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Assessment of epigenetic methylation changes in hop (*Humulus lupulus*) plants obtained by meristem culture

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Abstract: *In vitro* meristem cultures have been used for the production of hop (*Humulus lupulus* L.) virus-free rootstocks worldwide, because multipropagation is considered to preserve the genetic stability of the produced plantlet. Nevertheless, *in vitro* tissue cultures can cause genetic and epigenetic changes. Therefore, we studied the genetic and epigenetic variability of Saaz Osvald's clones, Sládek and Premiant cultivars on the DNA methylation level by methylation-sensitive amplification polymorphism (MSAP). *In vitro* propagated plants, acclimatised glasshouse rootstocks as well as derived mericlones and control plants under field conditions were used for the analyses. A total of 346 clearly and highly reproducible amplified products were detected in the MSAP analyses within the studied hop plants. We found 16 polymorphic products (4.6% of products) and 64 products with methylation changes (18.5% of products) in the analyses. The demethylation events were comparable to the *de novo* methylation events. Most demethylation changes were found in the *in vitro* plants, but only a few of them were found in the derived mericlones under field conditions. In contrast, the *de novo* methylation changes persisted in the acclimatised plants under glasshouse or field conditions. A hierarchical cluster analysis was used for the evaluation of the molecular genetic variability within the individual samples. The dendrogram showed that the individual samples of the same variety, more or less, clustered together. Because the methylation status varied during the virus-free rootstock production process, we suppose that de/methylation process is a natural tool of epigenetics and evolution in vegetatively propagated plants.

Keywords: demethylation and *de novo* methylation changes; epigenetic variability; hierarchical cluster analysis; mericlones; meristem *in vitro* tissue cultures; methylation-sensitive amplification polymorphism (MSAP)

In vitro tissue cultures have been used for the multiplication of plants, due to their totipotency, for more than fifty years worldwide. Their uses have been associated with the initiation and formation of somaclonal variability due to genetic and epigenetic changes caused by the development and stress conditions (Miguel & Marum 2011; Krishna et al. 2016). Meristem *in vitro* tissue cultures have been used for the production of virus-free hop rootstocks (*Humulus lupulus* L.) for more than 30 years in the Czech Republic (Svoboda & Kopecký 1996). Even though plant regeneration via an *in vitro* culture can induce genetic and epigenetic variation, genetic stability is assumed in the micropropagation process, but it has to be proven and confirmed. Krishna et al. (2016) reported no somaclonal variation for the almond (*Prunus dulcis*), banana (*Musa* sp.), gerbera (*Gerbera jamesonii*), turmeric (*Curcuma longa*), grapevine (*Vitis* spp.), *Hedychium coronarium*, *Kaempheria galanga* and *Swertia chirayita* plants, regenerated from an apical meristem. However, the genetic stability of clonally propagated multiple-shoot cultures established from meristems has been proven, some epigenetic changes can occur during the long-term cultivation (Smýkal et al. 2007). Epigenetic changes have been mainly found on the methylation level as

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reported by Miguel and Marum (2011) for the apple (*Malus* × *domestica*), common hazel (*Corylus avellana*), grapevine (*Vitis vinifera*), banana (*Musa* sp.), pea (*Pisum sativum*), lance asiabell (*Codonopsis lanceolata*) and the orchid *Doritaenopsis*. Methylation changes were also found within the *in vitro* regenerated hop plants of the cultivar Nugget from a callus (Peredo et al. 2006), after cryopreservation (Peredo et al. 2008) and during the micropropagation of nodal meristems (Peredo et al. 2009) by molecular methods. Therefore, we examined the DNA methylation levels and variability by methylation-sensitive amplification polymorphism (MSAP) within the Saaz Osvald's clones 31, 72 and 114, Sládek and Premiant cultivars in different stages of the virus-free rootstock production process (*in vitro* culture, glasshouse acclimatisation, field growth) in comparison to control plants from rhizomes under field conditions.

MATERIAL AND METHODS

Plant material. Individual mericlones (M) were derived from meristems of selected mother plants in the maintenance hop breeding garden (Hop Research Institute, Co. Ltd., Žatec) and preserved as clonally propagated virus-free *in vitro* multi-shoot cultures without heat therapy. Five mericlones of the Saaz Osvald's clone 72 (M1–M5) were established during 1988–1989, two mericlones each of the Saaz Osvald's clones 31 and 114, the Sládek variety (M1–M2) and one mericlone of the Premiant variety (M1) were developed during 1996–1997. The *in vitro* multi-shoot culture of the mericlones have been monthly subcultured and maintained on solid half-strength Murashige and Skoog (MS) media at 26 °C with a 16 h light period (2 500 lx) according to Svoboda and Kopecký (1996) up to date. Annually, around one thousand *in vitro* plants of each clone and variety are acclimatised in a glasshouse (TU-FLOR Co., Ltd., Tušimice, Czech Republic), always representing a mixture of mericlones. The acclimatised plants are propagated by cuttings and used for virus-free mother plant production. The mother plants are overwintered outside and used the following year for the hop rootstock production by using cuttings for propagation in the glasshouse (TU-FLOR Co., Ltd.). The hop rootstocks are rooted during the summer under field conditions and distributed to the hop growers. Leaf samples from all the process stages for each clone and cultivar in the glasshouse (five acclimatised cuttings, four mother plants and five rootstock samples) were collected in May, 2019. Nine leaf samples of the acclimatised individual Saaz Osvald's clone mericlones and five mixed mericlone samples under field conditions (research farm in Stekník) were also collected in May, 2019. For comparison, five control plant samples, derived from rhizomes in 2004, of each clone and variety under field conditions were collected from the maintenance breeding hop garden (Hop Research Institute, Co.Ltd., Žatec).

Molecular analyses. DNA was isolated from the young leaf samples by the cetyl trimethylammonium bromide (CTAB) method according to Patzak (2001). The methylation-sensitive amplification polymorphism analyses were performed according to Peredo et al. (2009) using both EcoRI-MspI and EcoRI-HpaII digests. The pre-selective and selective amplifications were carried out with classical amplified fragment length polymorphism (AFLP) cycling parameters (Patzak 2001). The primers used for the pre-selective amplification were EcoRI+0 (GACTGCGTACCAATTC) and HpaII/MspI+0 (ATCATGAGTCCTGCTCGG), while the fluorochrome-labelled (6-FAM) primers EcoRI+AAC, ACT and ACG and the non-labelled primers HpaII/MspI+ACT, AAT and TCC (Generi Biotech, Hradec Králové, Czech Republic) were used for the selective amplification in all nine combinations. At the end of the selective polymerase chain reactions (PCRs), the samples were electrophoresed (Patzak et al. 2017) in an automatic ABI PRISM 3130 sequencer (Applied Biosystems, Lincoln, USA). The PCR products were analysed by GeneMapper 5.0 (Applied Biosystems) based on the molecular standard ROX 400HD (Applied Biosystems). The products were scored for the presence or absence of fragments in each sample.

Data analyses. A hierarchical cluster analysis was used for the evaluation of the molecular genetic variability within the individual samples. It was based on Jaccard's similarity coefficient and Neighbour-Joining (NJ) clustering by the Unweighted Pair Group Method with Arithmetic means (UPGMA) in DARwin (Ver. 5.0.155; Dissimilarity Analysis and Representation for Windows, http://darwin.cirad.fr/darwin). The resulting dendrogram (Figure 1) was visualised by Geneious Pro (Ver. 4.8.2; Biomatters Ltd., Auckland, New Zealand).

RESULTS AND DISCUSSION

A total of 346 clearly detectable and highly reproducible amplified products were detected in the

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MSAP analyses within all the studied hop plants. The average number of products per primer combination was 38.4 with a maximum of 59 for the primer combination EcoRI+ACG /HpaII/MspI+AAT (Table 1). In other crop studies, e.g., Baránek et al. (2015) detected 64 products for grapevine and Gimenez et al. (2016) detected 46 products for garlic. However, it was lower than was found in previous works for hops by Peredo et al. (2006; 2009). They reported an average number of 54 and 50.9 products per primer combinations, respectively, with a maximum of 73 for the primer combination EcoRI+ACG/HpaII/MspI+TCC. These differences can be due to studying different hop cultivars and processes such as the *in vitro* regeneration from an organogenic callus. We found a similar level of polymorphism (4.6% of products) and methylation changes (18.5% of products) in comparison to previously published results where the total polymorphism and DNA methylation changes ranged from 8.72% to 28.73% (Peredo et al. 2009).

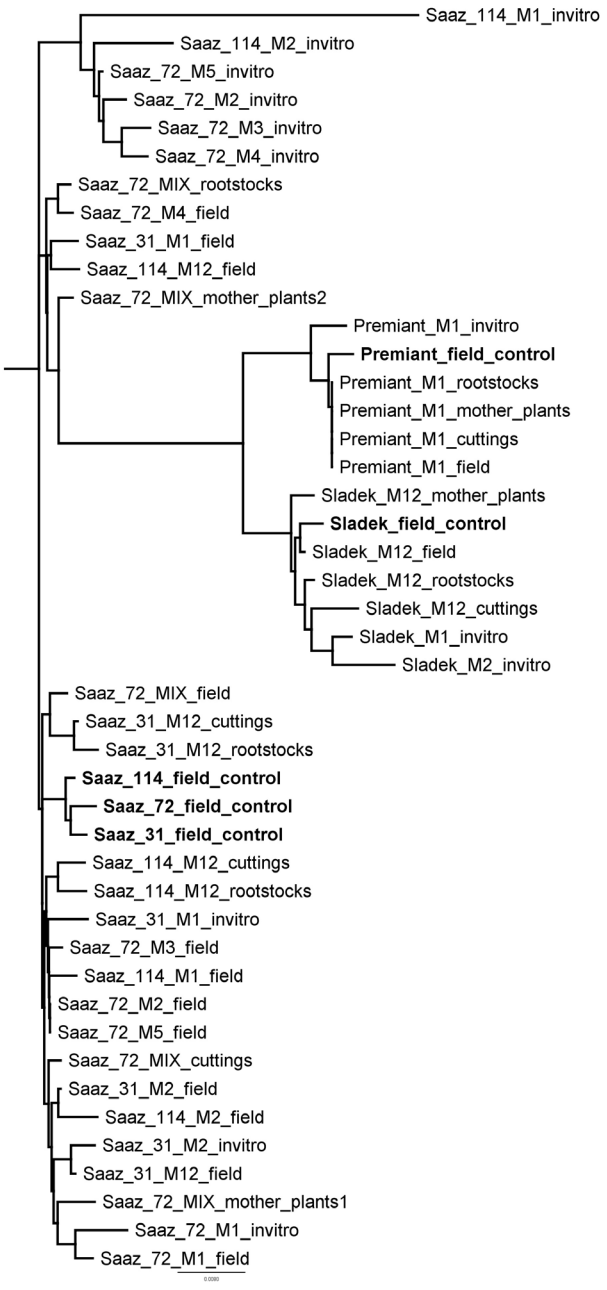

Figure 1. The dendrogram of the genetic distances of 12 *in vitro* mericlone plants, 14 acclimatised glasshouse samples (5 cuttings, 5 rootstocks and 4 mother plants), 14 acclimatised mericlones and 5 control plants under field conditions revealed by the unweighted Neighbour-Joining clustering based on Jaccard's similarity coefficient (DARwin, Ver. 5.0.155)

Table 1. The number of amplified products, polymorphism and methylation characteristics of the methylation-sensitive amplification polymorphism primer combinations

Combination E+M/H	No. of products	Polymorphic	Methylation change
ACT+AAT	39	0	2
ACT+TCC	31	0	8
ACT+ACT	41	3	6
ACG+AAT	59	4	12
ACG+TCC	31	2	6
ACG+ACT	35	2	6
AAC+AAT	35	3	8
AAC+TCC	42	2	9
AAC+ACT	33	0	7
Total	346 (100%)	16 (4.6%)	64 (18.5%)
Average	38.4	1.8	7.1

The demethylation events (Table 2) were comparable to the *de novo* methylation events (Table 3). The highest frequency (1.46%) in the demethylation changes was found for the primer combination EcoRI+AAC/HpaII/MspI+AAT (Table 2) and the highest frequency (1.544%) in the *de novo* methylation changes was found for the primer combination EcoRI+ACG/HpaII/MspI+AAT (Table 3). The demethylation changes were more frequent in the *in vitro* grown plants than in the acclimatised plants under the glasshouse or field conditions (Table 2). In contrast, the *de novo* methylation changes persisted in the acclimatised plants under the glasshouse or field conditions (Table 3). Our results were lower than the previously published results by Peredo et al. (2009). They found 22.23% of methylation changes within micropropagated plants. The demethylation events

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Table 2. The number of demethylation events in the restriction site (frequency in % in brackets) within the individual samples for the methylation-sensitive amplification polymorphism primer combinations in comparison to the control plants

Combination E+M/H	*In vitro* mericlones	Acclimatised glasshouse plants	Acclimatised plants under field conditions
ACT+AAT	1 (0.057)	0	0
ACT+TCC	1 (0.072)	0	0
ACT+ACT	4 (0.217)	4 (0.217)	2 (0.108)
ACG+AAT	4 (0.149)	2 (0.075)	2 (0.075)
ACG+TCC	4 (0.287)	1 (0.072)	1 (0.072)
ACG+ACT	2 (0.127)	0	0
AAC+AAT	23 (1.460)	18 (1.143)	5 (0.317)
AAC+TCC	16 (0.847)	1 (0.053)	1 (0.053)
AAC+ACT	6 (0.404)	0	1 (0.067)
Total	61 (0.392)	26 (0.167)	12 (0.077)
Average	6.8	2.9	1.3

occurred more often than the *de novo* methylation, up to 4.25 times, for the *in vitro* micropropagated plants (Peredo et al. 2009). Unfortunately, there was no data about the methylation status after the acclimatisation. Peredo et al. (2009) also reported about other epigenetic polymorphisms ranging from 3.8% to 9.83%. We suggested that it could be a single nucleotide polymorphism in the restriction sites, which we found in a comparable frequency of 0.2% to the other changes.

Most of the demethylation changes were found in the mericlone Saaz 114 M1 (27). However, only 19.6% of all the changes persisted in the acclimatised Saaz Osvald's clone plants (12) under field conditions (Table 4). Even though three of them were a new one. In contrast, the *de novo* methylation changes persisted in all of the studied mericlones (Table 5). The methylation and demethylation processes are a natural tool of plant epigenetics and evolution. Baránek et al. (2015) published that the methylation changes for *in vitro* grapevine plants were dynamic and reversible modifications one to three years after cultivation at least. Because all of the studied mericlones were kept in *in vitro* conditions a longer time (24–31 years), the *de novo* methylation changes were irreversible. Neither a visible phenotypical variation nor changes in the secondary metabolite contents in the hop cones (data not shown) were found between the acclimatised mericlone and the control plants under the field conditions.

We used a hierarchical cluster analysis (Figure 1) for the evaluation of the molecular genetic variability within the individual samples. The obtained dendrogram showed that the individual samples of the same variety, more or less, clustered together with some exceptions. The individual Saaz Osvald's

Table 3. The number of de novo methylation events in the restriction site (frequency in % in brackets) within the individual samples for the methylation-sensitive amplification polymorphism primer combinations in comparison to the control plants

Combination E+M/H	*In vitro* mericlones	Acclimatised glasshouse plants	Acclimatised plants under field conditions
ACT+AAT	0	0	0
ACT+TCC	3 (0.215)	0	0
ACT+ACT	0	0	0
ACG+AAT	35 (1.318)	36 (1.356)	41 (1.544)
ACG+TCC	2 (0.143)	2 (0.143)	3 (0.215)
ACG+ACT	6 (0.381)	4 (0.254)	6 (0.381)
AAC+AAT	7 (0.444)	7 (0.444)	7 (0.444)
AAC+TCC	0	0	0
AAC+ACT	1 (0.067)	0	0
Total	54 (0.347)	49 (0.315)	57 (0.366)
Average	6.0	5.4	6.3

Table 4. The number of demethylation events in the restriction site within the individual mericlones

Mericlone	*In vitro* plants	Acclimatised glasshouse plants	Acclimatised plants under field conditions	
Saaz 31 M1	1	7	2*	0
Saaz 31 M2	1		0	
Saaz 72 M1	4	6	3	2
Saaz 72 M2	4		0	
Saaz 72 M3	5		1	
Saaz 72 M4	4		2	
Saaz 72 M5	2		0	
Saaz 114 M1	27	5	0	1
Saaz 114 M2	2		1*	
Sládek M1	4	8	0	0
Sládek M2	5		0	
Premiant M1	2	0	0	0

*different event from *in vitro*

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Table 5. The number of de novo methylation events in the restriction site within the individual mericlones

Mericlone	*In vitro* plants	Acclimatised glasshouse plants	Acclimatised plants under field conditions	
Saaz 31 M1	5	8	4	4
Saaz 31 M2	4		4	
Saaz 72 M1	7	20	5	5
Saaz 72 M2	5		5	
Saaz 72 M3	6		5	
Saaz 72 M4	6		5	
Saaz 72 M5	5		5	
Saaz 114 M1	4	6	4	3
Saaz 114 M2	4		3	
Sládek M1	3	9	0	3
Sládek M2	3		0	
Premiant M1	2	6	0	2

clones in this study could not be distinguished, but there are intra- and inter-clonal variabilities between the Saaz Osvald's clones, as found by Patzak (2003) which may explain, to some extent, the variability of the various mericlones of the respective Saaz clones. In our analyses, six Saaz *in vitro* mericlones (Figure 1, first cluster) were more distinctive from the other Saaz Osvald's clone plants. Since a mixture of various mericlones was always used when starting the acclimatisation for the rootstock production process, we cannot exactly trace the origin of plants back. For example, sample Saaz_72_MIX_mother_plants1 was clustered to mericlone 72_M1 and sample Saaz_72_MIX_mother_plants2 was clustered to mericlone 72_M4 under the field conditions (Figure 1). This mericlone (M4_field) was the most distant from the Saaz Osvald's clone 72 control plants under the field conditions. Mericlone 31_M1_field was also more distant from the Saaz Osvald's clone 31 control plants under the field conditions, even if the original *in vitro* mericlone was clustered together (Figure 1). On the other hand, both the Saaz Osvald's clone 114 mericlones (M1 and M2) in the *in vitro* conditions significantly varied from the control plants under the field conditions, but the acclimatised mericlone plants were closely clustered under the field conditions, except for their mix sample (Saaz_114_M12_field) with a coupled methylation status (Figure 1). So far, we have not found any phenotypical variation under the field conditions of the meristem derived plants to the original rhizome derived plants (data not shown). We would suppose that the epigenetic variability within the Saaz Osvald's clones and plants is natural and a lot of methylation changes can be within the non-coding regions of the hop genome having no impact on the phenotype.

CONCLUSION

Methylation-sensitive amplification polymorphism is useful for assessing the epigenetic variability within different stages of the virus-free plant production process of hops. However, it cannot cover all of the methylation changes inside the DNA due to the limitation of MSAP to the restriction sites only of the HpaII/MspI enzymes. The demethylation events have been found to be comparable to the *de novo* methylation events. The demethylation changes were more frequent in the *in vitro* grown plants than in the glasshouse or field plants, most of them were restored to the previous status. In contrast, *de* the *novo* methylation changes persisted in the acclimatised plants under the glasshouse or field conditions. So far, no influence on the phenotype of the plant could be observed. The obtained results are useful for all hop rootstock producers worldwide being aware that methylation changes can occur , but not always affecting the appearance of plant, when using *in vitro* meristem culture.

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