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# 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 *Brassinosteorid-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. $F_2$-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.**

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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 (Sreenivasuluet 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* (*HvBRD*), *DIMINUTO* (*HvDIM*), *constitutive photomorphogenic dwarf* (*HvCPD*) and the gene encoding the brassinosteroid receptor is *brassinosteroid-insensitive1* (*HvBRI1*). 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 *HvBRI1*, the gene is located on chromosome 3H and the encoded brassinosteroid receptor is a transmembrane protein serine/threonine kinase (Gruszka et al, 2011). *HvBRD* encodes the enzyme brassinosteroid-6 oxidase and is located on chromosome 2H. Lastly, *HvDIM*, like *HvBRD*, encodes an enzyme in the synthetic pathway called $\Delta^5$-sterol-$\Delta^5$-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 *HvBRD* 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 *HvBRI1* 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 $F_2$-populations. The $F_2$-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 *HvBRD*), BW084 (*brh13.p* in *HvCPD*), BW333 (*ert-zd.159* in *HvDIM*) and BW885 (*uzu1.a* in *HvBRI1*) (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 $F_2$-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 $F_2$ 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 $F_2$ 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 *HvBRD*, *HvBRI1 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 $F_2$-plants were made using REDExtract-N-Amp Plant PCR Kit (Sigma). In one PCR tube 4 µl DNA template, 4 µl water,

10 µl REDExtract-N-Amp PCR ReadyMIX, 1 µl forward primer and 1 µl reverse primer were mixed together. For the fragments that were needed to be cut out from the 1% agarose gel (for separation of PCR-products, see below), the volume of the solution was doubled to 40 µl. The fragments that were cut out were those that did not have one clear band in the gel. For fragments 2, 3 and 4 of *ari-358* and *ari-402* the Thermo Specific Phire Plant Direct (Thermo Scientific) was used, one PCR tube containing 10 µl PCR MasterMix, 1 µl forward primer, 1 µl reverse primer, 0,5 µl DNA template and 7,5 µl water. To find the double mutants in the $F_2$-plants half the volume (10 µl in total) of REDExtract-N-Amp Plant PCR Kits was used. The identified double mutants in $F_2$ were then amplified using the REDExtract-N-Amp PCR Kit with the same volumes as written above.

**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/4s, 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/90 s repeated 32 cycles, followed by 72°C 5 minutes. A PCR gradient (52° C to 62° C) was 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 of 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.

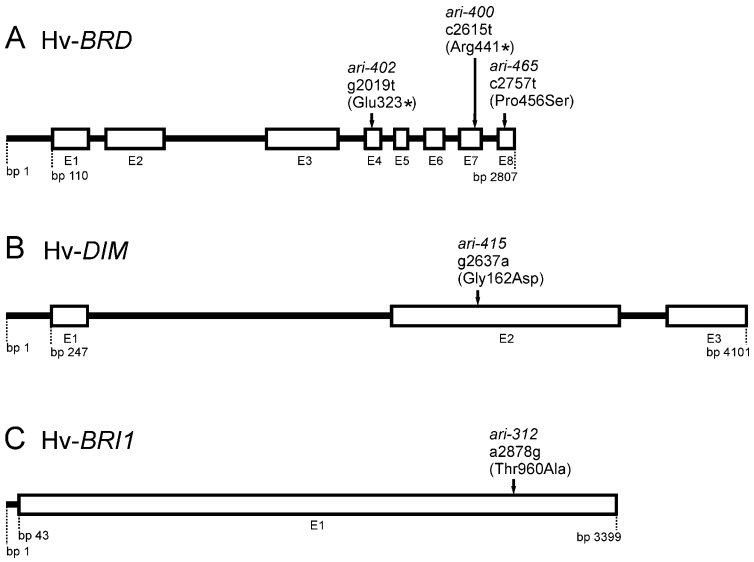


Fig. 1. Identified mutations in genes involved in brassinosteroid biosynthesis and signalling. *ari-402* has a mutation in exon 4 of *HvBRD* with the base substitution g2019t. This substitution gives a stop codon instead of glutamate at position 323 in the amino acid sequence of the enzyme brassinosteroid-6 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 c2757t 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 $\Delta^5$-sterol-$\Delta^5$-reductase (B). *ari-312* is found to have the mutation a2878g 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).

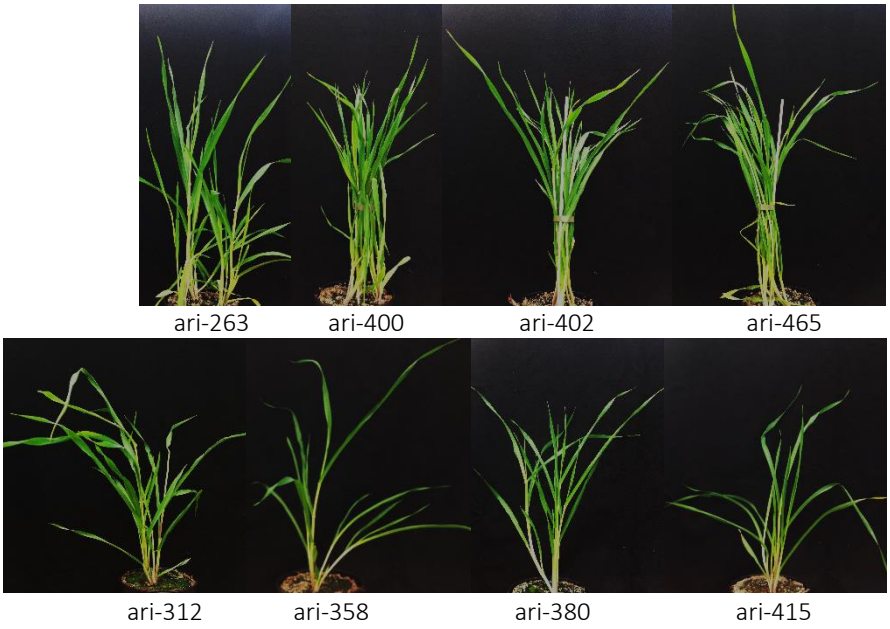


Fig. 2. Phenotypes of the *ari*-mutants. Plants had grown for approximately 7 weeks in greenhouse. No spikes had developed during this time. Three seeds were planted for *ari-263*, *ari-400*, *ari-402* and *ari-465* while the rest had one seed planted. These phenotypes are semi-dwarf and especially the mutants where only one seed were planted show a strong culm that is unlikely to lodge.

## 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 $\Delta^5$-sterol-$\Delta^5$-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 *BRI1* 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 *HvBRI1* 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 1. Crosses between *ari*-mutants studied in this project and known *ari*-mutants (BW-lines).

| Name of cross | Cross |
|---|---|
| MH155 | BW031 x *ari-312* |
| MH156 | BW084 x *ari-312* |
| MH157 | BW333 x *ari-312* |
| MH162 | BW885 x *ari-358* |
| MH164 | BW084 x *ari-358* |
| MH165 | BW333 x *ari-358* |
| MH175 | BW031 x ari-380 |
| MH176 | BW084 x *ari-380* |
| MH177 | BW333 x *ari-380* |
| MH203 | BW031 x *ari-415* |
| MH204 | BW084 x *ari-415* |

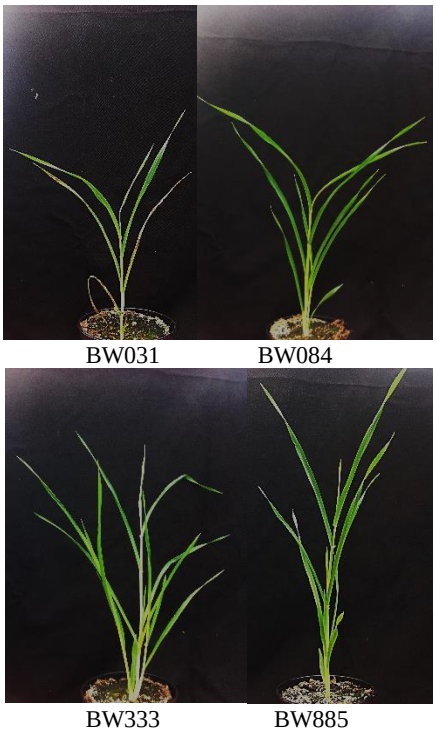


Fig. 3. Mother varieties for crosses. These mutants have already been sequenced and mutations were identified (Dockter et al. 2014). BW031 has a mutation in *HvBRD*, BW084 has a mutation in *HvCPD*, BW333 has a mutation in *HvDIM* and BW885 has a mutation in *HvBRI1*. They show a strong culm and semi-dwarf growth.

**Genotyping of $F_2$-plants.** In order to identify double mutants, 40 $F_2$ seeds from each of the 11 crosses were planted (Table 1). The most severe dwarf phenotypes were found among the crosses MH155, MH156, MH165, MH203 and MH204. The MH155 mother (BW031) has a known mutation in *HvBRD* and the father (*ari-312*) has an identified mutation in *HvBRI1* (Figs 2 and 3). Of the 40 MH155 plants, three of them showed a severe dwarf phenotype (Fig. 4) and these three are also confirmed to be homozygous for the mutation of BW031. Plant number 16 was sequenced and found to be homozygous for the *ari-312* mutation. Plants number 22 and 32 have not been sequenced yet. Heterozygous plants were also found in a greater number and are represented by plant number 27 (Fig. 4). The plant is homozygous for the mutation in BW031 while being heterozygous for the *ari-312* mutation. The phenotype is not as severe as the known (and suspected) double homozygous mutants.

MH156 has the same father as previous cross, but the mother is BW084, which has a mutation in *HvCPD* (Figs. 2 and 3). This cross had one plant showing severe dwarf phenotype, number 11 (Fig.4) and is homozygous for the mutations in both parents. Plant 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 BW031 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 $F_2$-populations, 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.

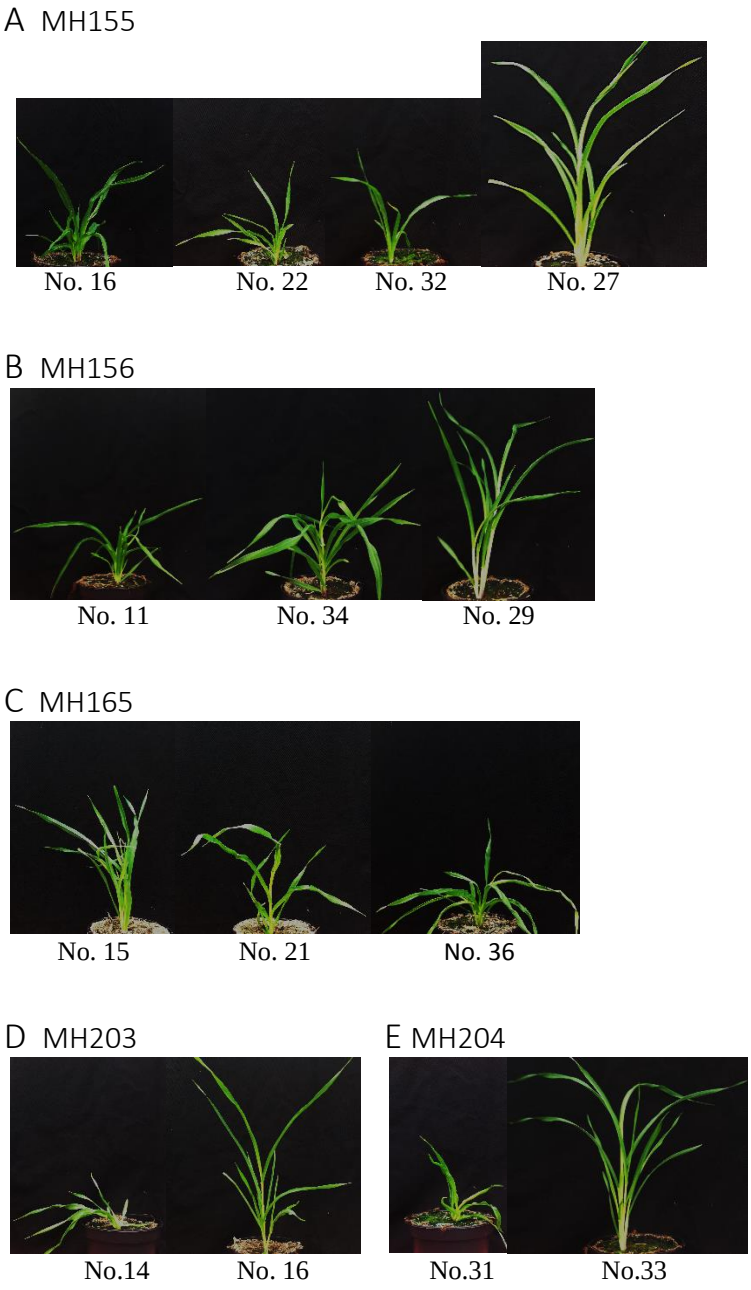


Fig. 4. $F_2$-cross MH155 (BW031 x *ari-312*). Plants number. 16, 22 and 32 show severe dwarf phenotype. All 3 plants are confirmed to be homozygous for the BW031 mutation. However, only plant number 16 has been sequenced for *ari-312* and is found to be homozygous for the mutation. Plants number 22 and 32 have not been sequenced yet. Plant number 27 is homozygous for mutation in BW031, but heterozygous for the mutation in *ari-312*. This plant, instead of having the severe dwarf phenotype, has a semi-dwarf phenotype (A). $F_2$-cross MH156 (BW084 x *ari-312*). Plant number 11 has severe dwarf phenotype and is found homozygous for the mutation in both BW084 and *ari-312*. Plant number 34 is heterozygous for the mutation in BW084 and has not been sequenced for the mutation in *ari-312*. Plant number 29 is homozygous for the mutation in BW084 but heterozygous for the mutation in *ari-312*. Comparing plant number 34 and number 29, 34 seems to have a more severe dwarf phenotype (although not as severe as plant number 11), while 29 has a semi-dwarf phenotype (B). $F_2$-cross MH165 (BW333 x *ari-358*). All plants show severe dwarf phenotype and are homozygous for the mutation in BW333. Mutation in *ari-358* has not been found so sequencing is not possible at the moment (C). $F_2$-cross MH203 (BW031 x *ari-415*). Plant number 14 shows severe dwarf phenotype and is found homozygous for the mutation in BW031 but has not been sequenced for mutation in *ari-415*. Plant number 16 shows semi-dwarf phenotype and is homozygous for mutation in BW031, but not sequenced for the mutation in *ari-415* (D). $F_2$-cross MH204 (BW084 x *ari-415*). Plant number 31 shows severe dwarf phenotype and is found to be homozygous for the mutation in BW0384 but has not been sequenced for the mutation in *ari-415*. Plant number 33 shows semi-dwarf phenotype and is homozygous for the mutation in BW084, but has been not sequenced for the mutation in *ari-415* (E).

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 $\Delta^5$-sterol-$\Delta^5$-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 $F_2$-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 $F_2$ 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 $F_2$ 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.

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