Identification of Mutations in Genes Regulating Culm Length in Malting Barley

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Lodging, the tendency in barley to lie down on the ground when exposed to hail and heavy fertilising, is one of the most important traits that barley breeding is trying to remove from the barley cultivars. This may be an even greater challenge when future climate changes may lead to more extreme weather leading to more severe storms with heavy rain and hail. In this project, 8 breviaristatum (ari)-mutants have been sequenced for mutations in three different genes Brassinosteroid-6 oxidase (HvBRD), Diminuto (HvDIM) and Brassinosteroid-insensitive1 (HvBRI1). These genes are involved in brassinosteroid biosynthesis and signalling. HvBRD and HvDIM encode enzymes taking part in the biosynthesis of brassinosteroids while HvBRI1 encodes the brassinosteroid receptor. Brassinosteroids are plant hormones, which among other things stimulate cell division and elongation. These genes regulate the length of the barley’s culm and a mutation in the genes leads to a shorter culm, a semi-dwarf phenotype, which is more tolerant against lodging. In 5 ari-mutants the mutation could be identified and these mutations can be used in marker-assisted barley breeding programs. These findings can be an important tool enabling more efficient barley breeding, which will be important in the future with predicted more extreme weather. F2-crosses between brassinosteroid mutants, with known mutation, and the ari-mutants sequenced in this project were also studied. A couple of the individuals that showed severe dwarf phenotype were sequenced in search of being homozygous for the mutations inherited from both parent lines.

One of the oldest crops that humans have cultivated is the grass barley and it was domesticated at least 10,000 years ago in the Fertile Crescent (Pourkheirandish and Komatsuda, 2007), today’s Lebanon, Syria and Iraq. The domesticated barley (Hordeum vulgare) derives from its wild relative Hordeum vulgare ssp. spontaneum (The International Barley Genome Sequencing Consortium, 2012) with the spike having seeds in two rows. Today’s barley has either two rows or six rows (Ramage, 1987), with the latter being attractive because of the potential to give a higher yield. barley is the fourth most cultivated crop after wheat, maize and rice (The international Barley Genome Sequencing Consortium, 2012). About 75% of the barley is used as animal feeding, 20% for malt and 5% for human consumption (especially where the climate is too tough for other cereals).

Barley has been adapted to many different climates and it is more tolerant to different environments than other cereals and is often grown in areas where other cereals cannot be cultivated. It grows on all continents, from Northern Scandinavia down to the equator, from moist Europe to the drier Asia and in lowlands as well as in higher mountains (Ramage, 1987). Although barley is very adaptable and its traits have been improved during 10,000 years, future climate changes can negatively affect barley yields for farmers because of the risk of more severe storms with heavier rain and hail (Dockter et al, 2014).

One of the most important traits that barley breeders are trying to improve is resistance against lodging; i.e. the tendency of barley plants to lie down when exposed to heavy rain, hail or high nitrogen fertilisation. Lodging in barley can in turn lead to fungus infections and seed germination before harvest, giving a lower yield. The lodged barley makes it also more difficult for the combine harvester, which also leads to loss of seeds (Dockter et al, 2014). The barley varieties with best resistance to lodging are the ones with shorter culms, giving a shorter plant height. The biochemical pathways regulating height have been identified, but genes and mutations affecting the plant architecture are still to be identified at the DNA level. If identified, these mutations would be beneficial, as these could be used in assisting barley breeding, leading to more high yielding barley cultivars.

The genome of barley is large (5.5 Gb) containing approximately 30,000 genes (Sreenivasulu et al, 2007). Barley has 7 chromosomes and is diploid (Liu et al, 2014). The short-culmed mutants, which have a reduced culm length, are gathered in several groups called breviaristatum (ari-mutants) erectoides, brachytic, uzu, slender-dwarf and semi-dwarf (Kucera et al, 1975). The different names seem to reflect different origin of the mutants rather than major
genetic differences (Dockter et al. 2014). The plant hormones brassinosteroids and gibberellin acid are the substances that affect the culm length (Raven, 2013) and this project is focusing on genes controlling brassinosteroid biosynthesis and signalling.

Brassinosteroids are found in the whole plant but especially in developing tissue. They act locally, either where they are synthesised or in near proximity of the synthesis. They control cell division, cell expansion, tissue differentiation and development of lateral roots. Around 50 different brassinosteroids have been identified (Dockter et al, 2014). The brassinosteroid that commonly has the highest activity is called brassinolide (Raven, 2013). However, in barley and other cereals castasterone, the predecessor of brassinolide, seems to be the finale active substance that controls plant growth, since brassinolide has not been found in cereals yet (Dockter et al, 2014). Mutations in the biosynthesis lead to smaller and fewer cells, giving a shorter culm length (Raven, 2013).

The genes encoding enzymes in the brassinosteroid biosynthetic pathway are brassinosteroid-6 oxidase (HvBD), DIMINUTO (HvDIM), constitutive photomorphogenic dwarf (HvCPD) and the gene encoding the brassinosteroid receptor is brassinosteroid-insensitive1 (HvBRII). The mutations in these genes lead to the semi-dwarf phenotype because of deficiencies in either the biosynthetic pathway, showing a lower castasterone level, or the receptor, leading to deficiencies in the hormone's signalling pathway (Dockter et al, 2014).

This project will focus on identifying mutations in 3 of these genes in two-rowed barley. Starting with HvBRII, the gene is located on chromosome 3H and the encoded brassinosteroid receptor is a transmembrane protein serine/threonine kinase (Gruszka et al, 2011). HvBD encodes the enzyme brassinosteroid-6 oxidase and is located on chromosome 2H. Lastly, HvDIM, like HvBD, encodes an enzyme in the synthetic pathway called Δ5-sterol-Δ5-reductase and is located on chromosome 7H. HvCPD is encoding the enzyme C-23α-hydroxylase cytochrome P450 90A1 (Dockter et al, 2014) but will not be studied in this project.

We hypothesised that 5 ari-mutants would have a mutation in the HvBD gene. These mutants are named ari-263, ari-358, ari-400, ari-402 and ari-465. Mutant ari-145 was thought to have a mutation in the HvDIM gene. Finally, ari-312 and ari-380 were thought to have a mutation in the HvBRII gene. The mother cultivars for these mutants are Bonus or Kristina. We also believed that it would be possible to find these mutations and already known mutations in descendants of diallelic crosses by examining the phenotype, choosing the shortest plants, in F2-populations. The F2-populations were obtained from crosses between ari-312, ari-358, ari-380 and ari-415, (studied in this project) and the already known mutants BW031 (carries the ari-u.245 mutation in HvBDR), BW084 (brh13.p in HvCPD), BW333 (ert-zd.159 in HvDIM) and BW885 (uzl.a in HvBRII) (The BW-lines are near isogenic lines generated in the cultivar Bowman by up to six recurrent back-crosses (Druka et al, 2011)). These two cases were studied by performing PCR to amplify the gene fragments of interest followed by sequencing of these fragments to find the mutations in the genes.

We successfully identified the base substitution along with amino acid substitution giving the semi dwarf phenotype in 5 of the 8 studied ari-mutants. The mutations were found to be in the coding sequence of each gene. The identified mutations in these ari-mutants could in the future be used in marker-assisted barley breeding when more effective breeding programs are needed because of more extreme weather caused by anthropogenic climate changes. Eleven F2-plants were selected and genotyping demonstrated that two of them were homozygous for mutations in two genes. These plants will be valuable tools for scientific studies aiming to further elucidate the mechanisms of brassinosteroid metabolism and signalling.

**MATERIALS AND METHODS**

**Planting of seeds:** 40 F2 seeds from each of eleven different crosses, were planted in 1,5 l pots in greenhouse G at the Department of Biology, Lund University. The mother and father varieties were also planted in 1,5 l pots. The established cultivars, Bonus and Kristina, along with ari-mutants were planted in 0,1 l pots in greenhouse A at the department of Biology, Lund University. Seeds were obtained from the Nordic Genetic Resource Center in Alnarp.

**DNA extraction:** When the plants had reached a height of approximately 10-20 cm, the leaves of the ari-mutants and the cultivars Bonus and Kristina were collected. Only a part of the leaf was cut from the plants of the F2 population. DNA was then extracted with the REDExtract-N-Amp Plant PCR Kit (Sigma) by cutting approximately 1 cm of the leaf and placed in 1,5 ml Eppendorf tube. 200 µl extraction buffer was then added followed by incubation at 95° C for 10 min. After this, 200 µl dilution buffer was added followed by vortex and centrifugation. These DNA stock solutions were then used as template in the PCR amplifications.

**Primer construction:** Forward and reverse primers for 5 fragments in the HvBDR, HvBRII and HvDIM genes were constructed as well as primers for the already known mutants BW031, BW084, BW333 and BW885. Two forward primers were made for each of the four BW-varieties, one primer with wildtype sequence and one with known mutant sequence.

**PCR-solutions:** PCR amplification for mother cultivars, ari-mutants and F2-plants were made using REDExtract-N-Amp Plant PCR Kit (Sigma). In one PCR tube 4 µl DNA template, 4 µl water,
used to find the appropriate temperature for the primers to find the homozygous mother mutants (BW-lines). For BW885, BW084 and BW031, the PCR program was: 94°C 2 min followed by 94°C/45s, 62°C/45s, 72°C/60s repeated 30 cycles, followed by 72°C 2 minutes. For BW333 the PCR-program was: 94°C 2 min followed by 94°C/45s, 59.9°C/45s, 72°C/60s repeated 30 cycles, followed by 72°C 2 minutes. PCR products were then separated on 1% agarose gel electrophoresis (GenAgaroseLE; GENAXXON Bioscience).

**Sample preparation for sequencing:** PCR clean-up Gel extraction (Machery-Nagel) was used to isolate the PCR-product that was cut out from agarose gel. Aliquot volume (200 µl per 100 mg of agarose gel) of Buffer NTI was added to 1.5 ml Eppendorf tube containing the gel fragment. The solution was then incubated 5-10 minutes in 50°C until the gel was completely dissolved. 700 µl of the sample was placed in NucleoSpin Gel and PCR Clean-up Column (in collection tube) followed by centrifugation for 30 s at 11,000 x g. Then 700 µl Buffer NT3 was added and centrifuged 30 s. Flow-through was discarded followed by centrifugation for 1 min to dry the membrane. The column was placed in 1.5 ml Eppendorf tube and 30 µl Buffer NE was added and incubated 1 min in room temperature. This was followed by 1 min centrifugation. The samples were then placed in tubes for sequencing, with volume water and PCR solution depending on the DNA concentration, together with 2 µl forward primer and 2 µl reverse primer. The PCR samples that gave clear bands were prepared with ExoProStar 1-Step (Illustra) for sequencing. 12.5 µl PCR reaction and 5 µl ExoProStar 1-Step was added together. This was then followed by incubation at 37°C for 15 minutes followed by incubation at 80°C for 15 minutes. The samples were then placed in tubes for sequencing with 7 µl sample solution together with 8 µl water and 2 µl forward primer and 2 µl reverse primer. All the samples were then sent to be sequenced at BMC, Lund.

**PCR programs:** For HvBRD fragments 3, 4, 5, HvDIM fragments 6, 7, 8 and HvBRI1 fragments 3, 5 and 6 the PCR program was: 94°C 3 min followed by 94°C/ 45s, 57°C/45s, 72°C/90s repeated 3 cycles, followed by 94°C/45s, 54°C/45s, 72°C/90s repeated 3 cycles, followed by 94°C/45s, 51°C/45s, 72°C/90s repeated 32 cycles, followed by 72°C 5 minutes. For HvBRD fragments 1 and 2 the program was: 94°C 3 min followed by 94°C/45s, 61.5°C/45s, 72°C/90s repeated 3 cycles, followed by 94°C/45s, 59.5°C/45s, 72°C/90s repeated 3 cycles, followed by 94°C/45s, 53°C/45s, 72°C/90s repeated 36 cycles, followed by 72°C 10 minutes. For HvDIM fragments 1 and 5 the program was 94°C 3 min followed by 94°C/30s, 57°C/45s, 72°C/105s repeated 40 cycles, followed by 72°C 10 minutes. For HvBRI1 fragment 2 the program was: 94°C 3 min followed by 94°C/45s, 62°C/45s, 72°C/90s repeated 3 cycles, followed by 94°C/45s, 60°C/45s, 72°C/90s repeated 3 cycles, followed by 94°C/45s, 51°C/57.5 s, 72°C/90s repeated 32 cycles, followed by 72°C 5 minutes. For HvBRI1 fragment 4 the program was: 94°C 3 min followed by 94°C/45s, 59°C/45s, 72°C/90s repeated 3 cycles, followed by 94°C/45s, 57°C/45s, 72°C/90 s repeated 3 cycles, followed by 94°C/45s, 55°C/45s, 72°C/90s repeated 32 cycles, followed by 72°C 5 minutes. A PCR gradient (52°C to 62°C) was

Fig. 1. Identified mutations in genes involved in brassinosteroid biosynthesis and signalling. *ari-402* has a mutation in exon 4 of *HvBRD* with the base substitution g2019t. This substitution gives a stop codon instead of glutamate at position 323 in the amino acid sequence of the enzyme brassinosteroid-6 oxidase. *ari-400* has a mutation in exon 7 of the same gene, giving c2615t. The amino acid arginine in the same enzyme is substituted by a stop codon at position 441 in the amino acid sequence. *ari-465* has the mutation c2757r in exon 8 and the amino acid proline is changed to serine at position 456 in the amino acid sequence (A). *ari-415* is found to have the mutation g2637a in exon 2 of *HvDIM*. This substitution results in aspartate instead of glycine at position 162 of the amino acid sequence in the enzyme Δ7-sterol-Δ7-reductase (B). *ari-312* is found to have the mutation a2876g in exon 1 of *HvBRI1* (the gene only has 1 exon). This mutation gives alanine instead of threonine at position 960 of the amino acid sequence in the brassinosteroid receptor (C).
RESULTS

Identification of mutations in HvBRD. All the fragments of the HvBRD gene were not successfully amplified in all mutants. Mutations were found in ari-400, ari-402 and ari-465 (Fig. 1). In ari-400, the base substitution is c2615t in exon 7. This substitution results in a nonsense mutation, replacing arginine with a stop codon at position 441 in the amino-acid sequence in the enzyme brassinosteroid-6 oxidase. Mutant ari-402 has the base substitution g2019t in exon 4, also resulting in a nonsense mutation where glutamate is replaced by a stop codon at position 323 in the amino-acid sequence. Lastly, ari-465 has the base substitution c2757t in exon 8. However, this substitution is a missense mutation, replacing proline with serine at position 456 in the amino-acid sequence. The mutations for all three ari-mutants are found in a conserved domain of the enzyme. No mutations were found in the DNA-fragments of ari-263 and ari-358. It should be noted that fragment 1 and 2 in ari-263 and fragment 1 in ari-358 could not be amplified.

Identification of mutations in HvDIM. The mutation in ari-415 was found to be g2637a in exon 2 of HvDIM (Fig.1), resulting in an exchange of glycine to aspartate in the amino-acid sequence of the enzyme Δ^5-sterol-Δ^3-reductase. The affected glycine is located in the FAD-binding domain. Fragment 1 of HvDIM could not be amplified in ari-415, but since the mutation is already found in another fragment this can be overlooked.

Identification of mutations in HvBRI. In ari-312 the base substitution is a2878g in the single exon encoding the brassinosteroid receptor (Fig. 1). This missense mutation causes a replacement of threonine 960 by an alanine in the amino-acid. This mutation matches the mutation bri1-T1039A (intermediate) (Dockter et al. 2014) in the orthologous BRII gene of Arabidopsis thaliana where the Thr1039Ala modification is in the activation loop of the receptor (Wang et al. 2005; Bojar et al. 2014). Fragment 1 of HvBRII in both ari-312 and ari-380 could not be amplified and no mutation was found in fragment 2-6 of ari-380.

Phenotype of ari-mutants. The ari-mutant plants had grown for 7 weeks at the deadline of this project report and no spikes had developed. Nonetheless, they showed a semi-dwarf phenotype with a strong culm that is unlikely to lodge (Fig. 2).

<table>
<thead>
<tr>
<th>Name of cross</th>
<th>Cross</th>
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<tbody>
<tr>
<td>MH155</td>
<td>BW031 x ari-312</td>
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<tr>
<td>MH156</td>
<td>BW084 x ari-312</td>
</tr>
<tr>
<td>MH157</td>
<td>BW333 x ari-312</td>
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<tr>
<td>MH162</td>
<td>BW885 x ari-358</td>
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<td>MH177</td>
<td>BW333 x ari-380</td>
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<td>MH203</td>
<td>BW031 x ari-415</td>
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<tr>
<td>MH204</td>
<td>BW084 x ari-415</td>
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number 34 is heterozygous for the mutation in BW084 and the ari-312 mutation has not yet been sequenced. Lastly, plant number 29 is homozygous for the BW084 mutation and is not yet sequenced regarding the ari-312 mutation, but when comparing plant number 34 and 29 (Fig.4), number 34 seemed to have a more severe dwarf phenotype (but not as severe as number 11) while number 29 had a more semi-dwarf phenotype.

The cross MH165 has BW333 as mother, which has a mutation in HvDIM and ari-358 as father, which has a mutation in HvBRD (Figs. 2 and 3). Three plants showed a severe dwarf phenotype (Fig. 4) and are homozygous for the mutation in BW333, but no mutation in ari-358 has yet been found as mentioned above.

The MH203 mother (BW031) has a mutation in HvBRD and the father (ari-415) has a mutation in HvDIM (Figs. 2 and 3). One plant, number 14 showed a severe dwarf phenotype (Fig. 4) and is homozygous for the mutation in BW031 though it has not yet been sequenced for the ari-415 mutation. Number 16 showed a semi-dwarf phenotype (Fig. 4) and is homozygous for the mutation in BW301 but has not been sequenced for the ari-415 mutation.

The MH204 cross has a mother (BW084) that has a mutation in HvCPD and a father (ari-415) with a mutation in HvDIM (Figs. 2 and 3). Plant number 31 showed a severe dwarf phenotype (Fig. 4) and is homozygous for the mutation in BW084 though it has not been sequenced for the ari-415 mutation. Plant number 33 showed semi-dwarf phenotype (Fig.4) and is homozygous for the mutation in BW084, while not being sequenced for the ari-415 mutation.

**DISCUSSION**

Of the five ari-mutants, which had suspected mutations in the HvBRD gene, ari-400, ari-402 and ari-465 were identified to have a mutation, while none were found in ari-263 and ari-358. ari-415 was identified to have a mutation in the HvDIM gene. Lastly, a mutation in the HvBRI1 gene was identified in ari-312, but no mutation in the same gene was found in ari-380. Regarding the F2populations, MH155, MH156, MH165, MH203 and MH204 had plants showing severe dwarf phenotype. These plants were found to be homozygous for the mutation inherited from the mother, while plant number 16 in MH155 and plant number 11 in MH156 were sequenced and identified to be homozygous for the mutation inherited from the father.
Although mutations could not be found in *ari*-263 and *ari*-358, they probably do have mutations. The mutations are likely to be located in the fragments of the *HvBRD* gene that were not amplified, although several different PCR-mixes and PCR-programs were tested. Furthermore, MH165 plant number 15, whose father is *ari*-358, had a severe dwarf phenotype and was found to be homozygous for the mother's mutation, indicating that there is a mutation in *HvBRD* in *ari*-358 as well. The *ari*-mutants whose mutation could be identified in *HvBRD* were found to be in a conserved domain of the brassinosteroid-6 oxidase. This domain is also conserved in the cytochrome P450 superfamily, which include proteins involved in degradation of various compounds (NCBI, 2016).

Both *ari*-400 and *ari*-402 had the amino-acid residues in this domain replaced by a stop codon, while *ari*-465 had proline changed to serine. Especially the mutations generating a stop codon would severely affect the effectiveness of the enzyme, but the change from proline (hydrophobic) to serine (polar) could also influence effectiveness. Since the domain is conserved it is likely that it plays a role in the enzyme’s activity and the mutations in this domain are likely to explain the semi-dwarf phenotype of these mutants.

The mutation of *HvDIM* in *ari*-415 could be identified and was found to be in the FAD-binding domain. The enzyme Δ5-sterol-Δ5-reductase uses FAD as a co-factor (NCBI, 2016) and mutations in this domain might change the binding site of FAD.
preventing the co-factor and enzyme to interact, leading to lowered effectiveness of the enzyme. The mutation in ari-415 replaced glycine with aspartate. Since aspartate is charged and much larger than glycine, it can be expected to change the structure and the hydrophobicity of the binding site of FAD. This can consequently lower the enzyme’s activity, leading to the semi-dwarf phenotype.

The mutation in ari-380 could not be found and like the previous case with ari-263 and ari-358 this could be explained by the failed amplification of one of the fragments in HvBRD, where the mutation is likely to be found. The mutation in ari-312 in the activation loop domain was found to be at the phosphorylation site of the domain. The threonine that was replaced is polar while alanine is hydrophobic. Alanine cannot be phosphorylated and thus the normal orientation of the loop will be prevented. This could lead to low or no activation of the BRI1 kinase, which in turn affects the interaction with the substrate (Bojar et al. 2014). This might explain the lower brassinosteroid signalling, leading to the semi-dwarf phenotype in ari-312.

Because of time constraints not every F2-cross showing severe dwarf phenotype was genotyped. Those who were sequenced are homozygous for the mutation in the BW-line as well as the mutation in the ari-mutants, indicating it to be likely that a severe dwarf phenotype correspond to being homozygous for the mutation in both parents. Comparing the different crosses’ phenotypes, there does not seem to be a difference if the F2 plant is homozygous for mutation in two enzymes active in the biosynthesis of brassinosteroids (for example MH203) or homozygous for one enzyme in the biosynthesis and the other mutation is in the brassinosteroid receptor. This could be open for further research and it would be interesting to explore which combinations of mutations that give the most severe phenotype. Comparing MH165 and MH203, their crosses are reciprocal and when observing the phenotypes they have the same size, but the MH203 dwarf do not stand up as good as the MH165 dwarf. Interestingly, the two semi-dwarfs in MH156 showed different degree of dwarfism. Plant number 34, which is heterozygous for the mutation in BW084 is shorter than number 29, which is homozygous for the same mutation. In turn, number 29 is heterozygous for the mutation in ari-312. It would be interesting to see if number 34 is homozygous or heterozygous for the ari-312. Then one might examine if being homozygous for mutation in HvBRI1 gives a more severe dwarf phenotype than being homozygous for the mutation in HvCPD.

Even though not all ari-mutants and the F2 plants with severe dwarf phenotype have been sequenced for their respective mutation, our results in this project can be used for further research on deficiencies in brassinosteroid synthesis and signalling. The found mutations can also be used in marker-assisted barley breeding to develop more varieties with resistance against lodging to improve the already well adapted barley to future climate changes.

REFERENCES:


12. The international barley genome sequencing consortium. 2012. A physical, genetic and functional sequence