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INHERITED PHENOTYPIC INSTABILITY OF BARLEY HOMEOTIC SINGLE AND DOUBLE MUTANTS AND ITS POSSIBLE CAUSES

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VILNIAUS UNIVERSITETAS

RAIMONDAS ŠIUKŠTA

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INTRODUCTION

Phenotypic instability is relatively frequent in natural conditions – it may be caused by various ecological factors such as temperature and illumination (Bonnett, 1966), abiotic stress (Boyko and Kovalchuk, 2011; Yao et al., 2011) or pathogen infections (Boyko et al., 2007; Ghareeb et al., 2011). Furthermore, intriguing phenotype variations have been also observed among several single and double mutants (Forster et al., 2007; Babb ir Muehlbauer, 2003; Trevaskis et al., 2007; Wang et al., 2010; Dreni et al., 2011; Li et al., 2011; Müller-Xing et al., 2014; Zheng et al., 2015; Šiukšta et al., 2015) and even epimutants (Zhang et al., 2012). Despite its widespread prevalence in nature, the mechanisms of such phenotype variations are not studied enough, especially of phenotypic instability, arising among double mutants when for constituent single mutants such variations are absent. There are only a few studies investigating this phenomenon (Müller-Xing et al., 2014).

The hybridization of homeotic barley *Hv-tweaky spike*-type mutants (allele *Hv-tw$_2$*), having a specific polar spike architecture and lodicules that are irregularly transformed into stamens and/or pistils, with different *Hv-Hooded/Kap1*-type (*Hv-Hd*) mutants, containing a 305-bp duplication in intron IV of the *BKn3* gene that causes the development of ectopic flower-like structures in the lemma/awn transition zone (Müller et al., 1995), led to *Hv-tw$_2$;Hd* double mutants with wide variations of the ectopic expression of both homeotic mutations (Vaitkūnienė et al., 2004a, b). While some of such double mutants are phenotypically stable and may be directly used as ornamental plants (Siuksta et al., 2012), the other portion of them develops new features, such as ectopic leafy/shoot-like outgrowths in part of tillers and long naked gaps on rachis, which are absent in the parental mutants. Variations of the *Hv-tw2;Hd* double mutants are so wide that cover even the phenotypes of many known mutants of barley and other grasses (Bonnett, 1966; Babb and Muehlbauer, 2003; Duan et al., 2003; Ikeda et al., 2007; Trevaskis et al., 2007; Whipple et al., 2010).

According to several characteristics of phenotype variations, *Hv-tw$_2$;Hd* double mutants resemble several mutants of the auxin-signalling pathway (Krizek, 2011; Gallavotti, 2013), whereas the character of the phenotypic variations in the *Hv-tw$_2$;Hd* double mutants suggests that their hormone pathway may be unbalanced. On the other hand, recently it has been shown that 305-bp duplication in intron IV of gene *BKn3* has regulatory elements that bind ethylene-responsive proteins, and the *Hv-Hooded* phenotype may be partially normalised by the ethephon, an ethylene releasing compound (Osnato et al., 2010). Thus, we hypothesised the possible relation of the phenotypic instability of *Hv-tw$_2$;Hd* barley double mutants with the ethylene pathway.

Moreover, when a wider spectrum of barley double mutants, such as *Hv-tw;laxatum-a* (*Hv-tw;lax-a*), *Hv-tw;tweaky No.18* (*Hv-tw;tw No.18*) and *Hv-tw;tweaky and missing kernels* (*Hv-tw;twmk*) was studied for the instability expression, it was noted that the phenotypic instability was triggered by *Hv-tw*-type alleles. In order to reveal the preliminary changes in the gene expression profile of the mutant *Hv-tw$_2$* that may be related to the phenotypic instability induction in barley double mutants, a differential display PCR analysis was performed.

Aim of the study

The aim of this study is to evaluate the character of the phenotype variation spectrum in barley homeotic double mutants, formed by hybridization of *Hv-tw*$_2$ and *Hv-Hd* single mutants, and to examine the possible causes of such phenotypic instability.

Tasks of research

1. To examine the phenotype variation spectrum of double mutants and to determine the definite trends of ectopic flower development.
2. To perform DNA sequencing of two allele-specific diagnostic markers in the barley *BKn3* gene and to identify the *BKn3* allele type in single and double mutants.
3. To evaluate the callus growth intensity of barley single and double mutants.
4. To determine the impact of exogenous application of the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) on spike development of barley single and double mutants.
5. To evaluate the effect of 2,4-D and auxin inhibitors on basic and ectopic flower structures of barley single and double mutants.
6. To determine the effects of the exogenous ethylene on the development of awns, basic and ectopic flowers in barley single and double mutants.
7. To investigate a wider spectrum of barley double mutants and to determine the mutation that specifically triggers the phenotypic instability in barley double mutants.
8. To carry out the preliminary analysis of differences in gene expression between the mutant *Hv-tw*$_2$ and *WT* (cv. 'Auksiniai II') using the differential display method, and to determine the genes-candidates that may be related to phenotypic instability.

The statements to be defended

1. The phenotype variation of barley double mutants *Hv-tw*$_2$*;Hd* covers a wide spectrum of known developmental mutations and even exceeds it in extent.
2. Despite their extremely different phenotype, all barley single and double *Hv-Hd*-type mutants have the same type *BKn3* allele (IIIc).
3. Barley *Hv-tweaky*-type mutations are associated with defects in the auxin function and serve as a trigger of phenotypic instability in barley double mutants.
4. The phenotypic instability of barley double mutants is caused by ectopic local disturbances in the auxin concentration.
5. The effect of ethylene on the flower/inflorescence structure of barley single and double mutants is mainly genetic background-dependent and is best pronounced in the ectopic flower-like structures.

Scientific novelty of the study

Based on the investigations of phenotypic instability modification by the synthetic auxin 2,4-D and auxin inhibitors HFCA and PCIB in barley double mutants *Hv-tw*$_2$*;Hd*, auxin imbalance has been described as a cause of phenotypic instability for the first time. The effects of 2,4-D on barley single and double mutants' flower/inflorescence structures and callus growth allowed us to link *Hv-tweaky*-type mutations with defects in the auxin function. The preliminary analysis of differences in gene expression between the mutant *Hv-tw*$_2$ and *WT* (cv. 'Auksiniai II') showed that the *Hv-tw*$_2$ mutant differs from *WT* by the expression of several genes, encoding the transcription factors and proteins that are involved in chromatin activity regulation, stress responses and

developmental events, and that may contribute to the phenotypic instability in barley double mutants.

The sequencing of two *BKn3* gene regulatory regions of fourteen *Hv-Hooded/Kap1*-type mutants preserved in the collection of the Vilnius University Botanical garden showed that, despite of the great difference in *Hv-Hd* phenotype expression, all the single and double *Hv-Hd*-type barley mutants have the same *BKn3* allele (IIIc).

Practical and scientific significance

We have selected a group of stable barley double *Hv-tw$_2$;Hd* mutants that may be directly used for ornamental purposes or may be employed as the initial material to create new decorative stocks. Moreover, according to the formation of leafy/shoot-like structures, some genotypes were selected from several hundreds of *Hv-tw$_2$;Hd* double mutants preserved in the collection of the Vilnius University Botanical Garden, and the genetic lines (offspring of an individual plant) from them were created. Such double-mutant lines are a valuable material for the further study of barley inflorescence and flower reversion. The results of the investigation of auxin and ethylene effects on floral organ development and flower/inflorescence reversions in barley double mutants will complement the fragmentary fundamental understanding about the role of both phytohormones in the regulation of reproductive processes of grasses.

The preliminary search of differentially expressed genes in the mutant *Hv-tw$_2$* led us to identify four new barley cDNA fragments that will be registered in the NCBI database.

The presentation and approbation of the results

Two scientific papers on the topic of the dissertation were published in the ISI Web of Science Journal List issues with the impact factor, plus one scientific publication in the ISI Conference Proceedings, two scientific papers in peer-reviewed international journals without the impact factor, and one scientific paper in Lithuanian peer-reviewed scientific periodical. The results of the research were presented at five international and one Lithuanian conferences.

The structure and volume of the dissertation

The dissertation contains the following chapters: List of acronyms, Introduction, Review of the literature, Materials and methods, Results, Discussion, Conclusions, List of publications and Conference abstracts, Acknowledgements, References (270 reference sources). The volume of the dissertation is 138 pages. The dissertation is illustrated with 12 figures and 21 tables. The dissertation is written in Lithuanian with a summary in English.

MATERIALS AND METHODS

A collection of dominant *Hv-Hooded* (*Hv-Hd*) barley (*Hordeum vulgare*) mutants, also a part of *Hv-laxatum*-type mutants, including *Hv-lax-ab* (GSHO 1573), *Hv-lax-ac* (GSHO 1574) and *Hv-lax-ae* (GSHO 2041), and *Hv-tweaky*-type mutants *Hv-tweaky No.18* (*Hv-tw No.18*; GSHO 111) and *Hv-tweaky and missing kernel* (*Hv-twmk;* GSHO1119) were obtained from the USDA-ARS National Small Grains Collection (Aberdeen, ID, USA). The recessive allelic *Hv-tweaky spike* (*Hv-tw* and *Hv-tw$_2$*) mutants (Fig. 1Q) were induced by chemical mutagenesis using ethylene imine in barley cv. 'Auksiniai II' (Fig. 1P), which in the present study was used as a *Wild Type* (*WT*). A part of *Hv-laxatum* collection, including *Hv-lax-a.434*, was obtained from the Nordic Gene Bank (Alnarp Sweden). To produce double mutants, the hybridization of the paternal mutants was performed in 2003 (Vaitkūnienė et al., 2004a, b). As the *Hv-Hd*-type mutants differ significantly in spike architecture, two alternatives of different expression of *Hv-Hd* phenotype were selected from 14 *Hv-Hd*-type mutants, namely, mutant *Hv-Hooded/Kap1.a* in Colsess II (*Hv-H*; GSHO 67; Fig. 1S) and mutant *Hv-Lemma hooded* (*Hv-Lh*; GSHO 932; Fig. 1R). *Hv-H* mutant was created by introduction of *Hv-Hooded/Kap1a* mutant gene into the cv. Atlas 46 from the cv. Colsess (Stebbins and Yagil, 1966). *Hv-Lemma hooded* mutant was selected by T. Tsuchiya from Clho 6868 whose pedigree is *Triple Bearded Mariout* (Clho *2523*)/*Hooded Awn* (Clho 6176//*Brittle Rachis* (Clho 6170)/Lyallpur (PI 57959) (http: www.ars-grin.gov/cgi-bin /npgs/acc/display. pl?103790).

All tested material was bred and studied in the Vilnius University Botanical garden and in the greenhouse of the Department of Botany and Genetics. For exposure to ethylene, auxin inhibitors or 2,4-D, only double-mutant lines (offspring from the initial individual plants) were used. Lines were created using F_6 material and were subject to preliminary testing for at least three generations before exposure to auxin inhibitors or 2,4-D. The selection of individual plants was based (1) on their flower/inflorescence structures according to both *Hv-Hd* and *Hv-tw* phenotypes, and (2) on their capacity to produce ectopic leafy/shoot-like structures in spikes.

Flowers and spikes were fixed in Carnoy's solution (ethanol: glacial acetic acid, 3:1) and analyzed under a stereomicroscope (Motic SMZ-143) connected to a photo camera (Moticam 2000).

Plant treatments with ethylene, auxin inhibitors and 2,4-D

Under both field and greenhouse conditions, treatment with ethylene, auxin inhibitors and 2,4-D was started at the beginning of the 5th leaf stage as it is regarded as the most critical stage for inflorescence/flower development (Yagil and Stebbins, 1969; Osnato et al., 2010). Plants were sprayed six times with 100 mM solutions of either the auxin transport inhibitor 9-hydroxyfluorene-9-carboxylic acid (HFCA) or the antiauxin p-chlorophenoxyisobutyric acid (PCIB). To avoid the necrotic injury, plants were sprayed with 2,4-D sodium salt (2 g·L^{-1}) only once. Treatment with ethephon (2-chloroethylphosphonic acid), an ethylene releasing compound, was conducted according to Yagil and Stebbins (1969) and Osnato et al., (2010). To facilitate the adhesion of the solutions, a surfactant Tween20 (final concentration of 0.1%) was added to all spray solutions. All chemical materials for plant treatment were obtained from Sigma-Aldrich (Germany).

Callus cultures and treatment with inhibitors

Seeds for embryo dissection were surface-sterilized with a commercial bleach ACE and water solution (1:1) for 18 min and washed 5× with sterile water. Water-imbibed embryos were aseptically isolated and cultivated in Petri dishes for 4 weeks at 25 °C in the dark (Incucell, MMM Medcenter, Einrichtungen, Germany). Preliminarily calli were induced and grown on the Murashige–Skoog (MS) medium with 3 $mg \cdot L^{-1}$ 2,4-D (Alfa Aesar, Germany). Then, the calli were individually divided into portions for treatment with the HC-toxin (10 $\mu g \cdot mL^{-1}$ dissolved in DMSO, final concentration 0.1 %), and for two controls – H_2O and DMSO. All portions were weighed on an analytical balance and placed on MS medium with a particular compound and grown in a thermostat at 25 °C in the dark for 4 weeks. After the 4-week growth period, the calli were once again weighed and the growth index (GI) was calculated according to the formula $GI = (m - m_0)/m_0$, where m is the final callus weight and m_0 is the initial callus weight. All manipulations, including callus weighing, were conducted under sterile conditions in a laminar box (SAFE 2020, Thermo Scientific). All solutions of thermolabile compounds were filter-sterilized using 0.22 μm pore diameter syringe filters (Roth, Germany).

DNA extraction and *BKn3* gene sequence analysis

DNA was extracted from young leaves using the Hk0512 Genomic DNA Purification Kit (ThermoFisher, Lithuania) according to the manufacturer's protocol. DNA was amplified with the MasterCycler Personal (Eppendorf), and all of the reagents for PCR were obtained from ThermoFisher (Lithuania). For DNA sequencing in two polymorphic regions of the *BKn3* gene, two pairs of primers were used (dir./rev.): 5'-GGCATTGTGTGGCAATGCGTGACA-3'/5'-ACACTACTCTCGAGCACCTTCCTC-3' (for the *BKn3* promoter region) and 5'-CCATGCAGCTACCTCTTGTCCTCG-3'/5'-GGAAAGCATCCAACGTCTCTTGAC-3'(for the region in intron IV). The primer pair used to determine the 305-bp duplication in *BKn3* intron IV was 5'-TTCTTTGTGTGTGTTCTGGGG-3'/5'-AGGTTTGAACTTGGACTCGCC-3'. Each 50-μL polymerase chain reaction contained 5 μL of 10× PCR buffer (+$MgSO_4$), 200 μM $MgCl_2$, 160 μM dNTPs, 3 units of *Taq* polymerase, 0.25 μM each primer and 100 ng of DNA. The PCR was conducted for 5 min at 97 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 65 °C, and 2 min at 72 °C and a final extension step of 5 min at 72 °C. In parallel to each amplification reaction, a negative control PCR without DNA template was performed. The PCR products were fractionated in 0.8 % agarose gel, and the proper fragments were purified with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA) according to the manufacturer's recommendations. Purified amplicons were sequenced by BaseClear (Netherlands). The selection of *BKn3* polymorphic regions and the determination of the *BKn3* allele were performed according to Badr et al. (2000). Multiple sequence alignment was conducted using MEGA version 4.0.2 software (Tamura et al., 2007).

Gene expression analysis using differential display method

Total RNA from *Hv-tw*$_2$ and *WT* (cv. 'Auksiniai II') plants was extracted from the apical stem segments containing inflorescence primordium at the 5th leaf stage using innuPREP Plant RNA Kit (Analytik Jena, Germany) according to the manufacturer's recommendations. All steps of the differential display procedure were performed using RNAspectra™ Red Kit 7 (GenHunter, USA) following the manufacturer's protocol. In

brief, reverse transcription of the mRNR was performed using three oligo-dT primers anchored with C, G and T at the 3'-end. The resulting three cDNA subpopulations were amplified in parallel PCRs by using 24 combinations of three 3'-anchored oligo-dT primers with 8 arbitrary 13mer primers. DD-PCR products were separated by electrophoresis on 6% denaturing polyacrylamide gels in 1× TBE buffer and stained with silver according to Benbouza et al. (2006). Differentially expressed bands were cut out of the gel, reamplified in two rounds of PCR, purified and sequenced by BaseClear (Netherlands). Homology searches were performed using the BLAST algorithm (Altschul et al., 1990) in the NCBI database.

Statistical analysis

The mean values ± SE are given in the tables. The significance of the differences between the means was evaluated using Student's t-test.

RESULTS

Phenotypic instability of *Hv- tw$_2$;Hd* double mutants

Simultaneous variations in the structure of the basic flowers and in the ectopic transformation of the awns to floral structures may be naturally expected in *Hv-tw$_2$;Hd* double mutants originated from the hybridization of two different type homeotic *Hv-tw$_2$* and *Hv-Hd* mutants. However, the phenotypic variations significantly exceeded those of the single mutants in basic and ectopic flower organs, and new types of the spike (inflorescence) variation were observed (Fig. 1). The phenotype variations in double-mutant lines were so wide that covered the phenotypes of many known mutants in barley and other grasses.

The most peculiar new features that were absent in the parental mutants were bract/leafy-like and shoot-like structures inside a spike and long naked gaps on the rachis (spike axis), varying in the degree of expression (Fig. 1M–O). To separate group were attributed plants simultaneously having two features – the bract/leafy-like structures and the long naked gaps without florets on the rachis (Fig. 1A–F). The other phenotypic group consisted of plants forming shoot-like structures within the spike (Fig. 1H, I, K, L). Rarely, ectopic shoots also developed extra spikes (Fig. 1M). Though the ectopic leafy/shoot-like phenotype is also a feature of several other barley mutants and hybrids (Babb and Muehlbauer, 2003; Forster et al., 2007; Trevaskis et al., 2007; Curaba et al., 2013), but plants with long, naked gaps on rachis were most intriguing in our study. The latter phenotype, or even fully naked pin-formed structures, is specific to mutations in cereal genes that are involved in auxin synthesis, transport or response (McSteen et al., 2007; Morita and Kyozuka, 2007; Gallavotti, 2013).

A great extent of variation was also observed in the degree of the ectopic outgrowths in the lemma/awn transition zone and more distantly along the awn (Fig. 1). The high variation amongst ectopic flower-like structures suggests a definite developmental trend of flower development, varying from insignificant outgrowths on the awn to a tube (of non-inverted/inverted positions) and finally to flowers with sterile sexual organs.

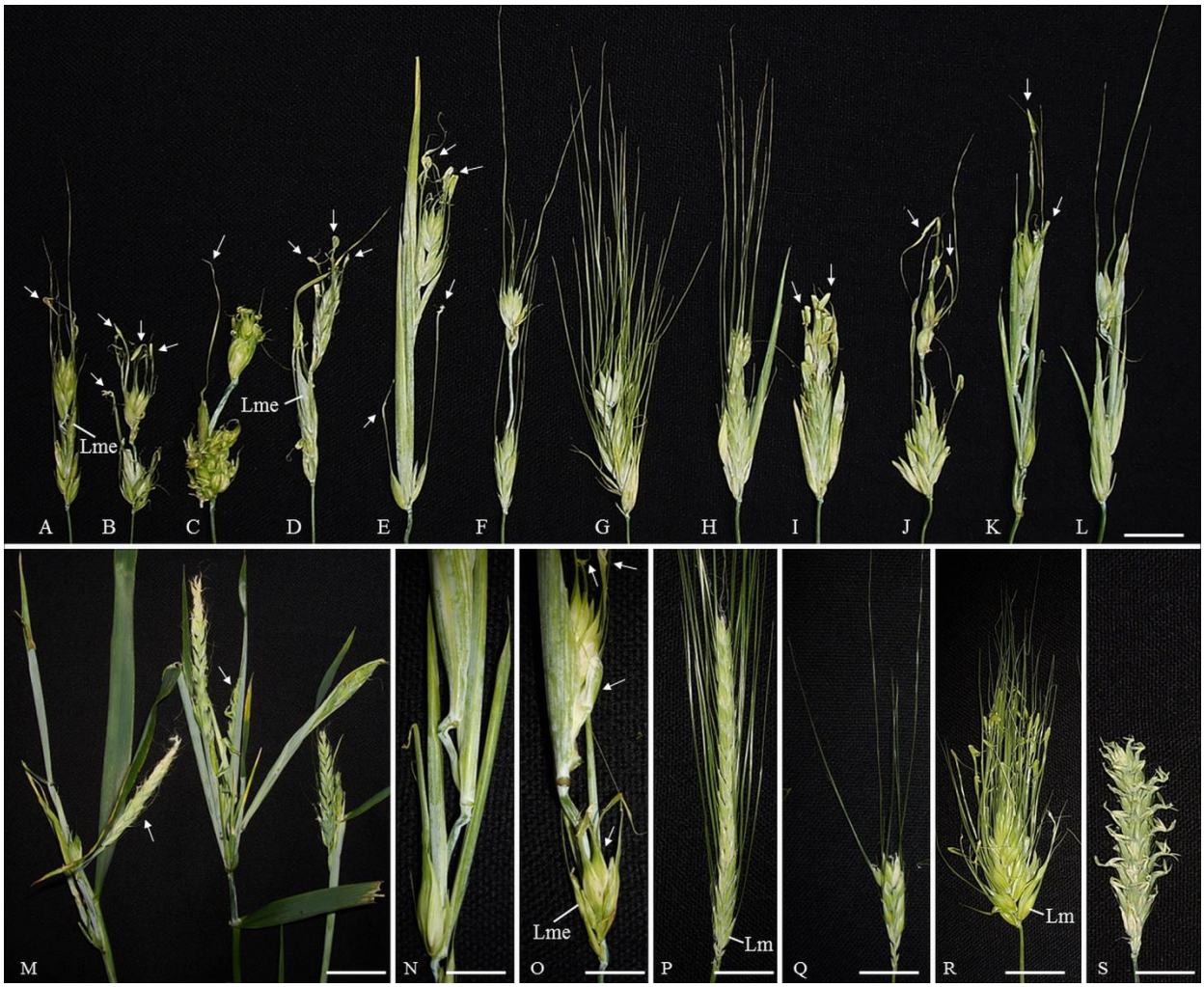

Fig. 1. Inflorescence variations in barley double mutants. (A-L) – inflorescence variations in *Hv-tw$_2$;Lh*: (A-E) bract/leafy-like spikes with long naked gaps on rachis; (F) long gaps with longer bract/lemma; (G) short gap; (H, I, K, L) shoot-like spike structures of various extents (arrows denote insignificant outgrowths on the awn); (M-O) leafy-like phenotype of *Hv-tw$_2$;H* N6 double mutant (arrows indicate the ectopic spikes); (P-S) spikes of parents: (P) *Hv-WT*, (Q) *Hv-tweaky spike 2* (*Hv-tw$_2$*), (R) *Hv-Lemma hooded* (*Hv-Lh*), (S) *Hv-Hooded/Kap1* (*Hv-H*). Lm – lemma with awn; Lme – lemma elongation. Scale bars = 20 mm, 40 mm, 8 mm, 8 mm, 20 mm, 20 mm, 20 mm, 20 mm, respectively (Šiukšta et al., 2015)

Comparison of meristem growth intensity in callus cultures

The data presented above suggest that the phenotypic instability of *Hv-tw$_2$;Hd* double mutants may be caused not only by the ectopic expression of organ identity genes but also by more intensive division of meristematic cells. This presumption was verified by examining callus cultures from the initial mutants and double mutant lines that had varying expressions of leaf/shoot-like inflorescence phenotypes (Table 1). A very weak callus growth intensity of plants with the *Hv-tw* allele was noted, while plants with the *Hv-tw$_2$* allele, used as mother plants, did not differ significantly from the *WT* (Table 1). In general, lower callus growth intensity was a common feature of all *Hv-Hd* mutants involved in the hybridization, as well as *Hv-tw$_2$;Hd* double mutants. Callus growth was slightly more intensive in several double-mutant lines than in the respective parental mutants. However, the growth intensities of the intact plants were not as uniform under field conditions. The height of the *Hv-Hcs* mutant and the double mutant lines *Hv-H;Hcs*

N9 and *Hv-tw$_2$;Dwh* N25 significantly exceeded that of *Hv-wt* (cv.'Auksiniai II') despite their low callus growth under baseline conditions (without DMSO and HC toxin; Table 1). Despite the fact that DMSO was only used for dissolving HC toxin in water, it unexpectedly caused a stimulatory effect on callus growth in plants with the strongest *Hv-tw* allele. However, the stimulatory effect of HC toxin on the growth of *Hv-tw* callus was still stronger, ~2.0-fold greater than that of DMSO. In addition to *Hv-tw*, DMSO only affected one of the *Hv-Hd* type mutants, *Hv-Hcs* but, in contrast to *Hv-tw*, in this case the effect of DMSO was inhibitory. The effect of the HC toxin clearly depended on the plant genotype. The HC toxin increased callus growth in two *Hv-Hd* type mutants, *Hv-Dwh* and *Hv-Hcs*, and in several double-mutant lines of different origin: *Hv-tw$_2$;Lh* N11, N19; *Hv-tw$_2$;Dwh* N25 and *Hv-H;Hcs* N9. Notably, all these double mutants had 'leaf/shoot'-forming phenotypes (Table 1).

Table 1. Intensity of callus meristem growth (GI) of barley single and double mutants with and without DMSO or HC-toxin

Genotype	Control (H_2O)	Control (DMSO)	DMSO + HC	Height of intact plant (cm)
***WT* (-)**	4.18 ± 0.57	3.18 ± 0.50	4.26 ± 0.66	75.2 ± 1.2
Hv-tw (-)	0.35 ± 0.08^{c3}	0.70 ± 0.16^{c3}	1.30 ± 0.24^{c3}	71.7 ± 0.9
***Hv-tw$_2$* (-)**	3.90 ± 0.58	3.97 ± 0.54	2.82 ± 0.30^{a}	66.4 ± 1.3^{3}
***Hv-Lh* (-)**	1.46 ± 0.24^{c3}	1.91 ± 0.35^{a2}	1.61 ± 0.30^{c2}	50.3 ± 1.0^{3}
Hv-tw$_2$;Lh N13 (-)	2.00 ± 0.33^{c2}	1.84 ± 0.32^{a3}	1.30 ± 0.22^{c3}	49.3 ± 0.8
Hv-tw$_2$;Lh N17 (+-)	2.61 ± 0.52^{a}	2.08 ± 0.34^{2}	1.48 ± 0.19^{c3}	57.9 ± 1.0^{3}
Hv-tw$_2$;Lh N11 (+)	2.17 ± 0.40^{b1}	2.41 ± 0.22^{2}	2.86 ± 0.35	67.4 ± 1.3^{3}
Hv-tw$_2$;Lh N19 (+)	1.11 ± 0.18^{c3}	1.65 ± 0.27^{b3}	2.39 ± 0.48^{a}	70.6 ± 1.3^{3}
***Hv-H* (-)**	2.03 ± 0.28^{c2}	2.17 ± 0.25^{2}	2.00 ± 0.31^{b}	71.4 ± 1.2
Hv-tw$_2$;H N8-20 (-)	2.14 ± 0.25^{b2}	1.51 ± 0.18^{b3}	1.63 ± 0.19^{c3}	75.2 ± 0.8
***Hv-Dwh* (-)[1]**	2.08 ± 0.42^{b1}	2.35 ± 0.26^{2}	3.85 ± 0.39^{1}	30.4 ± 0.8^{3}
Hv-tw$_2$;Dwh N25 (+)	2.38 ± 0.30^{b1}	2.55 ± 0.33^{1}	3.15 ± 0.50	80.8 ± 1.0^{3}
***Hv-Hcs* (-)[1]**	1.59 ± 0.28^{c3}	0.68 ± 0.11^{c3}	2.82 ± 0.29^{a}	91.8 ± 1.1^{3}
Hv-H;Hcs N9 (+)	2.10 ± 0.20^{c2}	2.07 ± 0.25^{2}	3.57 ± 0.45	89.8 ± 1.1
***Hv-Mf* (-)[1]**	2.01 ± 0.51^{b1}	2.23 ± 0.77	1.41 ± 0.38^{c2}	44.2 ± 0.9^{3}
Hv-tw$_2$;Mf N14 (+)	2.45 ± 0.25^{b1}	2.13 ± 0.25^{2}	2.77 ± 0.79	66.6 ± 0.9^{3}

[1]Accession numbers in USDA-ARS National Small Grains Collection: for *Hv-Dwh* (*Hv-Dense wing hood*) GSHO 928, for *Hv-Hcs* (*Hv-Hoods on center spikelet*) GSHO 666 and for *Hv-Mf* (*Hv-Multiflorous*) GSHO 79.

(+,-) – formation of leafy/shoot-like structures: (+) – present, (-) – absent; (+-) – present under field conditions, absent in greenhouse; a – $P < 0.05$, b – $P < 0.01$, c – $P < 0.001$ – in comparison with *WT* (*wt*) (cv. 'Auksiniai II'); 1 – $P < 0.05$, 2 – $P < 0.01$, 3 – $P < 0.001$ – in comparison with the mother plant *Hv-tw$_2$*; for plant height – the same in comparison of respective mutant with *WT* (*wt*) or of father mutant with respective double mutant

Allelism test of *BKn3* gene in barley single and double mutants

Because extremely wide morphological and callus growth variations were determined among the *Hv-tw$_2$;Hd* double-mutant lines, it was necessary to verify that all of the single and double mutants that were used in our study have the same *BKn3* allele, which causes the development of the ectopic flowers. A distinctive feature of *BKn3*

alleles I, II and IIIa, b, and c is the 20-bp insertion in the *BKn3* promoter, which is absent in the European barley stocks, containing *BKn3* allele I (*k*), while the absence of the other 33-bp insertion in *BKn3* intron IV specifically marks the *BKn3* allele IIIc (*K*) among the other alleles of type III (Badr et al., 2000).

Table 2. Summarized sequencing results of two fragments in barley *BKn3* gene

Genotype \ Position, nt	Promoter										Intron IV						*BKn3* allele[4]
	877	930	20 bp ins.[1]	1080	1085	1230	1265	1315	1425	1499	5059	33 bp ins.[2]	5342	5491	5553	305 bp dup.[3]	
Hv-tw$_2$, WT	T	C	-	C	C	C	A	C	C	C	G	+	A	T	C	-	I
Hv-Hd mutants[5] and *Hv-tw$_2$;Hd* double-mutant lines	-	T	+	T	T	T	G	G	T	T	C	-	G	G	T	+	IIIc (*K*)

[1,2,3]ins. – insertions and duplication: (+) – present, (-) – absent; [4]*BKn3* alleles, determined according to Badr et al. (2000); [5]A collection of 14 different *Hv-Hd*-type single mutants preserved in Vilnius University Botanical garden was used for the *BKn3* allele determination, namely, *Hv-2-row hooded* (acessions 1441 and 1445), *Hv-Brittle rachis*, *Hv-Hooded lemma*, *Hv-Hood awn*, *Hv-Hooded 2-row*, *Hv-Elevated hood*, *Hv-Hooded/Kap1.a* in Colsess II, *Hv-Dense wing hood*, *Hv-Hooded*, *Hv-Hoods on center spikelet*, *Hv-Sessile hood*, *Hv-Lemma hooded* and *Hv-Multiflorous*

The results of DNA sequencing in the two regions including both of the diagnostic molecular markers have demonstrated that the *WT* and *Hv-tw$_2$* mutant contain the European *BKn3* allele I, while all of the tested *Hv-Hd*-type single and double mutants have diagnostic molecular markers (presence of 20-bp and absence of 33-bp insertions) specific for allele IIIc (*K*) (Table 2) as well as a 305-bp duplication in intron IV (Fig. 2). This result does not exclude the possibility of differences in the other sites of the *BKn3* gene for the mutants *Hv-Hd* and *Hv-Lh*.

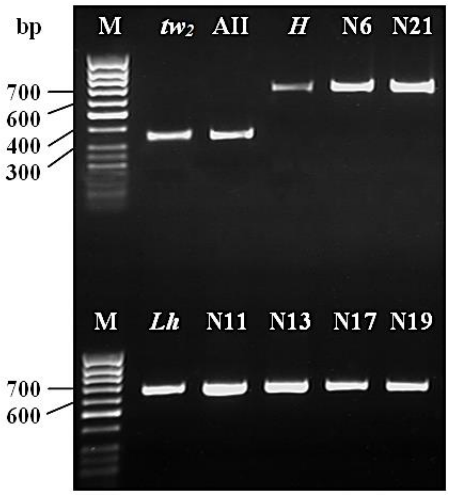

Fig. 2. PCR products containing a 305-bp duplication of *BKn3* gene in barley single mutants *Hv-Hooded/Kap1.a* in Colsess II (*Hv-H*) and *Hv-Lemma hooded* (*Lh*) and all tested double-mutant lines or without 305-bp duplication in mutant *Hv-tweaky spike 2* (*Hv-tw$_2$*) and *WT* (cv. 'Auksiniai II') (649-bp and 335-bp fragments, respectively); M – DNA molecular weight marker

Ethephon effect on awn and ectopic flower development

As the *Hv-tw$_2$;Hd* double mutants express both *Hv-tw$_2$* and *Hv-Hd* parental features (variations in flower/inflorescence structure and ectopic awn transformations, respectively), we used different groups of chemical compounds to modify both homeotic parental features separately.

The successful results of the partial *Hv-Hd* phenotype normalization in Osnato et al. (2010) study gave us a pretext to investigate the effects of ethephon as a source of ethylene on the development of ectopic structures in the lemma/awn transition zone of single and double barley mutants (Table 3). Significant differences in response to exogenous ethylene were observed among single and double mutants (Table 3). In the *Hv-H* mutant, in which awns are fully transformed into ectopic flower-like structures, several ethephon-induced the increase of awned flowers, while any effect of ethephon on this feature was observed in its double-mutant line *Hv-tw$_2$;H* N6 (+) (Table 3). A well-pronounced effect of ethephon on awn development was determined in the *Hv-Lh* mutant and in its *Hv-tw$_2$;Lh* double-mutant lines: in all of the representatives of this group, the frequency of normally awned flowers increased after exposure to ethephon (Table 3).

Table 3. Development of awns and ectopic flower-like structures after the exposure of barley single and double mutants to ethephon

Single/double mutant line	Treatment	n	Normal awns	Ectopic structures on lemma, % ± SE			
				With small awns	Wings	Rudimentary outgrowths	Ectopic flowers
Hv-Lh (-)	0	300	33.3 ± 2.7	25.0 ± 2.5	2.3 ± 0.9	5.7 ± 1.3	33.7 ± 2.7
	E	155	53.5 ± 4.0^c	0^c	0^a	0^c	46.5 ± 4.0^b
Hv-tw$_2$;Lh N11 (+)	0	86	62.8 ± 5.2	12.8 ± 3.6	4.7 ± 2.3	4.7 ± 2.3	15.1 ± 3.9
	E	85	89.4 ± 3.4^c	3.5 ± 2.0^a	1.2 ± 1.2	4.7 ± 2.3	1.2 ± 1.2^c
Hv-tw$_2$;Lh N13 (-)	0	250	11.2 ± 2.0	56.0 ± 3.1	3.2 ± 1.1	20.4 ± 2.6	9.2 ± 1.8
	E	253	17.8 ± 2.4^a	50.2 ± 3.1	3.6 ± 1.2	18.2 ± 2.4	10.3 ± 1.9
Hv-tw$_2$;Lh N19 (+)	0	111	38.7 ± 4.6	27.0 ± 4.2	7.2 ± 2.5	8.1 ± 2.6	18.9 ± 3.7
	E	126	68.3 ± 4.2^c	11.1 ± 2.8^b	0.8 ± 0.8^a	4.8 ± 1.9	15.1 ± 3.2
Hv-H (-)	0	128	0	3.9 ± 1.7	0.8 ± 0.8	0.8 ± 0.8	94.5 ± 2.0
	E	148	2.7 ± 1.3^a	6.8 ± 2.1	4.7 ± 1.7^a	3.4 ± 1.5	82.4 ± 3.1^b
Hv-tw$_2$;H N6 (+)	0	109	10.1 ± 2.9	23.9 ± 4.1	4.6 ± 2.0	1.8 ± 1.3	59.6 ± 4.7
	E	107	11.2 ± 3.1	25.2 ± 4.2	4.7 ± 2.1	6.5 ± 2.4	52.3 ± 4.9

In *WT* and *Hv-tw$_2$* ectopic flowers are absent and in double-mutant line *Hv-tw$_2$;H* N21 (+) ectopic structures are rare and very elevated; (+,-) – development of leafy/shoot-like structures in spike; E – ethephon; a – $P < 0.05$; b – $P < 0.01$; c – $P < 0.001$ – compared to the respective control (0)

If the decrease in the spectra of ectopic flowers with sexual organs is considered as the phenotype normalisation process in the developmental trend of ectopic flower-like structures, the partial ethephon-induced normalisation effect on ectopic flower structures was noted also by a comparison of ectopic structures with and without reproductive organs (Fig. 3). However, this effect was observed only in some of tested mutants.

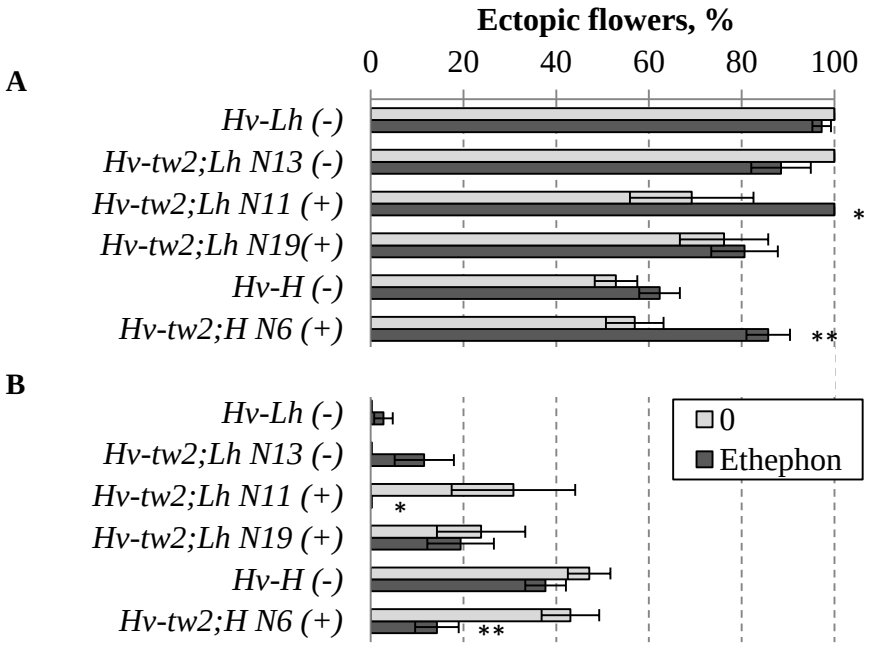

Fig. 3. Effects of ethephon on ectopic flower structures without sexual organs (A) and with sexual organs (B) in different barley single and double mutants Only single and double mutants developing ectopic structures are shown. Capacity to develop leafy/shoot-like structures is indicated as (+,-). Differences between inhibitor and corresponding control (0): **P* < 0.05; ***P* < 0.001

In conclusion, the action of ethephon was directed mainly into the ectopic structures caused by the mutant *BKn3* gene, and the effects of partial *Hv-Hd* phenotype normalization in single and double mutants *Hv-tw$_2$;Hd* were observed twice: (1) on awn development and (2) on the sexual organs inside of the ectopic flowers, and these effects strongly depended on the genetic background.

Impacts of 2,4-D and auxin inhibitors PCIB and HFCA on the structures of basic and ectopic flowers in barley single and double mutans

Although pleiotropic recessive *Hv-tweaky*-type mutants have been known for a long time (Reid and Wiebe 1968; Søgaard and Wettstein-Knowles 1987), they are still not fully genetically characterised. One of the peculiarities of the newly induced *Hv-tw*-type mutants at the early generations was genetic instability which resulted in a variety of revertants differing in separate quantitative features, such as a higher protein content, changed composition in amino acids, elevated resistance to some phytopathogenes (Rančelis ir kt., 2004; Vaitkūnienė ir kt., 2006; Šiukšta ir kt., 2008; Žvingila ir kt., 2012).

Nevertheless, it has been shown that the application of some phenoxy herbicides (including 2,4-D) leads to the specific inflorescence malformations (‘tweaking’ ears) in wheat and barley, when used before the completion of spikelet differentiation (Derscheid, 1952; Derscheid et al., 1952; Staniforth, 1952; Kumar and Singh, 2010). To determine if 2,4-D was a suitable chemical compound to modify the *Hv-tweaky* phenotype, we firstly investigated the effects of 2,4-D on spike development of *WT* plants (cv. ‘Auksiniai II’). Our experimental data have shown that 2,4-D induces a specific type of spike morphoses in *WT* plants, some of which are the phenocopies of *Hv-tweaky*-type mutations (Fig. 4). On the ground of the latter observation, the modification of *Hv-tweaky* phenotype features of barley single and *Hv-tw$_2$;Hd* double

mutants was performed using the synthetic auxin 2,4-D and two selected auxin inhibitors – the auxin polar transport inhibitor 9-hydroxyfluorene-9-carboxylic acid (HFCA) or the antiauxin p-chlorophenoxyisobutyric acid (PCIB).

Fig. 4. The diversity of morphoses induced by the synthetic auxin 2,4-D. (A) Branched spikes and the opposite position of spikelets (indicated by arrows) in *WT* (cv. 'Auksiniai II') spikes; (B) 2,4-D-induced stem deformation in mutant *Hv-Hv-twmk*; (C−E) spikes with gaps of different localization in mutant *Hv-tw* No. 18; (F−H) 2,4-D-induced phenocopies of *Hv-tw* No. 18 mutation in *WT* spikes; (I) 2,4-D-induced loss of spikelets in *WT* spike; (J) 2,4-D-induced long gap which consists of seven nodes without spikelets (indicated by arrowheads) in *WT* spike

The application of exogenous auxin inhibitors PCIB and HFCA to single mutant *Hv-tw*$_2$ caused a partial normalisation of the basic flower structure (Fig. 5A and B). This effect was expressed as an increase in the fraction of normal structure basic flowers with 2 lodicules, 3 stamens and 1 pistil (2L+3S+1P) (Fig. 5A) and by a decrease in flowers with the typical to *Hv-tw*-type mutant lodicule transformation to stamens and/or pistils (Lt) in which the total number of L+S+P was not altered (Fig. 5B).

For the *Hv-tw*$_2$*;Hd* double mutants, the effects of PCIB and HFCA on the basic flower structure differed even for leaf/shoot-forming double-mutant lines (Fig. 5A and B). Similarly to mutant *Hv-tw*$_2$, a partial normalisation of basic flower structure occurred in the double mutant *Hv-tw*$_2$*;Lh* lines N11 (+) and N13 (-) and it was expressed as an increased fraction of flowers with normal structure (Fig. 5A), but a statistically significant decrease in Lt flowers was observed only for line N13 (Fig. 5B). No effects of HFCA or PCIB on the structure of the basic flowers were evident for genotypes *WT*, *Hv-Lh*, and *Hv-tw*$_2$*;H* N6 (+) in which Lt was not observed at all.

In regard to ectopic flower structures, *Hv-Hd*-type mutants differed significantly amongst themselves and representatives of the two alternative *Hv-Hd* mutants, *Hv-Hooded* (*Hv-H*) and *Hv-Lemma hooded* (*Hv-Lh*), were tested. For the mutant *Hv-H*, ectopic transformation of awns into flower structures occured, and a significant portion of the ectopic flowers developed sterile reproductive organs. In contrast, the rudimentary flower-like structures constituted a significant portion of the ectopic outgrowths on the awns of the mutant *Hv-Lh* (cf. Fig. lR and S).

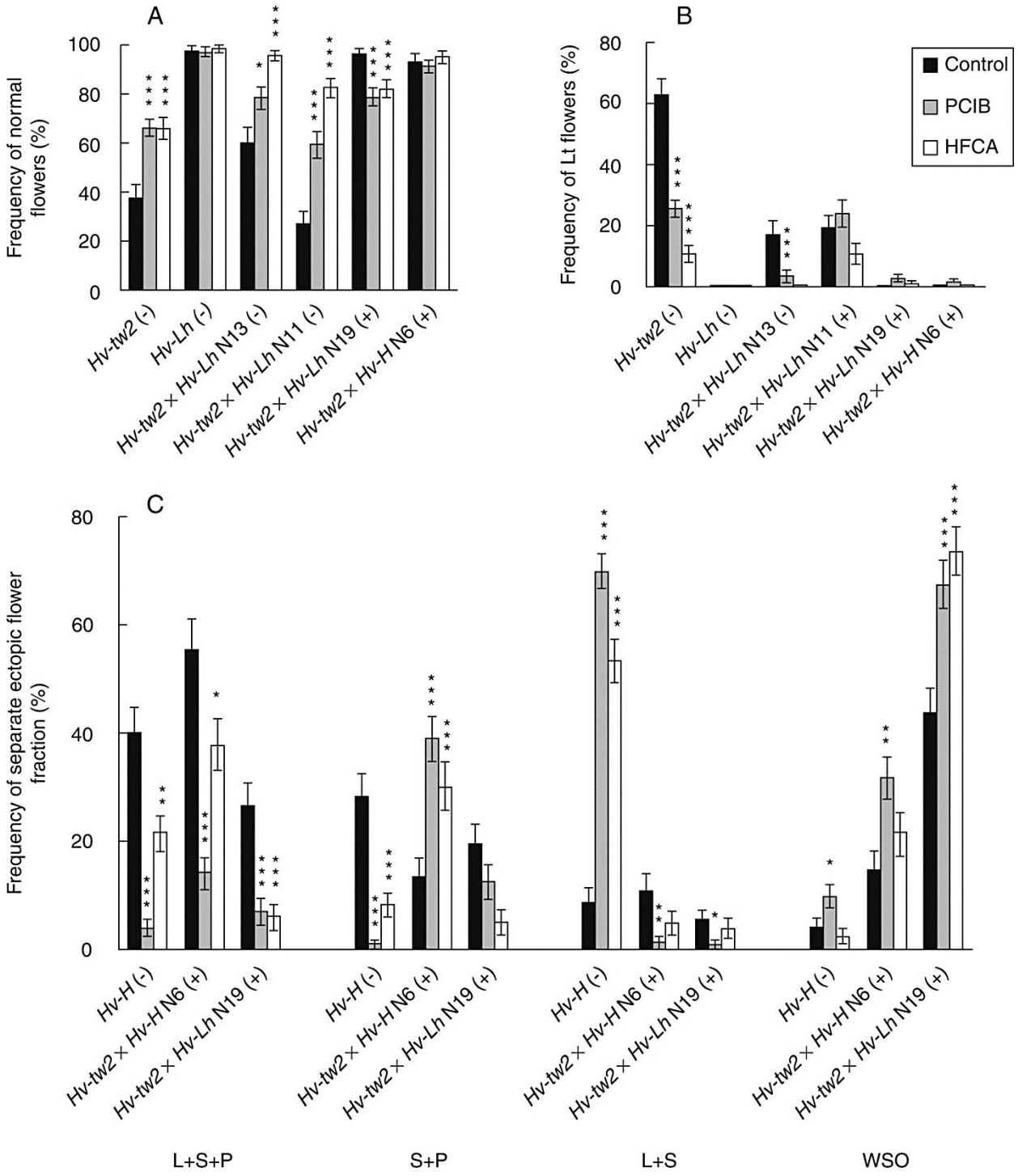

Fig. 5. Effects of PCIB and HFCA on fractions of normal flowers (A), flowers with lodicule transformations (Lt) to stamens and/or pistils (B) in basic flowers and on several fractions of ectopic flowers (C) cultivated under greenhouse conditions. In (C), only genotypes with a wide spectrum of ectopic outgrowths are shown. L, lodicule; S, stamen; P, pistil; WSO, without sexual organs. Differences between inhibitor and corresponding control: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$

The effects of auxin inhibitors were also clearly expressed on the development of the ectopic awn transformations, but only in genotypes, that are characterised by a wide spectrum of ectopic outgrowths, such as mutant *Hv-H* and its double-mutant lines *Hv-tw$_2$;H* N6 (+) and *Hv-tw$_2$;Lh* N19 (+) (Fig. 5C). A significant portion of the flower

spectrum of the latter group comprised ectopic flowers with both reproductive organs, L+S+P and S+P. Both flower types may be considered as highly advanced towards the final development of flowers because the organ identity genes specifying the fourth floral whorl (representatives of class C, according to the ABC model of flower development) must be expressed for these phenotypes.

The effects of HFCA and PCIB were most evident on these fractions of ectopic flowers (Fig. 5C). However, while the fraction of L+S+P flowers decreased for all representatives of this group, the effect of auxin inhibitors on the S+P fraction was more genotype-specific. For the mutant *Hv-H* and line *Hv-tw$_2$;Lh* N19 (+), both auxin inhibitors decreased the S+P portion of the spectrum, while the opposite effect of auxin inhibitors was observed for *Hv-tw$_2$;H* N6 (+) (Fig. 5C).

If rescue of several phenotypic instability features in *Hv-tw$_2$;Hd* double mutants with auxin inhibitors was really caused by auxin concentration deficiencies, an additional treatment with an exogenous auxin should induce the opposite effects. This presumption was tested on two different features of the phenotypic instability of double-mutant lines: on leaf/shoot-like structures (Table 4) and on variations in the frequencies of basic and ectopic flower fractions (Fig. 6).

Table 4. Effects of 2,4-D on the spike structure of selected double-mutant lines (F_{10}) under the field conditions

Single/double mutant	Treat-ment	n	Phenotypic groups*			
			I	II	III	Others
Hv-tw$_2$ (-)	0	282	0	17.7 ± 2.3	0	0
	2,4-D	288	0	0^c	0	0
Hv-tw$_2$;Lh N1 (-)	0	420	13.3 ± 1.7	0.7 ± 0.4	0	0
	2,4-D	215	1.4 ± 0.8^c	0	0.5 ± 0.5	0
Hv-tw$_2$;Lh N2 (+)	0	326	3.1 ± 1.0	2.8 ± 0.9	12.0 ± 1.8	1.5 ± 0.7
	2,4-D	322	0.6 ± 0.4^a	1.6 ± 0.7	11.2 ± 1.8	1.2 ± 0.6
Hv-tw$_2$;Lh N11 (+)	0	262	20.2 ± 2.5	10.3 ± 1.9	15.3 ± 2.2	0
	2,4-D	210	16.7 ± 2.6	7.1 ± 1.8	5.2 ± 1.5^c	0
Hv-tw$_2$;Lh N17 (+-)	0	324	9.3 ± 1.6	9.9 ± 1.7	18.5 ± 2.2	4.0 ± 1.1
	2,4-D	200	6.5 ± 1.7	15.5 ± 2.6	15.0 ± 2.5	9.0 ± 2.0^a
Hv-tw$_2$;Lh N19 (+)	0	533	9.4 ± 1.3	12.4 ± 1.4	11.6 ± 1.4	0.4 ± 0.3
	2,4-D	435	3.4 ± 0.9^c	17.2 ± 1.8^a	2.5 ± 0.7^c	0
Hv-tw$_2$;H N21 (+)	0	437	3.7 ± 0.9	14.9 ± 1.7	5.9 ± 1.1	0
	2,4-D	352	2.3 ± 0.8	2.0 ± 0.7^c	9.7 ± 1.6	0
Hv-tw$_2$;H N6 (+)	0	147	3.4 ± 1.5	10.2 ± 2.5	2.7 ± 1.3	0.7 ± 0.7
	2,4-D	75	0^a	2.7 ± 1.9^a	1.3 ± 1.3	0

*Phenotypic groups: I – with long naked gaps on rachis (see Fig. 1A, F); II – with short gaps (see Fig. 1G); III – leafy/shoot-like (see Fig. 1H, I, K, L); for *Hv-wt*, *Hv-Lh* and *Hv-H* the above variations of spike structure are absent; (+,-) – as in Table 3; n – the number of tested spikes; n for *Hv-wt*: 0 – 201; 2,4-D – 215; for *Hv-Lemma hooded* (*Hv-Lh*): 0 – 155; 2,4-D – 147; for *Hv-Hooded* (*Hv-H*): 0 – 193; 2,4-D – 173; a – $P < 0.05$, b – $P < 0.01$, c – $P < 0.001$ – in comparison with the respective control (0)

The effect of 2,4-D was rather specific to ectopic malformations. Exposure to 2,4-D lowered the frequency of spikes with long naked gaps (Fig. 1A, F; Table 4, group I).

However, statistically significant effects were observed in only 4 of the 7 double-mutant lines tested. This feature is rather characteristic of an auxin deficiency because it is similar to phenotypes of auxin-pathway mutants or to phenocopies induced by auxin inhibitors. Polymorphism of double-mutant lines was also clearly expressed for other tested features, such as short gaps (Table 4, group II) and leafy/shoot-like structures (Table 4, group III). After exposure of the mutant *Hv-tw*$_2$ to 2,4-D, short gaps in spikes completely disappeared. Polymorphism of this feature was clearly expressed amongst most of the tested double-mutant lines.

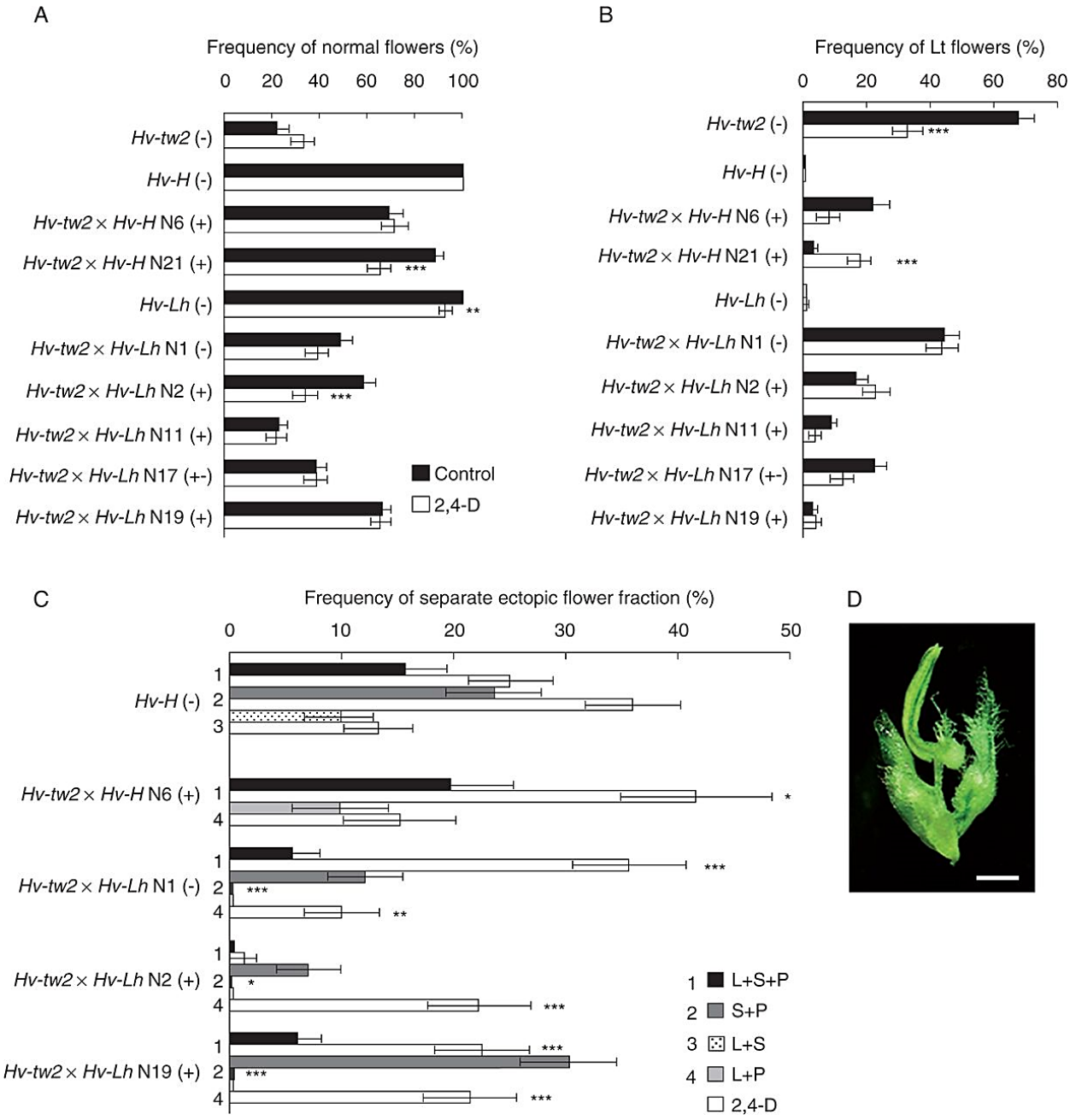

Fig. 6. Effects of 2,4-D on the frequencies of (A) basic normal flowers and (B) flowers with lodicule transformations to stamens and/or pistils (Lt) and (C) on the spectrum of ectopic flower structures in place of awn or on awns, cultivated under field conditions. (D) Specific chimeric structures observed only after 2,4-D exposure. L, lodicule; S, stamen; P, pistil. Scale bar (D) = 1 mm. Differences compared with 2,4-D treatment: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

A significant amount of polymorphism in the response to 2,4-D was also evident for the leaf/shoot-like spike structures. In two double-mutant lines, *Hv-tw$_2$;Lh* N11 (+) and N19 (+), the frequency of such variations observed after exposure to 2,4-D decreased, and in other tested lines, the effect of 2,4-D was absent (Table 4, group III).

The effects of 2,4-D on the spectra of basic and ectopic flowers were not uniform (Fig. 6). For the mutant *Hv-tw$_2$*, 2,4-D decreased the frequency of basic flowers with Lt (Fig. 6B) as well as in double-mutant lines *Hv-tw$_2$;H* N6 (+) and *Hv-tw$_2$;Lh* N17 (+-), but the opposite effect was observed in line *Hv-tw$_2$;H* N21 (+). However, for 4 genotypes, including the *Hv-Lh* (-) mutant, the frequency of normal basic flowers decreased to various extents (Fig. 6A). This effect may be considered as opposite to the action of auxin inhibitors (cf. Fig. 5A and B). It is paradoxical that, in the *Hv-tw$_2$* mutant, the frequency of normal flowers increased after treatment with 2,4-D as well as after treatment with both auxin inhibitors PCIB and HFCA (cf. Figs. 5A and 6A).

2,4-D also induced a specific type of stamen malformation – a chimeric stamen (Sc) with a rudimentary carpel (Fig. 6D). However, this effect was observed in only a portion of the double-mutant lines.

A more uniform effect of 2,4-D was noted on ectopic flower structures. Plant exposure to 2,4-D increased the frequency of L+S+P flowers in all genotypes in which such flowers were developed (Fig. 6C). This effect may be considered to some extent as opposite to the effect of auxin inhibitors on the same fraction of ectopic flowers (cf. Figs. 6C and 5C). Additionally, a significant modification of the fraction of ectopic S+P flowers occurred: their proportion decreased in cases where *Hv-Lh* was used as the father plant (Fig. 6C). In contrast, the fraction of L+P ectopic flowers increased in those genotypes.

Nevertheless, the responses of the double-mutant lines to 2,4-D varied across a wide range for both the basic and ectopic flower structures and specifically concerned the development of reproductive organs in ectopic flowers and of long naked gaps. A specific effect of 2,4-D was also expressed on the short gaps in the spikes of the *Hv-tw$_2$* mutant. However, the effects of auxin inhibitors on this spike feature were not investigated.

Putative differentially expressed genes in barley mutant *Hv-tw$_2$*

Despite the fact that inherited phenotypic instability is a feature only of barley *Hv-tw$_2$;Hd* double mutants, and this peculiarity is not observed in single parental mutants *Hv-tw$_2$* or *Hv-Hd*, the trigger of instability is the mutant allele of *Hv-tw*-type. This conclusion is suggested by the fact that similar and even more pronounced phenotypic instability was also observed in *Hv-tw;lax-a* double mutants that originated from the hybridization of the other *Hv-tweaky spike* allele *Hv-tw* with series of allelic barley mutants *Hv-laxatum-a* (*Hv-lax-a*), that have reduced spike density and lodicules that are regularly transformed into stamens (Larsson, 1985; Laurie et al., 1996; Vaitkūnienė et al., 2004b) (Table 5).

Furthermore, the same type of inherited phenotypic instability was also observed in double mutants derived from the hybridization of *Hv-tw* mutant with other *Hv-tweaky*-type mutants, namely, *Hv-tweaky No.18* (*Hv-tw No.18*, GSHO111) and *Hv-tweaky and missing kernel* (*Hv-twmk*, GSHO1119) (Table 5), that are characterized by variation in number of flower organs.

Table 5. The instability features of spike structure in *Hv-tw;lax-a* (of different alleles), *Hv-tw;tw No.18* and reciprocal *Hv-tw;twmk* and *Hv-twmk;tw* barley double mutants (F_8) under the field conditions

Double mutant	n	Phenotypic groups*					
		I	II	III	IV	V	WSV/*tw*
Hv-tw;lax-a.434	169	21.3 ± 3.2	32.0 ± 3.6	5.9 ± 1.8	0	0	40.2 ± 3.8
Hv-tw;lax-ab	1119	0.6 ± 0.2	14.1 ± 1.0	26.1 ± 1.3	0	0	52.1 ± 1.5
Hv-tw;lax-ac	289	0.4 ± 0.4	21.8 ± 2.4	9.3 ± 1.7	0	0	54.7 ± 2.9
Hv-tw;lax-ae	815	0.6 ± 0.3	12.3 ± 1.2	12.0 ± 1.1	0	0	55.8 ± 1.7
Hv-tw;twNo.18	132	0.9 ± 0.8	11.9 ± 2.8	14.2 ± 3.0	0	0	63.6 ± 1.0
Hv-tw;twmk	1860	12.7 ± 0.8[1]	10.5 ± 0.7	16.2 ± 0.9	13.2 ± 0.8[1]	5.0 ± 0.5[1]	31.2 ± 1.0[1]
Hv-twmk;tw	1039	9.2 ± 0.9	20.0 ± 1.2[a]	5.7 ± 0.7[a]	1.3 ± 0.3[a]	0.1 ± 0.1[a]	37.2 ± 1.0[a]

*Only a part of variations is shown; phenotypic groups: I, II and III – as in Table 4; IV – spikes with elongated and (V) especially elongated glumes and lemma/palea; WSV/*tw* – without spike variations or of phenotype, typical for *Hv-tw* mutant; n – number of tested spikes; [1]P<0.001 in comparison with *Hv-tw;twNo.18*, [a]P<0.001 in comparison with reciprocal combination *Hv-tw;twmk*

Although the results of the morphological analysis suggest that the triggers of phenotypic instability in barley double mutants are mutant alleles of *Hv-tw*-type, it is also obvious that phenotypic instability is induced by *Hv-tw*$_2$ or *Hv-tw* gene products via their pleiotropic interaction with products of other homeotic genes. To screen the possible gene-candidates for such role, the analysis of differences in gene expression between *WT* (cv. 'Auksiniai II') and *Hv-tw*$_2$ mutant (both of the same genetic background) was performed using the differential display (dd-PCR) method. Although the results of the dd-PCR analysis are considered to be preliminary, it allowed us to reveal several differences in gene expression between *WT* and *Hv-tw*$_2$, which may be associated with the inherited phenotypic instability induction in all the double mutants tested, with the obligate participation of *Hv-tw*$_2$ or *Hv-tw* mutant alleles.

From 49 cDNA fragments differentially expressed in *WT* and *Hv-tw*$_2$, 43 cDNA fragments were successfully reamplified and 34 of them were sequenced (Table 6). The majority of sequenced cDNAs were homologous to *Hordeum vulgare* subsp. *vulgare* mRNA, cDNA or gDNA sequences registered in the NCBI database, but only a part of them encoded proteins of the annotated function. Part of cDNA fragments (10 in total) were homologous to genes encoding proteins found in grasses, such as *Aegilops*, *Brachypodium*, *Oryza*, *Triticum*, *Zea*). Only four of differentially expressed cDNA fragments were homologous to barley genes of the annotated function, namely f.N3/4 (homologous to the immunorepressive *BCI-5* (*Barley Chemically-Induced5*) gene), f.N19 (homologous to the putative *IDS4/ERF* (*Iron-Deficiency Specific4/Ethylene Responsive Factor*) gene), f.N28 (homologous to the *GSL7* (*Glucan Synthase-Like7*) gene) and f.N34 (homologous to the *GLP1* (*Germin-Like Protein1*) gene). F.N41 was homologous even to three barley protein-coding genes, *Lks2* (*Short awn2*), *Vrs1* (*Six rowed spike1*) and *eIF4E* (*Translation initiation factor4E*).

While the *Hv-tw*$_2$ mutant is of pleiotropic nature and triggers phenotypic instability in barley double mutants, the main attention was paid to the differentially expressed cDNA fragments that were homologous to various regulatory genes, such as transcription factors, chromatin activity regulators, developmental genes and genes participating in phytohormone (especially ethylene and auxin) pathways.

Table 6. Putative differentially expressed genes in barley mutant *Hv-tw$_2$* and *WT* (cv. 'Auksiniai II') at the developmental stage, critical for the inflorescence meristem induction

cDNA frag-ment	Size, bp	Putative protein/gene	Accession	E	%
		Down-regulated in Hv-tw$_2$			
N1/17	190	*A. thaliana Embryonic Flower 1* (*EMF1*)	AF319968.1	0.13	85
N10	390	Predicted: *Se.italica* 50S ribosomal protein L6, chloroplastic-like	XM_004984284.1	8e-7	87
N21	400	Predicted: *Se. italica* lysophospholipid acyltransferase LPEAT1-like	XM_004971457.1	7e-2	91
N38	290	*T. rubrum* CBS 118892 imidazole glycerol phosphate synthase (TERG_04658)	XM_003235554.1	0.34	86
		Up-regulated in Hv-tw$_2$			
N3/N4	150	*H. vulgare* immunoresponsive protein bci-5	AJ250661.1	3e-3	99
		D. antarctica plastid-specific ribosomal protein 2 precursor	AY090534.1	1e-1	88
		Predicted: *B. distachyon* 30S ribosomal protein 2, chloroplastic-like	XM_003577869.1	0.2	83
N19	280	*H. vulgare* putative iron-deficiency specific 4 protein and putative ethylene responsive factor	AP009567.1	7e-3	99
		PREDICTED: *Brachypodium distachyon* SPX domain-containing protein 1-like	XM_003563615.1	5e-30	83
N20	230	Predicted: *B. distachyon* PHD finger protein ALFIN-LIKE 8-like, transcript variant	XM_003577601.1	9e-3	79
N26	160	*Ae. tauschii* chromosome 1 *Ds* prolamin gene locus	JX295577.2	7e-5	82
N29	370	Predicted: *O. brachyantha* cystathionine gamma-synthase, chloroplastic-like	XR_423359.1	8e-4	93
N34	160	*T. turgidum* subsp. *durum Pm3* locus	AY146587.2	4e-8	88
		H. vulgare germin-like protein 1	Y15962.1	1e-2	100
N35	160	*Sa. hybrid* ubiquitin-conjugating enzyme (UBc E2)	KJ577594.1	7e-4	89
		Predicted: *B. distachyon* ubiquitin-conjugating enzyme E2 28-like	XM_003580724.1	5e-2	88
N39	350	*T. aestivum Vrn-B1-a* allele	HQ130483.2	5e-7	97
		T. carthlicum retrotransposon *VRN*, complete sequence and *VRN-B1* gene	JN817430.1	5e-7	97
		T. aestivum 5-methylcytosine DNA glycosylase (DME-5A)	JF683316.1	5e-7	97
		T. aestivum ABI3-interacting protein 2-1 (AIP2-2)	FJ643533.1	5e-7	97
		T. aestivum malate dehydrogenase (*Mdh4B*) gene	EF109232.1	4e-3	92
		A. tauschii retroposon *Au* element sequence	AY674984.1	4e-3	97
N40	300	Predicted: *B. distachyon* 60S ribosomal protein L13a-4-like, transcript variant 2	XM_003557639.1	2e-3	86
N41	320	*Hordeum vulgare* subsp. *vulgare Lks2* gene for putative short internodes family transcription factor	AB678347.1	1e-78	94
		Hordeum vulgare subsp. *vulgare eIF4E* gene locus	AY661558.1	5e-57	86
		Hordeum vulgare vrs1 locus, complete sequence; and *Hox1* gene	EF067844.1	2e-55	85

Plant species: *A. – Arabidopsis, Ae. – Aegilops, B. – Brachypodium, D. – Deschampsia, H. – Hordeum, O. – Oryza, Po. – Populus, Pr. – Prunus, Sa. – Saccharum, Se. – Setaria, T. – Triticum.* E – Expect value, the lower the E value, or the closer it is to zero, the more significant the match is; % − sequence homology

Apparently, it is not a coincidence that the significant part of differentially expressed genes in *Hv-tw$_2$* was involved in chromatin activity regulation or encoded transcription factors. In this aspect, the most exclusive was f.N1/17 which showed a weak homology to the *Arabidopsis EMBRYONIC FLOWER1* (*AtEMF1*) gene. The

EMF1 protein is among the main floral repressors and has a similar repressive function as the PRC-1 complex (Calonje et al., 2008; Derkacheva and Henning, 2014; Wang et al., 2014). The floral organ identity genes *AGAMOUS*, *APETALA3* and *PISTILLATA* are direct targets for the EMF1 protein (Calonje et al., 2008; Kim et al., 2010; Pu et al., 2013). The *EMF1* gene is involved not only in the regulation of flowering time and organ identity, but also in the pathways of some phytohormones (Moon et al., 2003; Kim et al., 2010; Pu et al., 2013). Recently, the *DEFORMED FLORAL ORGAN 1* (*OsDFO1*) gene which is orthologous to *AtEMF1* has been found in the rice genome (Zheng et al., 2015).

As mentioned above, cDNA f.N19 was homologous to the barley putative *iron-deficiency specific protein 4* (*IDS4*)/*ethylene responsive transcription factor* (*ERF*) which links the plant metabolism, development and integrative hormone regulation. *IDS4* is a regulatory gene, encoding the transcription factor of a multiple function. The IDS4 protein contains the SPX domain which indicates the IDS4 protein to be involved into the inorganic phosphate (Pi) metabolism (Duan et al., 2008).

F.N20 is homologous to the *Brachypodium distachyon* PHD (*Plant Homeodomain*) sequence containing the *ALFIN-LIKE 8-LIKE* gene. The PHD motif is found in many regulatory plant proteins that are frequently associated with the chromatin-mediated transcriptional regulation (Sanchez and Zhou, 2011).

F.N39 cDNA was homologous to even four sequences in *Triticum* and *Aegilops*, namely, the *Vrn-B1* gene, *Vrn* retrotransposon in the *Vrn-B1* gene, 5-methylcytosine DNA glycosylase (DEMETER-5A), AIP2-2 and malate dehydrogenase (Mdh4B) genes, and the retrotransposon *Au* element sequence. Although all these genes participate in a variety of processes in plants, they all contain an insertion of a transposible *Au* element which was first determined in the genome of *Aegilops umbellulata* (Yasui et al., 2001) and is widely shared among grasses and other families of plants (Fawcett et al., 2006; Ben-David et al., 2013).

DISCUSSION

Ethephon applied on to two different barley homeotic mutants of *Hv-Hooded/Kap1*-type and *Hv-tweaky spike*-type and their double-mutant lines allowed us to reveal the specific effects of ethylene on the development of ectopic floral structures. The effect of exogenous ethylene was specific in three respects. First, the effect of ethephon was specifically restricted mainly to phenotypic effects of the *Hv-Hooded*/*Kap1* mutation in the barley gene *BKn3* in the lemma/awn transition zone despite the fact that plants of different genotypes, phenotypes and backgrounds were tested. In this respect, the results of the present study agree with the observations of Osnato et al. (2010). No effect was noted on lodicule transformation into sexual organs, which is typical for *Hv-tw*$_2$ mutants.

Although according to the DNA sequencing results of the two regions in the *BKn3* gene, allowing the differentiation of *BKn3* alleles to I–IIIa, b, and c (Badr et al., 2000), both of the tested *Hv-Hooded/Kap1*-type single mutants and all of the double-mutant lines have molecular markers that are specific to *BKn3* allele IIIc and the 305-bp duplication in the intron IV of the *BKn3* gene, significant differences in the phenotypic expression of *Hv-Hooded/Kap1* mutation features among single mutants and among the double-mutant lines of the same origin were observed not only in response to exogenous ethylene but also in ethephon-untreated plants. A more-uniform response to exogenous

ethylene was noted only for the single mutant *Hv-Lemma hooded*, and all of the tested double-mutant lines originated from this single mutant, which was expressed by the increased frequency of normally awned flowers. However, this effect was absent for the other mutant *Hv-Hooded/Kap1.a* in Colsess II. The phenotypic differences among the single mutants *Hv-Hooded/Kap1.a* in Colsess II and *Hv-Lemma hooded* may be explained not only by the different genetic background but also by the possible differences in the other sites of the *BKn3* gene because only fragments of this gene were studied.

Differences among double-mutant lines, especially within the same cross-combination, may be explained mainly by the different genetic background that resulted from segregation, resembling the observations in inbred lines of maize (Vollbrecht et al., 2000). Modifier genes may also be involved. Indeed, the interaction of the *BKn3* gene with other genes from the TALE superfamily was demonstrated (Müller et al., 2001), and suppressor genes for the *Hv-Hooded/Kap1* mutation were determined in at least five loci (Roig et al., 2004). Especially interesting is the fact that the interaction of even three of these genes with the other gene *lks2* which is non-allelic to *BKn3* and determines a short awn, produced elevated and even very elevated *Hooded* phenotypes, very similar to the double-mutant line *Hv-tw$_2$;H* N21 (+). Furthermore, the phenotypic instability of some of the suppressor gene *suK* mutants was also noted by Roig et al. (2004), as well as in the present study for the double mutants *Hv-tw$_2$;H.*

The modifications of phenotypic instability features with the auxin inhibitors PCIB and HFCA and the synthetic auxin 2,4-D are of principle significance because, to date, auxin imbalance has not been described as a cause of phenotypic instability. The inherited forms of phenotypic instability across all of the tested generations of barley *Hv-tw$_2$;Hd* double mutants, which originated from the hybridization between homeotic mutant *Hv-tweaky spike* (*Hv-tw$_2$* allele) and various *Hv-Hooded/Kap1* mutants, showed the varying effects on inflorescence/flower development, that were an intriguing and unexpected phenomenon. DNA sequencing of two regions of the barley *BKn3* gene that serve as specific markers (20 and 33 bp insertions) differentiating the *BKn3* gene into alleles I, II, IIIa, IIIb, and IIIc (Badr et al., 2000) demonstrated that all of the tested *Hv-Hooded/Kap1*-type mutants and hybrids used in the present study have the molecular markers of the same allele IIIc (*Kap*), while the *WT* and the *Hv-tw$_2$* mutant contain the European *BKn3* allele I.

Our presumption that the phenotypic instability of the double-mutant lines may be caused by an imbalance in auxin distribution, based on the similarity of the long naked inflorescence gaps to the pin-like phenotype of auxin transport mutants (McSteen et al., 2007; Morita and Kyozuka, 2007; Gallavotti, 2013) and on the fact that the *LEAFY* gene, the mutations of which are expressed as a phenotype similar to that of the leafy/shoot spike structures of double mutants *Hv-tw$_2$;Hd*, has auxin response elements in its promoter and may be recognised as an ARF (Auxin Response Factor) (Bai and DeMason, 2008), was confirmed by the significant decrease in this type of spike variations after exposure to 2,4-D, also by the partial normalisation of the structure of basic flowers and by the effects on ectopic flowers of the auxin inhibitors HFCA and PCIB. Moreover, the effects of 2,4-D, HFCA and PCIB on the flower spectra were considerably greater for the flower fractions in which both sexual organs (stamens and pistils) were developed.

The gene *BKn3*, the mutation of which results in the *Hv-Hd* phenotype (Müller et al., 1995), is a representative of the *Kn1* class of genes that exhibit mutual interactions with auxin (Woodward and Bartel, 2005; Tabata et al., 2010; Rast and Simon, 2012) and form complex developmental modules with auxin pathway genes in the flower meristem (Hay and Tsiantis, 2010). We have hypothesised that the disturbance of such modules, as well as of the ectopic auxin maxima (Krizek, 2011) or of the auxin gradients (Benková et al., 2003; Tanaka et al., 2006), may lead to the phenotypic instability in the inflorescences and flowers of double-mutant lines. This supposition was also strengthened by the described pleiotropic effects in transgenic barley plants ectopically expressing the maize gene *Zm-Kn1* (Williams-Carrier et al., 1997) and in mutant *Zm-semaphore1* with a disturbed auxin/*Kn1* gene interaction (Scanlon et al., 2002). The pleiotropic effects of the awn transformation into flower structures may also contribute to the phenotypic instability because the awn is the main photosynthetic organ in a barley spike (Abebe et al., 2009). Photosynthesis is also one of the main regulators of numerous plant processes and genes.

However, the various phenotype normalisation effects caused by the auxin inhibitors PCIB and HFCA indicate that ectopic auxin hyperaccumulation has occurred or that the ectopic auxin maxima have arisen. This conclusion is sustained by the contrasting effect of 2,4-D on the frequency of the normal basic flowers. Frequently, auxin inhibitors cause phenocopies of auxin pathway mutations (Morita and Kyozuka, 2007; Krizek, 2011; Gallavotti, 2013). The opposite effect – the rescue of the mutant phenotype by auxin inhibitors – is a rare event (Morita and Kyozuka, 2007; Staldal et al., 2008) and the inherited phenotypic instability in double homeotic mutants (i.e., *Hv-tw$_2$;Hd)* is a suitable model system for the further investigation of ectopic auxin maxima formation, its consequences, and the possible mechanisms of modifications. According to Krizek (2011), it is difficult to determine the auxin concentration in developing flower organs, and the phenomenon of phenotypic instability in the *Hv-tw$_2$;Hd* barley double mutants may be a useful system for investigating the integrative processes that occur at the organ initiation sites of a flower.

The specific effects of 2,4-D on spikes (short gaps in *Hv-tw$_2$* and long naked gaps in double mutants) and of the auxin inhibitors HFCA and PCIB on flower fractions with both sexual organs may indicate that various regulatory modules are involved with varying relationships to auxin distributions in definite cells of the spike/flower primordium. The results also suggest that certain features characterised by the phenotypic instability of double mutants may be of different natures.

This variation in the nature of phenotypic instability was especially clear for ectopic flowers. For the development of sexual organs, the expression of organ identity genes (classes B and C) in the third and fourth whorls is necessary, which may indirectly suggest that unbalanced overexpression of B/C genes may have occurred in barley hybrids and this unbalanced expression may be caused by occasional auxin accumulations. In turn, this situation may induce phenotypic variations in organ initiation. In the present work, the involvement of different regulatory modules in relation to auxin responses also indicates significant differences between the effects of auxin inhibitors on callus meristematic cells and on the flower structure of the *WT* and the *Hv-tw$_2$* mutant.

The high polymorphism of the response to 2,4-D and auxin inhibitors in tested double-mutant lines is noteworthy. This polymorphism of response to auxin, as well as

the unexpected effect of 2,4-D on short spike gaps in the *Hv-tw$_2$* mutant and several other double-mutant lines, deserves further study. Our presumption is that differences among double-mutant lines, especially within the same origin, may be explained mainly by the different genetic background that resulted from segregation, resembling the observations in inbred lines of maize. The penetrance of the maize *Kn1* gene was genetic background-dependent (Vollbrecht et al., 2000). In turn, the pleiotropic action of auxin was involved in an extraordinary variety of plant growth and developmental processes (Woodward and Bartel, 2005; Vanneste and Friml, 2009; Sauer et al., 2013). It may have variable effects on the different genetic background, creating variations in auxin concentrations, deficiency or ectopic maxima, in different single and double mutants. Furthermore, natural variations in auxin response were observed (Delker et al., 2010; Laskowski, 2013).

The polymorphic response of callus growth after the exposure to histone deacetylase inhibitor HC-toxin in some double mutants (in comparison to the parental single mutants) indicates that epigenetic factors may also be involved into the phenotypic instability induction in barley double mutants. However, similar phenotype variations are attributed to floral/inflorescence reversions in *Arabidopsis* (Tooke et al., 2005). The majority of genes, mutations in which lead to floral/inflorescence reversions, encode transcription factors of MADS superfamily (Yu et al., 2004; Tooke et al., 2005; Fornara et al., 2008; Liu et al., 2009; Wang et al., 2010), nevertheless, the Polycomb-group (Pc-G) proteins were demonstrated to play a key role in floral reversion events (Müller-Xing et al., 2014).

Among the cDNA fragments identified by dd-PCR, the nearest to genes of MADS group is f.N39, which is homologous even to 6 cDNA sequences of annotated function, including 2 transposable elements and wheat vernalization *VRN-B1* gene which is orthologous to *Arabidopsis* MADS genes *APETALA1* and *FRUITFULL*. *VRN-B1* gene is also involved into the specification of floral organs (Ferrandiz et al., 2000; Distelfeld et al., 2009; Trevaskis, 2010; Kobayashi et al., 2012) and is expressed in all developing floral organs (Kinjo et al., 2012). Over 500 genomic regions were identified as potential *VRN1*-binding targets, including genes involved in spike-architecture and auxin, ethylene and other hormone metabolism (Deng et al., 2015) and has multiple regulatory regions of itself (Distelfeld et al., 2009).

CONCLUSIONS

1. A high variation amongst the ectopic flower-like structures of barley (*Hordeum vulgare* subsp. *vulgare* L.) *Hv-tw$_2$;Hd* double mutants suggests a definite developmental trend in ectopic flower development, varying from insignificant outgrowths on the awn to a tube (in non-inverted/inverted positions) and finally to flowers with sterile sexual organs.
2. A great variation was determined in the callus growh intensity (GI) of different single and double *Hv-tw$_2$;Hd* mutants. The GI of single mutants was lower of that in *wt*, while GI of double mutants in most cases exceeded the GI of parental single mutants. Great differences in callus growth were also observed in response to 2,4-D, auxin inhibitors and HC-toxin.
3. According to sequence analysis of two diagnostic markers in the *BKn3* gene, all the *Hv-Hooded/Kap1*-type single mutants (14 in total) and double-mutant lines tested in the present study have the same *BKn3* allele (IIIc).

4. In *WT* plants, the synthetic auxin 2,4-dichlophenoxyacetic acid (2,4-D) induced a slight variation in the floral organ number and specific spike morphoses that mimic the *Hv-tweaky*-type mutations. 2,4-D also decreased the frequency of *Hv-tw$_2$*-specific short gaps in spikes, indicating that a specific phenotype of *Hv-tweaky*-type is caused by defects in the auxin function.
5. 2,4-D decreased the frequency of normal structure basic flowers and induced a specific transformation of statmens into chimeric organs in some double-mutant lines. The opposite effect on basic flowers was observed for the auxin inhibitors HFCA and PCIB: both inhibitors increased the fraction of normal structure basic flowers in some of tested double mutant *Hv-tw$_2$;Lh* lines.
6. 2,4-D increased the frequency of ectopic flowers with stamens and pistils in some double-mutant lines, while in some cases both auxin inhibitors HFCA and PCIB showed the opposite effects.
7. The results of 2,4-D and the auxin inhibitors HFCA and PCIB allowed to conclude that the phenotypic instability of barley double mutants may also be linked with defects in the auxin function.
8. The effects of ethephon, an ethylene-releasing compound, were specifically restricted mainly to the lemma/awn transition zone: treatment with ethephon increased the frequency of normally awned basic flowers in most tested single and double mutants. Ethephon showed no effect on lodicule transformation, but in some double-mutant lines (mostly in *Hv-tw$_2$;Lh*) it decreased the frequency of flowers with the altered number of floral organs.
9. When a wider spectrum of barley double mutants, originated not only from the hybridization of *Hv-tw$_2$* with different *Hv-Hd,* but also *Hv-tw* with other mutants (*Hv-lax-a, Hv-tw No. 18* ir *Hv-twmk*) was studied for the instability induction, it has been determined that the triggers of phenotypic instability are mutant alleles of *Hv-tw*-type.
10. Preliminary results of the differential display analysis showed that the mutant *Hv-tw$_2$* differs from *WT* in the expression of several cDNA fragments that may be related to phenotypic instability induction and inflorescence/flower reversions in barley *Hv-tw$_2$;Hd* double mutants. Among such fragments there were f.N1/17 (homologous to the *Arabidopsis* chromatine activity regulator gene *EMF1*) and two transcription factor-encoding fragments – f.N39 (homologous to the wheat *VRN-B1* gene) and f.N41 (homologous to the barley *Lks2* gene and the *Vrs1/Hox1* locus).

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Wir haben die Kunst, damit wir nicht an der Wahrheit zugrunde gehen
Friedrich Nietzsche

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SANTRAUKA

Fenotipo nestabilumas gamtoje yra gana paplitęs reiškinys – natūraliomis sąlygomis jį gali sukelti įvairūs išoriniai veiksniai, tokie, kaip temperatūros ir apšvietimo pokyčiai (Bonnett, 1966), abiotinis stresas (Boyko ir Kovalchuk, 2011; Yao ir kt., 2011) bei įvairūs patogenai (Boyko ir kt., 2007; Ghareeb ir kt., 2011). Fenotipo variacija būdinga ir kai kuriems viengubiems bei dvigubiems mutantams (Forster ir kt., 2007; Babb ir Muehlbauer, 2003; Trevaskis ir kt., 2007; Wang ir kt., 2010b; Dreni ir kt., 2011; Li ir kt., 2011; Müller-Xing ir kt., 2014; Zheng ir kt., 2015; Šiukšta ir kt., 2015) ir netgi epimutantams (Zhang ir kt., 2012). Nepaisant plataus fenotipo nestabilumo paplitimo, jo mechanizmas nėra pakankamai ištirtas. Dar mažiau žinoma apie fenotipo nestabilumą, kuris atsiranda dvigubuose mutantuose, kurių tėviniams viengubiems mutantams toks kintamumo tipas nebūdingas – šia tema iki šiol pasaulyje atlikti tik pavieniai tyrimai (Müller-Xing ir kt., 2014).

Sukryžminus pleiotropinį *Hv-tw$_2$* mutantą, kuriam būdinga nereguliari lodikulių transformacija į reprodukcinius organus, su įvairiais *Hv-Hooded/Kap1* (*Hv-Hd*) tipo mutantais, kuriems dėl *BKn3* geno IV-jame (didžiausiame) introne įvykusios 305 bp duplikacijos vietoje akuotų vystosi ektopinės į žiedą panašios struktūros (Müller ir kt., 1995), buvo gautos dvigubų *Hv-tw$_2$;Hd* mutantų linijos, pasižyminčios ypač didele fenotipo variacija (Vaitkūnienė ir kt., 2004a, b). Kai kurie tokie savitos išvaizdos dvigubi mutantai gali būti naudojami dekoratyviniais tikslais (Siuksta ir kt., 2012), tačiau didelei jų daliai be įvairiu laipsniu išsivysčiusių tėvinių (viengubų) mutantų požymių yra būdingas fenotipo nestabilumas, pasireiškiantis pradiniams mutantams nebūdingais pokyčiais, kurių spektras ženkliai pralenkia žinomų miežio ir kitų miglinių šeimos augalų mutantų fenotipus (Šiukšta ir kt., 2014; Šiukšta ir kt., 2015). Fenotipo nestabilumu pasižyminčių miežio dvigubų *Hv-tw$_2$;Hd* mutantų žiedo/žiedyno struktūros variacijos apima daugelį žinomų miežio ir kitų miglinių šeimos augalų mutantų fenotipų (Bonnett, 1966; Ambrose ir kt., 2000; Babb ir Muehlbauer, 2003; Duan ir kt., 2003; Ikeda ir kt., 2007; Trevaskis ir kt., 2007; Thompson ir kt., 2009; Whipple ir kt., 2010), tačiau didžiausias šio reiškinio išskirtinumas miežio dvigubuose *Hv-tw$_2$;Hd* mutantuose yra jo paveldima forma. Panašaus pobūdžio, tik siauresnio spektro generatyvinės fazės grįžimo į vegetatyvinę reiškiniai – žiedyno ir žiedo reversijos – nustatyti ir tiriant dvigubus vairenio Polycomb grupės genų mutantus (Müller-Xing ir kt., 2014).

Dvigubų *Hv-tw$_2$;Hd* mutantų fenotipo nukrypimų pobūdis primena kai kuriuos žinomus dvigubus auksino signalinės sistemos mutantus (Krizek, 2011; Gallavotti, 2013), o tai leido *Hv-tw$_2$;Hd* mutantų fenotipo nestabilumą susieti su fitohormonų, pirmiausiai su auksino, balanso sutrikimais. Kita vertus, neseniai nustatyta, kad *BKn3* geno IV-ąjame introne esančioje 305 bp duplikacijoje, lemiančioje *Hv-Hooded/Kap1* fenotipą (ektopinio žiedo susidarymą apatinio žiedažvynio/akuoto pereinamojoje zonoje), yra reguliacinių *cis*-elementų, atrankiai sąveikaujančių su etileno atsako baltymais, o *Hv-Hooded/Kap1* fenotipą iš dalies normalizuoja egzogeninis etilenas (Osnato ir kt., 2010). Remiantis šiais faktais buvo iškelta prielaida, jog dvigubų *Hv-tw$_2$;Hd* mutantų fenotipo nestabilumas taip pat gali būti susijęs ir su sutrikusia etileno kelio funkcija.

Į tyrimus įtraukus didesnį skaičių dvigubų mutantų, tokių, kaip *Hv-tw;laxatum-a* (*Hv-tw;lax-a*), *Hv-tw;tweaky No.18* (*Hv-tw;tw No.18*) ir *Hv-tw;tweaky and missing kernels* (*Hv-tw;twmk*), paaiškėjo, kad miežio dvigubų mutantų paveldimo fenotipo nestabilumo paleidiklis (trigeris) yra būtent mutantiniai *Hv-tweaky spike* tipo aleliai,

todėl buvo aktualu nustatyti genų raiškos pokyčius *Hv-tw* tipo mutantuose, kurie galėtų lemti dvigubų mutantų fenotipo nestabilumą.

Darbo tikslas ir uždaviniai

Įvertinti paprastojo miežio dvigubų žiedo/žiedyno raidos mutantų, gautų sukryžminus *Hv-tw* ir *Hv-Hd* tipo mutantus, fenotipo variacijos ribas ir ištirti galimas šių dvigubų mutantų fenotipo nestabilumo priežastis.

1. Ištirti paprastojo miežio dvigubų mutantų fenotipo variacijų spektrą ir juo remiantis nustatyti žiedo raidos kryptis.
2. Remiantis miežio *BKn3* geno reguliacinių regionų sekoskaitos rezultatais, identifikuoti miežio viengubų ir dvigubų mutantų *BKn3* alelio tipą.
3. Įvertinti ir palyginti miežio viengubų ir dvigubų mutantų kaliaus augimo intensyvumą.
4. Nustatyti sintetinio auksino 2,4-dichlorfenoksiacto rūgšties (2,4-D) poveikį miežio viengubų ir dvigubų mutantų žiedyno (varpos) morfologiniam spektrui.
5. Ištirti auksino 2,4-D ir auksino inhibitorių poveikį miežio viengubų ir dvigubų mutantų pagrindinio ir ektopinio žiedo struktūrai.
6. Nustatyti egzogeninio etileno įtaką miežio viengubų ir dvigubų mutantų akuoto raidai ir pagrindinio bei ektopinio žiedo struktūrai.
7. Ištirti platesnį dvigubų mutantų spektrą ir nustatyti, kuri mutacija yra miežio dvigubų mutantų paveldimo fenotipo nestabilumo paleidiklis (trigeris).
8. Diferencinio vaizdinimo metodu atlikti preliminarią *Hv-tw*$_2$ mutanto ir jo pradinės veislės ‘Auksiniai II’ genų raiškos skirtumų paiešką bei nustatyti su fenotipo nestabilumo indukcija galimai susijusius genus-kandidatus.

Ginamieji teiginiai

1. Miežio dvigubų *Hv-tw*$_2$*;Hd* mutantų fenotipo variacija apima platų spektrą žinomų žiedo/žiedyno raidos mutacijų ir savo mastu jas pralenkia.
2. Skirtingo *Hv-Hooded/Kap1* fenotipo viengubi ir dvigubi *Hv-Hd* tipo miežio mutantai pagal tirtus žymenis turi tą patį *BKn3* geno alelį.
3. Miežio *Hv-tweaky* tipo mutacijos yra susijusios su sutrikusia auksino funkcija ir yra dvigubų mutantų fenotipo nestabilumo paleidiklis (trigeris).
4. Miežio dvigubų mutantų žiedo/žiedyno fenotipo nestabilumą lemia lokalūs ektopiniai auksino koncentracijos nuokrypiai.
5. Etileno poveikis miežio viengubų ir dvigubų mutantų žiedo/žiedyno raidai labiausiai priklauso nuo jų genetinio fono ir stipriausiai pasireiškia ektopinėms į žiedą panašioms struktūroms.

Darbo mokslinis naujumas

Remiantis miežio dvigubų *Hv-tw*$_2$*;Hd* mutantų fenotipo nestabilumo modifikavimo sintetiniu auksinu 2,4-D ir auksino inhibitoriais tyrimų rezultatais, auksino disbalansas pirmą kartą aprašytas kaip fenotipo nestabilumo priežastis. 2,4-D poveikio viengubų ir dvigubų mutantų žiedo/žiedyno raidai ir kaliaus augimui tyrimai leido susieti miežio *Hv-tweaky* tipo mutacijas su auksino fiziologijos defektais. Atlikta preliminari *Hv-tw*$_2$ mutanto pakitusios raiškos genų, galimai susijusių su šios mutacijos sukeliamu fenotipo nestabilumu dvigubuose mutantuose, paieška, leidusi *Hv-tw*$_2$ mutante preliminariai

identifikuoti kelis epigenetiniame genų raiškos reguliavime, streso atsakuose ir raidos procesuose dalyvaujančius pakitusios raiškos genus.

Taip pat pirmąsyk atliktas visų (iš viso keturiolikos) VU Botanikos sodo kolekcijoje saugomų *Hv-Hooded/Kap1* (*Hv-Hd*) tipo mutantų *BKn3* geno dviejų reguliacinių sričių sekvenavimas, parodęs, kad nepaisant didelės fenotipo įvairovės, visi *Hv-Hd* tipo mutantai turi vienodą IIIc tipo alelį.

Mokslinė ir praktinė darbo reikšmė

Darbų metu atrinkta grupė miežio dvigubų mutantų, kurie dėl savo savitos išvaizdos gali būti tiesiogiai pritaikyti dekoratyviniams tikslams arba būti naudojami kaip pradinė selekcinė medžiaga kuriant naujas veisles. Be to, iš kelių šimtų VU Botanikos sodo kolekcijoje saugomų miežio dvigubų *Hv-tw$_2$;Hd* mutantų atrinkta keliolika stabiliai ektopines į lapus/stiebus panašias išaugas sudarančių ir nesudarančių genotipų, iš kurių tyrimų metu gautos genetinės linijos, sudarytos iš vieno augalo palikuonių. Tokios dvigubų mutantų linijos yra vertinga medžiaga tolimesniems miežio žiedo/žiedyno reversijos tyrimams, o auksino ir etileno poveikio miežio mutantų žiedo organų raidai ir žiedo/žiedyno reversijoms tyrimų rezultatai prisidės prie gana fragmentiškų fundamentinių žinių apie šių fitohormonų reikšmę miglinių šeimos augalų reprodukciniams procesams.

Preliminari *Hv-tw$_2$* mutanto pakitusios raiškos genų paieška diferencinio vaizdinimo metodu leido identifikuoti keturis naujus pakitusios raiškos cDNR fragmentus, kurių sekos bus užregistruotos NCBI duomenų bazėje ir ją papildys

Išvados

1. Ištyrus vasarinio miežio (*Hordeum vulgare* subsp. *vulgare* L.) mutantų ektopinių į žiedą panašių darinių pokyčius, galima išskirti tokią žiedo raidos kryptį (trendą): nežymios ektopinės išaugos ant akuotų → vamzdelio pavidalo struktūros (neinvertuotos/invertuotos padėties) → žiedai su steriliais reprodukciniais organais.
2. Nustatyta didelė viengubų ir dvigubų mutantų įvairovė pagal kaliaus augimo indeksą (GI). Viengubų mutantų GI buvo mažesnis nei *wt*, o kai kurių dvigubų mutantų GI lenkė jų tėvinių mutantų GI. Dideli kaliaus augimo skirtumai nustatyti ir pagal reakciją į 2,4-D, auksino inhibitorius ir HC toksiną.
3. Visi VU Botanikos sodo kolekcijoje saugomi *Hv-Hooded/Kap1* tipo mutantai (iš viso 14) ir visos dvigubų *Hv-tw$_2$;Hd* mutantų linijos pagal tirtus žymenis turi vienodą *BKn3* geno IIIc tipo alelį.
4. Sintetinis auksinas 2,4-dichlorfenoksiacto rūgštis (2,4-D) laukinio tipo augalams sukėlė nedidelius žiedo organų skaičiaus pokyčius ir varpos morfozes, kurios yra *Hv-tweaky* mutacijų fenokopijos. Be to, po poveikio 2,4-D sumažėjo trumpų varpos trūkių *Hv-tw$_2$* mutanto varpose. Tai leidžia susieti *Hv-tweaky* tipo mutantams būdingą fenotipą su auksino kelio pokyčiais.
5. 2,4-D sumažino kai kurių dvigubų mutantų linijų normalios struktūros pagrindinių žiedų dažnį ir indukavo pagrindinio žiedo kuokelių transformaciją į chimerinius organus. Auksino inhibitorių HFCA ir PCIB poveikis buvo priešingas, abu inhibitoriai

padidino dvigubo *Hv-tw$_2$;Lh* mutanto linijų normalios struktūros pagrindinio žiedo dažnį.

6. Kai kurių dvigubų mutantų linijose 2,4-D paskatino pilnos struktūros ektopinio žiedo raidą, o auksino inhibitoriai PCIB ir HFCA, atvirkščiai, kai kurių genotipų pilnos struktūros ektopinių žiedų dažnį sumažino.
7. Dvigubų mutantų fenotipo nestabilumas irgi iš dalies susijęs su auksino funkcijos sutrikimais, nes 2,4-D ir auksino inhibitoriai HFCA ir PCIB pastebimai modifikavo dvigubų *Hv-tw$_2$;Hd* mutantų pagrindinių bei ektopinių žiedų sandarą.
8. Etefono poveikis daugiau pasireiškė ektopinėms žiedo struktūroms: beveik visiems tirtiems viengubiems ir dvigubiems mutantams etefonas padidino žiedų, kuriems išsivysto akuotas/akuotėlis, dažnį, tačiau lodikulių transformacijai įtakos neturėjo. Kai kurioms dvigubų mutantų linijoms (dažniausiai *Hv-tw$_2$;Lh*) etefonas padidino žiedų su reprodukcinių organų skaičiaus variacijomis dažnį.
9. Ištyrus didesnį skaičių dvigubų mutantų, gautų sukryžminus ne tik *Hv-tw$_2$* su *Hv-Hd*, bet ir *Hv-tw* su kitais mutantais (*Hv-lax-a*, *Hv-tw No.18* ir *Hv-twmk*) nustatyta, kad paveldimo nestabilumo paleidikliai (trigeriai) yra mutantiniai *Hv-tw* arba *Hv-tw$_2$* aleliai.
10. Diferencinio vaizdinimo metodu *Hv-tw$_2$* mutante preliminariai nustatyti keli pakitusios raiškos cDNR fragmentai, galimai susiję su *Hv-tw$_2$;Hd* dvigubų mutantų žiedo/žiedyno reversija ir fenotipo nestabilumu. Tai fragmentas N1/17, homologiškas vairenio chromatino pertvarkos *EMF1* genui ir fragmentai, homologiški genams, koduojantiems transkripcijos veiksnius: fragmentas N39, homologiškas kviečio *VRN-B1* genui bei fragmentas N41, homologiškas miežio *Lks2* genui ir *Vrs1;Hox1* lokusui

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