measured in the second internode (Fig. 3B, D). In contrast, in cultivar Jaran the expression of both genes was the lowest in the second internode and higher in the first and third internode. In cultivar Scarlett the expression pattern of these two genes was opposite, *CCR* had the highest expression in the third internode and

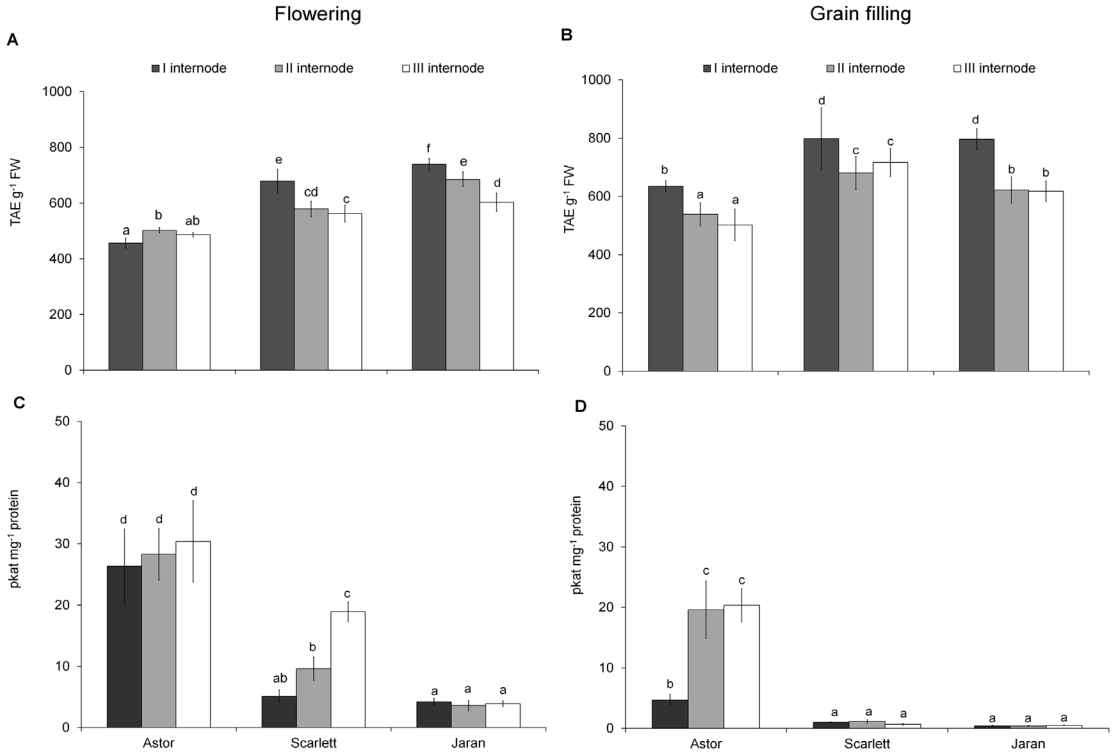

Fig. 2. Total soluble phenolics content (A, B) expressed as tannic acid equivalents per g of fresh weight (TAE g^{-1}FW) and PAL activity (C, D) expressed as pkat per mg of protein (pkat mg^{-1} protein) in the internodes of three spring barley cultivars at flowering and grain filling stage. Vertical bars represent mean values ± standard deviation ($n = 5$). Different letter represents significance at $P \leq 0.05$ for each growth stage separately.

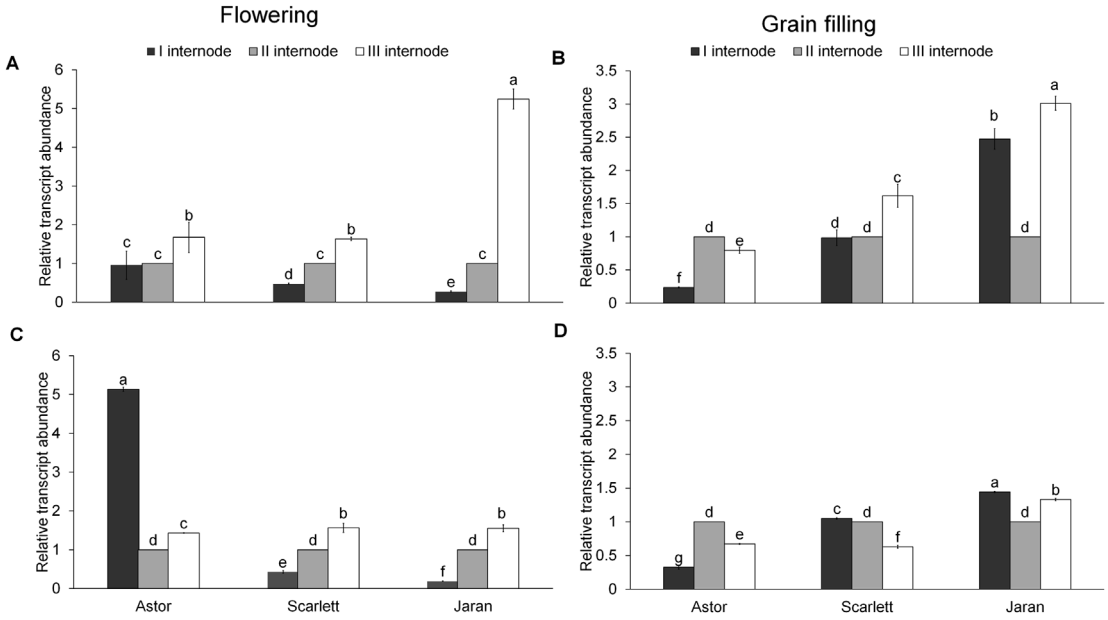

Fig. 3. Real-time PCR analyses (qRT-PCR) of (A, B) cinnamoyl-CoA reductase (*CCR*) and (C, D) cinnamyl alcohol dehydrogenase (*CAD*) genes in the internodes of three barley cultivars (Astor, Scarlett and Jaran) at flowering and grain filling stage. The gene expression level of *CCR* and *CAD* was normalized against the reference gene (*GAPDH*) and presented as fold change relative to that of the second internode within each cultivar at flowering and grain filling stage. Mean values of three replicates are presented ± standard deviation (SD).

CAD in the first (Fig. 3B, D).

Different letter represents significance at $P \leq 0.05$ for each growth stage and gene separately.

3.4. The screening of SSR markers in three barley cultivars differing in their resistance to lodging

Twenty SSR markers were used for screening 48 spring barley cultivars. The obtained PIC values ranged from 0.247 to 0.786 with an average value of 0.549. Bmag7 (7H) showed the highest PIC value of 0.786 as well as the highest N_a followed by Bmag120 (7H) with PIC value of 0.740 (Table S4). Dendrogram derived from genetic distance matrix based on 20 SSR markers showed a close relationship between Jaran and Scarlett which were both genetically distant from cultivar Astor (Fig. S1). Indexing of SSR profiles showed total value of allele similarity between Scarlett/Jaran of 12 over 20 while Scarlett/Astor showed 9 over 20 and Jaran/Astor 9 over 20 as well (Table S5). Number of different alleles was congruent and SSR markers were also analogous from both Jaran and Scarlett towards Astor, meaning that differing alleles were put to closer inspection considering the trait of interest such as resistance to lodging (Bmag337, Bmag7 and HVM54) and stem height (Bmag223, Bmag120 and EBmac679). Scarlett and Jaran discriminated from Astor through following SSR probes: Bmag337 (5H) and HVM054 (2H) (Table 1).

The most detectable and important was the difference at the locus Bmag337, connected to resistance to lodging (Von Korff et al., 2006), where Scarlett produced 131 bp allele as well as Jaran, however, Astor showed a 146 bp allele. A similar result was obtained for locus HVM54 positioned close to the QTL for lodging resistance (Von Korff et al., 2006). Scarlett and Jaran produced 161 bp and Astor 157 bp allele. SSR markers Bmag7 and EBmac679 (4H) did not exhibit any allelic similarities between cultivars while Bmag120, related to stem height, showed similarities between cultivars Astor and Scarlett (Table 1).

Table 1
Allelic similarity matrix based on fragment length (shared alleles within varietal combination are designated with number "1") with index calculation of 6 microsatellite markers in three cultivars of spring barley.

SSR marker	Astor/Scarlett	Jaran/Astor	Jaran/Scarlett
Bmag337	0	0	1
HVM54	0	0	1
Bmag120	1	0	0
Bmag223	1	1	1
Bmag7	0	0	0
EBmac679	0	0	0
Indexing totals	2/6	1/6	3/6

4. Discussion

Lignification is an important part of development and differentiation in plant cells and tissues and as such it depends on many factors (Boerjan et al., 2003). The amount of lignin, as well as its composition and structure, affects the adaptive value of the plant in the processes of selection and breeding of different genotypes (Schlindwein, 2002). In agronomy, the plant's adaptive value is estimated through germination, herbicide resistance, survival, growth, lodging and yield (Pedersen et al., 2005) and includes economically profitable production and stable yield of a particular crop. Lignin synthesis and deposition in plants has been a subject of comprehensive investigation in recent years (for review see Barros et al., 2015). Because of its complexity, it is still unexplored in many segments. In crops, the mechanical strength of a plant's stem contributes to stem lodging resistance, an important agronomic trait with direct influence on yield and grain quality (Berry et al., 2004). It is controlled by changes in cell wall composition and structure (Cosgrove, 2000).

Our previous findings, related to anatomical changes associated with cell wall thickening as a result of lignin deposition in the internodes of three barley cultivars, indicated that most of the lignin is deposited at flowering stage in all cultivars, while in cultivar 'Astor' additional cell wall thickening was also visible in the grain filling stage (Begović et al., 2015). In the present study, we further evaluated three barley cultivars by analyzing lignin and cellulose content, the activity of PAL enzyme, total soluble phenolics content as well as expression of *CCR* and *CAD* genes involved in lignin biosynthesis pathway in the stem. In addition, analysis of SSR markers for resistance to lodging and/or plant height was evaluated for three barley cultivars.

Cultivar Astor did not exhibit stem lodging, whereas cultivars Jaran and Scarlett exhibit 38.3 and 34.2% of stem lodging, respectively (Table S1). The cellulose content remained similar at both growth stages (Fig. 1), indicating a decrease in cellulose synthesis and thus the beginning of intensive lignin synthesis in the non-elongating internodes (Bosch et al., 2011; Jung and Casler, 2006). This confirms previous findings that lignin deposition in the cell walls follows cellulose and hemicellulose deposition (Bidlack and Buxton, 1992; Morrison and Buxton, 1993). Differences in total lignin content were observed between flowering and grain filling stage when comparing three cultivars. Increased lignin content at the reproductive stage was also reported by Chen et al. (2002) where lignification process in the internodes of tall fescue (*Festuca arudinacea* Schreb.) was described and by Ma (2009) in wheat (*Triticum aestivum* L.) cultivars differing in lodging resistance.

PAL enzyme catalyzes deamination of phenylalanine and represents a starting point in phenylpropanoid pathway and as such, it has an important role in the regulation of phenylpropanoid compounds, including lignin (Whetten and Sederoff, 1995). Our data showed a connection between PAL activity and total soluble phenolics content regarding internodes and cultivars (Fig. 2). When comparing two growth stages overall PAL activity significantly decreased at grain filling stage (Fig. 2C and D), at the same time total soluble phenolics content increased (Fig. 2A and B). The exception to that trend is lodging resistant cultivar Astor in which, already high at flowering, PAL activity additionally increased particularly in the second but also in the third internode at grain filling stage, while it decreased significantly in the first internode. Moreover, in lodging resistant Astor, PAL activity was higher compared to more susceptible cultivars Scarlett and Jaran and remained high at both growth stages. Of particular importance for lodging resistance might be the significantly higher PAL activity at grain filling stage in the second and the third internodes of Astor, compared to Scarlett and Jaran (Fig. 3D). This finding underlines previously established statements about the important role of PAL enzyme in lignin biosynthesis pathway (Boudet, 2007) and lodging resistance (Peng et al., 2014; Wang et al., 2014a).

Depending on the cultivar, PAL activity could be associated with lignin content during development from flowering to grain filling stage. Namely, in cultivar Scarlett PAL activity and the lignin content were lower at grain filling than flowering stage (Figs. 1 and 2C, D). Furthermore, in cultivar Jaran PAL activity and lignin content in the internodes followed similar trends at flowering (Figs. 1A and 2C). Despite the fact that at grain filling stage PAL activity in Jaran significantly decreased, lignin content in the internodes did not change (Figs. 1B and 2D). In cultivar Astor, obtained results indicated that PAL activity could be related to total lignin content between the internodes. In Astor, decreased PAL activity was observed in the more lignified first internode and increased activity in less lignified second and third internodes (Figs. 1 and 2C, D). Generally, total lignin content was significantly higher in the first internode than in the second and the third at grain filling stage in all cultivars (Fig. 1B) and it did not correspond with PAL activity (Fig. 3D). These results were expected as it was previously noted that developmentally older internodes are more lignified due to constant biosynthesis and aging (Jung and Casler, 2006). However, lignification related to lodging resistance could mostly depend on timely and spatially regulated lignin biosynthesis which influences relative strength between internodes (Peng et al., 2014). Basal internodes are short while the second and the third internodes undergo elongation process to a certain extent depending on the cultivar/genotype (Pinthus, 1973; Tripathi et al., 2003). It could be hypothesized that prior to and during grain filling stage, the lignification of the second and the third internodes has to become more intensive than lignification of the most basal internode. This is in concordance with the study of Berry et al. (2006) who showed that lower internodes, from the second to the sixth, significantly influence lodging characteristics of barley stem. The stable and high PAL activity in cultivar Astor during the flowering and grain filling stages, in contrast to significantly lower PAL activity especially at grain filling of Scarlet and Jaran, supports this hypothesis. Moreover, observed intensive increase of PAL activity in the second and the third internodes of Astor proves the hypothesis that lodging resistance in barley depends mostly on lignification in the second and the third internodes (Berry et al., 2006).

The hypothesis that lodging resistant cultivar Astor exhibits different dynamics of lignin synthesis and deposition, in the stem, compared to lodging susceptible cultivars Scarlett and Jaran, is further corroborated by expression analysis of *CAD* and *CCR* genes involved in the last step of specific monolignol pathway. When comparing two growth stages, higher expression of *CCR* and *CAD* genes can be observed at flowering than grain filling (Fig. 3). Also at flowering stage in all three barley cultivars the highest expression of *CCR* gene was measured in the youngest, third internode (Fig. 3A). The expression of *CAD* gene showed a similar pattern to *CCR* in cultivars Scarlett and Jaran while in Astor the highest *CAD* transcript abundance was observed in the first internode (Fig. 3C).

We had previously suggested that the significant lignification of internodes involved in lodging resistance might occur prior and during grain filling. The data presented here showed that expressions of *CCR* and *CAD* genes at grain filling stage were higher in the second internode of cultivar Astor. By contrast, in lodging susceptible cultivar 'Jaran' the expression of both genes was lowest in the second internode while in lodging susceptible cultivar Scarlett the expression pattern of two genes was opposite with the highest CCR expression in the third, and *CAD* in the first internode. These results indicate that lodging resistance of the stem depends on temporally and spatially regulated control of genes that are directly involved in final steps of lignification.

Molecular marker-assisted technology has been successfully used for identification of QTLs related to agronomic and physiological traits (Varshney et al., 2006). Three spring barley cultivars were screened with 20 SSR markers and showed specific variability but only those markers relevant for traits such as resistance to lodging and plant height were subjected to further evaluation and are discussed here. Analysis of SSR markers related to stem height, such as Bmag223 (5H), showed the lowest PIC value which could be associated with higher allelic similarity (H_e) between cultivars. This indicates polymorphic characteristics of the Bmag223 (5H) marker, as opposed to more monomorphic markers Bmag7 (7H) and EBmac679 (4H) that showed higher PIC and lower H_e values. On the other hand, Bmag120 (7H) indicated higher allelic similarity between cultivars Astor and Scarlett compared to Jaran which is in concordance with lower stem phenotype observed in those two cultivars. Recent findings confirmed that plant height is controlled by several QTLs (Pillen et al., 2003) which are distributed throughout the barley genome (Wang et al., 2014b).

Among markers of interest the most detectable was Bmag337, previously reported as being linked to QTL associated with resistance to lodging located on chromosome 5H (Von Korff et al., 2006). Bmag337 significantly discriminates cultivar Astor from Scarlett and Jaran genotype-wise. This marker is known specifically for elongation and increase in the measure of the second internode (Ren et al., 2014). Furthermore, Unweighted Pair Group Method with Arithmetic Mean analysis (UPGMA) revealed that cultivars Scarlett and Jaran represented more similar genotypic backgrounds in contrast to Astor (Fig. S1), which is congruent to a large variability survey of 48 spring barley cultivars (Abičić, 2014).

5. Conclusion

This study provides new insights into the correlation between dynamics of lignin synthesis, deposition and genetic variability of three barley cultivars differing in their stem lodging phenotype. We showed that stem lodging resistance depends on timely and spatially regulated increase of lignin formation. Moreover, our data showed that enhanced lignin synthesis, PAL activity and *CCR* and *CAD* gene expression in the second internode during flowering but also grain filling stage contribute

to lodging resistant phenotype of cultivar Astor.

Contributions

LB performed most of the experiments and wrote the manuscript. IA performed the SSR marker analyses and wrote the manuscript. AL contributed with the data analysis and set up field experiment. HL contributed to data analyses. VC took part in interpretation of the results and manuscript preparation. DLL designed and supervised the study, prepared the manuscript.

LB and IA equally contributed to this work.

All authors approved the final version of the manuscript.

Acknowledgments

This work was supported by the Ministry of Science, Education and Sports of the Republic of Croatia (grant numbers 073-0731674-0841 and 073-0730718-0550) and the University of Zagreb (grant number 202688). We thank Miss Andreja Škiljaica for proof reading the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2018.10.036.

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