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CHANGES IN THE EXPRESSION OF THREE COLD-REGULATED GENES IN 'ELSANTA' AND 'SELVIK' STRAWBERRY (*FRAGARIA* × *ANANASSA*) PLANTS EXPOSED TO FREEZING

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ABSTRACT

Cold temperatures in midwinter and late-spring frosts cause severe damages to strawberry plants cultivated in temperate climate regions. Despite the seriousness of the problem, the plant mechanism of defense against cold stress has not been fully elucidated yet, especially in its molecular aspect. The presented investigations were conducted on the cold-susceptible cultivar 'Elsanta' and the cold-tolerant cultivar 'Selvik'. Expression profiles of three genes (*CBF4*, *COR47* and *F3H*) were determined at three time-points: 0, 6 and 12 weeks after sub-zero treatment at -12 °C. The *CBF4* gene was very strongly up-regulated in 'Selvik' plants and the highest value of the transcript level was detected just after the treatment (time-point 0). The *F3H* transcript in the treated 'Selvik' plants reached the level 4 times higher than in control plants in the 12th week after treatment (time-point 3). In 'Elsanta' plants, the *CBF4* and *COR47* genes were slightly up-regulated (time-point 2), while the *F3H* gene showed stability of expression. High positive correlation between the transcript level of *COR47* and transcript level of *CBF4* genes was observed for both cultivars.

Key words: strawberry, cold stress, gene expression

INTRODUCTION

Frost injury to perennial plants is one of the most important problems in the horticulture of temperate regions, where significant changes in temperature are frequent during the transition between the cold and warm seasons (Boyer 1982). Prolonged cold winters and late spring ground-frosts can have a negative influence on the growth and development of plants, which can result in significant reductions in fruit yield (Vij & Tyagi 2007; Shokaeva 2008). A harsh winter without snow cover is likely to cause damage to whole cold-susceptible plants, while a ground-frost in the spring can damage generative organs, such as flower buds and developing fruits of early blooming members of the *Rosaceae* (e.g., strawberry, apple, cherry, and apricot) (Rodrigo 2000). According to averaged statistical data, negative stress conditions are able to reduce fruit yield by more than 50%. However, these values are strongly dependent on the season. For instance, following the 2011-2012 winter, losses in apple orchards in the Netherlands were estimated at the level of 85 million euro, while the extent of damage to flower buds on Polish strawberry plantations reached 80% (www. eco-uprawy.pl 2012; www.minrol.gov.pl 2011). Thus, intensive studies on cold acclimation and tolerance to frost have been conducted since the 1970s to determine the physiological, biochemical, and later on also molecular mechanisms developed by plants during their evolution (Merymann 1971; George et al. 1974; Burke et al. 1976; Wisniewski & Davis 1989; Steponkus & Webb 1992; Thomashow 1998; Wiśniewski et al. 1999; Miura & Furumoto 2013).

The molecular fundamentals of plant response to low temperature stress were analysed mainly in the model plant *Arabidopsis thaliana*, and then, in

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a relatively narrower range, in cereals, *Brassica* spp., tobacco, peach and apple (Danyluk et al. 1994; Thomashow 1994; Stockinger et al. 1997; Gilmour et al. 1998; Liu et al. 1998; Ouellet at al. 1998; Medina et al. 1999; Wiśniewski et al 1999, 2006; Haake et al. 2002; Dubouzet et al. 2003; Miura & Furumoto 2013). Based on the model plant studies, several clusters of genes have been identified as being involved in the induction of the acclimation process and cold hardiness in plants. These main clusters contain genes encoding late embryogenesis abundant (LEA) proteins, a large class of molecular chaperons known as heat shock proteins (HSP), antifreeze proteins (AFP), and enzymes that remove damaging reactive oxygen species (ROS, SOD, APX) (Thomashow 1998). A special role in the process of plant adaptation to low temperatures is played by: cold-regulated genes (*COR*), transcription factor (TF) activated *COR* genes, and (MAP)-kinase-mediated cascades (Haake et al. 2002; Puhakainen et al. 2004; Kume et al. 2005; Medina et al. 2011).

In general, the stress-responsive genes have been divided into two groups: genes encoding substances taking part directly in the protection against cell dehydration (Wiśniewski et al. 1999), and genes encoding components of the signal transduction pathways and proteins that regulate gene expression in response to stress (Medina et al. 1999; Kmieć et al. 2005; Chinnusamy et al. 2006). Long-term studies on model-plants suggest that some steps of the mechanism of cold-tolerance might be conservative (Jaglo et al. 2001; Zhang et al. 2004). However, examinations of a wider spectrum of plant species have shown the possibility of additional components being induced to activate diverse molecular anti-freezing pathways (Hughes & Dunn 1996; Benedict et al. 2006).

Strawberry (*Fragaria* × *ananassa* Duch. ex Rozier), one of the evolutionary youngest representatives of *Fragaria*, is the most widely cultivated species within the genus. The annual world production of strawberries exceeds 4.5 million tons. The broad range of distribution and cultivation of dessert strawberry is associated with its genetic diversity and, as a result, the high adaptive capacity of the species (Hancock et al. 2008). Strawberry cultivars are also diverse in terms of cold-hardiness (Hürsalmi & Säkö 1991; Luby 1991; Rugienius & Sasnauskas 2005; Masny & Żurawicz 2007; Shokaeva 2008; Lukoševičiūtė 2013). However, only a few investigations on the genes regulating the cold-tolerance of strawberry were conducted (Owens et al. 2002; Schwab et al. 2009). In recent years, three key enzymes (chalcone synthase, CHS; flavonoid 3’-hydroxylase, F3H; dihydroflavonol 4-reductase, DFR) from the phenylpropanoid biochemical pathway (Koehler et al. 2012), as well as the dehydrins, galactinol and alcohol dehydrogenase (ADH) (Davik et al. 2013) were described as associated with cold tolerance in cultivated *F.* × *ananassa* and wild diploid strawberry spp., respectively.

In the presented study, we compared the expression profiles of three candidate genes: *CBF4*, *COR47* and *F3H* from different functional groups in two genotypes of strawberry – ‘Elsanta’ and ‘Selvik’, differing in their capacity of resistance to freezing (Rugienius & Sasnauskas 2005; Masny & Żurawicz 2007; Lukoševičiūtė 2013). These genes were selected based on the evaluation of the transcription level of different genes analysed in our earlier study (data unpublished). Two of the analyzed genes, *CBF4* and *COR47* encoding the AP2-type transcription regulator and dehydrin protein, respectively, are well known genes associated with acclimation too cold in *Arabidopsis* and many other higher plant species. The third one, *F3H*, encoding the flavonoid 3’-hydroxylase belonging to the phenylpropanoid pathway, is associated with response to low temperatures and freezing in some species; however, the role of this gene in strawberry response to cold temperatures has not been elucidated yet.

The aim of the investigations was to assess the involvement of the selected genes in defence responses induced in ‘Elsanta’ and ‘Selvik’ plants exposed to frost.

MATERIALS AND METHODS

Plant material. Two strawberry cultivars ‘Elsanta’ (cold susceptible) and ‘Selvik’ (cold tolerant) were used in the experiment. Runner plants of these cultivars were kept in a cold store at a temperature of -2.0 ± 0.3 °C at the end of November. Just before storage, the plants had been acclimated under natural conditions in a temperature range from +4 °C to –2 °C. The 'frigo' plants were planted into 0.8 l pots with a peat substrate and sand (4 : 1), and frozen at –12 °C for 3 hours (Masny & Żurawicz 2014) in a freezing chamber (BINDER GmbH, Germany). The temperature in the chamber was lowered at a rate of 1 °C per hour, and after freezing, it was raised at the same rate. After freezing, the plants were transferred to a heated greenhouse and maintained there at 20 ± 2 °C. Two experimental groups, control and frozen, consisting of 15 plants each (3 replications with 5 plants) were prepared for each cultivar. Samples of young leaves were collected for the molecular study at 3 time-points: immediately after the cold treatment, and then 6 and 12 weeks after exposing the plants to low temperatures. Three samples (100 mg of leaf tissue per sample) from each control and treated plant were collected. The collected tissues were stored at –80 °C before being used in the molecular study.

RNA extraction. Total RNA was extracted from leaf samples as described by Zeng & Yang (2002). Plant tissues were ground in liquid nitrogen and incubated for 10 minutes at 65 °C in an extraction buffer containing: CTAB (2%), PVP100 (2%), Tris-HCl (100 mM, pH 8.0), EDTA (25 mM); NaCl (2 M), spermidine trihydrochloride (0.05%), and ß-mercaptoethanol (2%). Nucleic acids were purified with chloroform : isoamyl alcohol (24 : l v/v) twice and then precipitated with 10 M LiCl. RNA was pelleted by centrifugation (30 min/30,000 rpm /4 °C), washed with 75% ethanol, air dried for 10 minutes and dissolved in DEPC-treated water. The RNA concentration and quality were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). Only samples with an RNA integrity number (RIN) > 7 were used for quantitative PCR.

Reverse transcription. First-strand cDNA from the total RNA (600 ng/sample) was synthesised with AffinityScript cDNA Synthesis Kit (Agilent Technologies) according to the manufacturer's instructions. The reaction volume (20 µl) contained: 2 × cDNA Synthesis Master Mix, oligo (dT) primer, AffinityScript RT/ RNase Block enzyme mixture. The reactions were performed at 25 °C/5 min, 42 °C/5 min, 55 °C/15 min., 95 °C/5 min. The cDNA samples were diluted 1:20 for use in quantitative PCR.

Real Time qPCR. Real-time quantitative polymerase chain reactions were run in a Rotor-Gene 6000 (Corbett Life Science) and quantified using the manufacturer's software. RT-PCR amplification was performed in a total volume of 10 µl containing 2x KAPA SYBR® FAST qPCR Master Mix2 Universal, 10 µM Forward and Reverse Primers, and cDNA (600 ng·µl^{-1}). Four primer pairs specific for strawberry sequences were synthesised based on GenBank data: *CBF4* (acc. No. HQ910515), *COR47* (acc. No. C0817504), *F3H* (AB201760), and *Actin* (acc. No. AB116565) (Table 1). All samples were amplified in triplicate from the same RNA preparation.

Table 1. Primer sequences used in the PCR

Primers	Primer F Forward sequence (5'-3')	Primer R Reverse sequence (5'-3')
CBF4	ttcaaggagacgaggcac	cgcagccatttcggta
COR47	gaggaaggagacgatgaagg	ccttcttctgctcctctgtgtag
F3H	acctcactctcggactcaaac	gagctgggttctggaatgtc
Actin	gggtttgctggagatgatg	cacgattagccttgggattc

The thermal profile of amplifications was as follows: cDNA was denatured at 95 °C for 5 min, followed by 40 cycles of 95 °C/10 s, 60 °C/20 s and 72 °C/20s. Melt curve analysis was programmed at the end of the run, 72-90 °C with temperature increments by 0.5 °C each step and 5 s at each degree, to determine reaction specificity and avoid contamination, mispriming and primer-dimmer formation. Each PCR product had a single melt curve. A negative control was included for each primer set. PCR products were subsequently analysed by agarose gel electrophoresis. *FaActin* was utilized as a reference sequence for normalization of qPCR.

RESULTS

The exposure of plants to a temperature of –12 °C influenced the activity of all the genes analysed, but the range of changes was dependent on the cultivar and sequence examined. The level of *CBF4* expression measured just after cold-treatment (time-point 0) was 5-fold and about 90-fold higher than its expression in non-frozen control plants of 'Elsanta' and 'Selvik', respectively. In the case of *COR47* gene, the influence of low temperature was not so spectacular, and the expression level was only twice as high in 'Elsanta' and 4 times as high in 'Selvik' as in the respective control plants. A similar change was noted for the *F3H* gene in 'Elsanta', whereas no significant difference in the expression level was observed between the frozen (treated) and non-frozen 'Selvik' plants.

At the next time-points, the expression levels of the *CBF4* and *COR47* genes significantly decreased and reached the level of the non-frozen plants. For 'Selvik', an almost 200-fold suppression of the transcription factor was noted 6 weeks after plant exposure to low temperature, which then reached a stable expression level. A five-fold suppression was observed for the same sequence at the 2nd time-point for 'Elsanta', but during the subsequent weeks the cold-treated plants died (Fig.1). In the case of the *COR47* gene, the transcript level of 'Selvik' reached slowly the values of non-frozen plants in the period between the 2nd and 3rd time-point (~3-fold suppression). The suppression level of the same gene in 'Elsanta' was lower (2-fold at the 2nd time-point) (Fig.2).

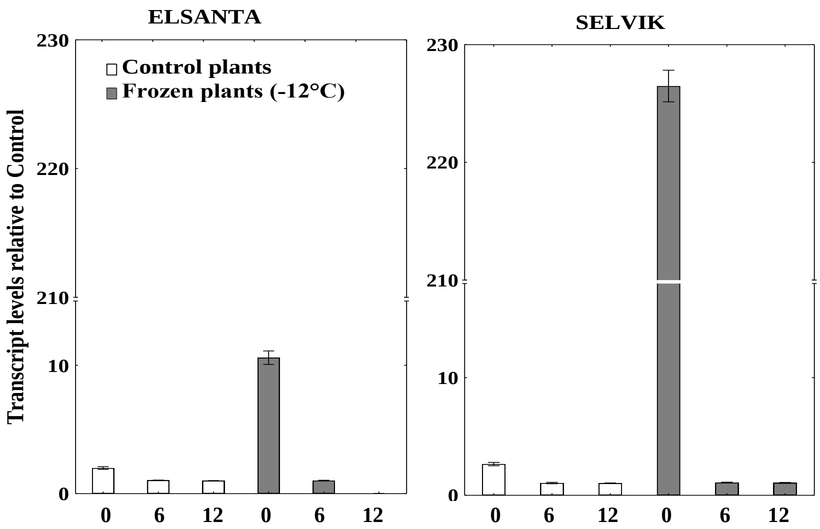

Fig. 1. Transcription profile of the *CBF4* gene obtained in Real Time qPCR at three time-points: 0, 6 and 12 weeks after cold treatment, for frozen (–12 °C) and non-frozen control plants of 'Elsanta' and 'Selvik'. Vertical bars represent ± SD

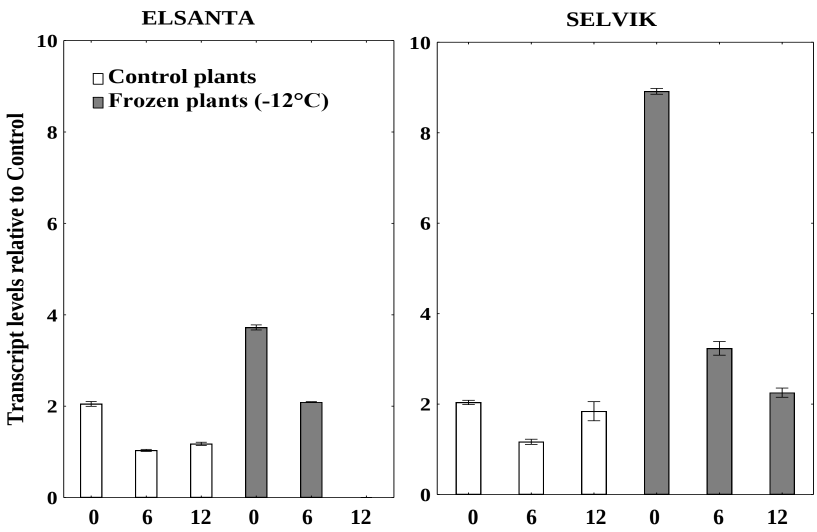

Fig. 2. Transcription profile of the*COR47* gene obtained in Real Time qPCR at three time-points: 0, 6 and 12 weeks after plant treatment, for frozen (–12 °C) and non-frozen control plants of 'Elsanta' and 'Selvik'. Vertical bars represent ± SD

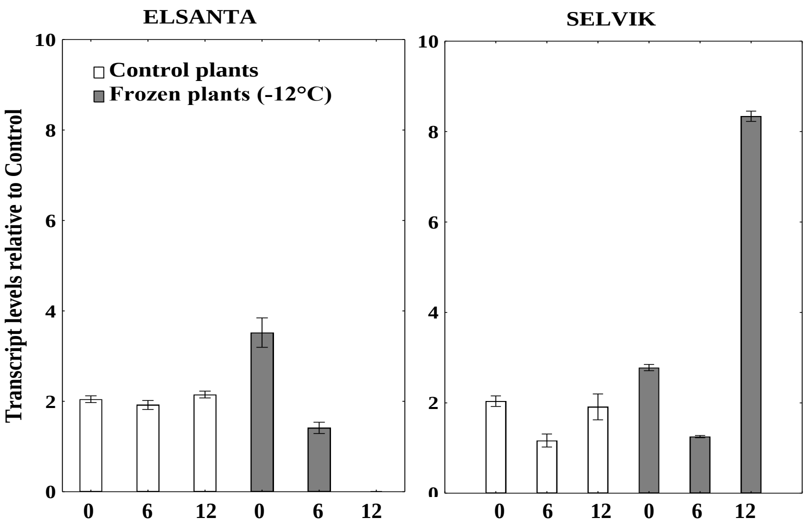

Fig. 3. Transcription profile of the *F3H* gene obtained in Real Time qPCR at three time-points: 0, 6 and 12 weeks after plant treatment, for frozen (–12 °C) and non-frozen control plants of 'Elsanta' and 'Selvik'. Vertical bars represent ± SD

The expression of the *F3H* gene in the tolerant cultivar 'Selvik' was different in comparison with the last two genes. Just after treatment at –12 °C, the level of transcript was comparable with that observed in the non-frozen plants, while in the period of 12 weeks after plant exposure to frost, induction of this gene's activity (4x) was noted. No changes in the level of *F3H* transcript were noted in the cold-treated 'Elsanta' plants (Fig. 3). High positive correlations for both cultivars were observed between transcript levels of *COR47* and *CBF4*, and additionally for 'Elsanta' between *F3H* and *CBF4*, as well as *F3H* and *COR47* (Table 2).

Table 2. Correlation coefficients between transcript levels of *COR47* and *CBF4*, *F3H* and *CBF4*, and *F3H* and *COR47* genes for 'Elsanta' and 'Selvik' strawberry cultivars (n = 18)

	COR47 × *CBF4*	*F3H* × *CBF4*	*F3H* × *COR47*
Elsanta	0.911***	0.931***	0.741**
Selvik	0.923***	-0.122	0.132

***; ** significance level at $p < 0.001$; 0.01 probability level

DISCUSSION

Cold injury is one of the major factors responsible for reducing the yield and quality of fruit crops in temperate regions. Thus, the derivation of cold-hardy cultivars has been a priority in many breeding programmes. However, the traditional approaches to breeding crop plants with improved tolerance to low temperature stress have so far met with limited success because of the unusual complexity of the phenomenon of cold hardiness. Cold hardiness encompasses various tolerance or damage avoidance mechanisms dependent on the stage of plant development, examined tissues, and genetic potential of each genotype (Callahan et al. 1991). Additionally, plant response to cold stress is strongly dependent on G (genotype) × E (environment) interactions (Tester & Bacic 2005). Thus, despite all the studies on cold hardiness that have been conducted on several model plants since the mid-twentieth century, the mechanisms of resistance to low temperatures and the role of individual cold-induced genes in many plant species are still unclear.

The ability of plants to tolerate low temperatures depends on the degree of hardening they have achieved. In the presented study, the acclimation procedure, which is known to induce proteins relevant to freezing survival (Zhu et al. 2007), was applied to the plants of both test cultivars. However, unlike in the cultivar 'Selvik', the adaptation process to tolerate low temperature was not successful in 'Elsanta', and all the plants of this cultivar died in the period between the 6th and 12th week after being frozen at –12 °C. This difference in the response of the test cultivars may be an effect of different interactions between cold-inducible proteins and the proteins which are associated with tolerance to low temperature but are not cold-inducible during plant acclimation (Takahashi et al. 2006).

The cultivars ‘Elsanta’ and ‘Selvik’ were also different in terms of the activity of three candidate cold-regulated genes. The *CBF*, belonging to the group of transcription factors in cold-response pathways, was originally described in *A. thaliana*. The genes from the *CBF* family were found to be induced by low temperature and dehydration stresses (Haake et al. 2002). The transcript level of this gene reached its maximum in 2 hours after model-plant exposure to a temperature of 2 °C (Gilmour et al. 1998). In woody plants, such as *Populus* spp., the mRNA of *CBF1-4* genes peaked in the range from 3 to 9 hours (Benedict et al. 2006). The transcripts of *CBF*-like genes in *Brassica napus*, wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.), and tomato (*Lycopersicon esculentum*) accumulated rapidly in response to low temperature and increased cold tolerance of both non-acclimated and cold-acclimated plants (Jaglo et al. 2001). In our study, the *CBF4* transcript was accumulated immediately after the cold treatment (time-point 0), but much stronger up-regulation was observed in tolerant ‘Selvik’ plants than in those of susceptible ‘Elsanta’. The same phenomenon was described by authors investigating *A. thaliana* and *Brassica* spp. (Jaglo et al. 2001). On the other hand, relatively small differences in the level of the CBF4 factor in plants of strawberry cultivars ‘Frida’ and ‘Jonsok’, which differ in cold tolerance, were found by Koehler et al. (2012), after exposing their plants to –2 °C. The strong induction of the *CBF4* in ‘Selvik’ plants in our experiments affirms the thesis that the gene was correlated with cold tolerance. However, it can be also correlated with dehydration of cells, being a consequence of osmotically active water movement from the cells to the intracellular spaces, and occurring at –10 °C but not at 2 °C (Thomashow 1998).

The *FaCOR47* belongs to the group of *COR* (cold responsive) genes. The *CORs* encoding cold-regulated dehydrins are dependent in their activity on cold-induced transcription factors (*CBF*) (Stockinger et al. 1997; Liu et al. 1998; Thomashow 1999). *COR* genes contain in their promoters *cis*-elements which are recognised by *CBF* (Chinnusamy et al. 2006; Medina et al. 2011). The influence of –9 °C on the expression of both *CBF1* and *COR47* genes was observed in plants from the genus *Thellungiella*. Expression of the *CBF1* gene in these plants reached the maximum level after 3 hours, while the accumulation of *COR47* transcripts started after 7 hours. Zalunskaitė et al. (2008) reported up-regulation of the *COR47* gene in strawberry plants even after 30 days of cold acclimation at 2 °C. However no regularity in respect of *COR47* homologue expression in cold-resistant and cold-susceptible cultivars was noted by these authors. The up-regulation of the *COR47* gene was also observed in plants of the two cultivars tested in our experiments. The trend of *COR47* activity was similar to that of *CBF4*, while the lower expression level at the same time-point can be explained by *COR*-dependence on the cascade of *CBF* transcription factors. Several research teams have shown that *COR*-gene expression is delayed towards CBF (Jaglo et al. 2001; Haake et al. 2002; Griffith et al. 2007). The correlation coefficients were calculated to estimate the relationship between transcript levels of genes relative to the control in both strawberry cultivars. The high positive correlation between the transcript level of *COR47* gene and the transcript level of *CBF4* gene ($r = 0.911^{***}$ for ‘Elsanta’ and $r = 0.923^{***}$ for ‘Selvik’) confirmed the close interdependence of these genes.

Flavanone 3-hydroxylase (*F3H*) is a key enzyme at a diverging point of the flavonoid pathway leading to production of different pigments: phlobaphene, proanthocyanidin, and anthocyanin. It has also been reported that the flavonoids are accumulated in the leaves and stem of plants in response to low temperature (Koehler et al. 2012; Theocharis et al. 2012). The size of our *F3H* amplicon was comparable with the size of the ortholog genes specific to *A. thaliana*, wheat, and *Gingko biloba* (Shen et al. 2006; Himi et al. 2011). The data concerning *F3H* expression in ‘Elsanta’ correspond to the results of Koehler et al. (2012) for ‘Frida’. In both cases, transient increment of *F3H* transcript for cold-sensitive strawberry was observed. By contrast, the freezing did not affect the mRNA level in our genotype ‘Selvik’, while in the tolerant cultivar ‘Jonsok’ the level of *F3H* transcript significantly decreased (Koehler et al. 2012). The increase in *F3H* expression noted in our investigation in the twelfth week after cold treatment can be difficult to explain because of the long period which had elapsed since the activation of stress.

The significant statistical differences obtained in our study had not influence on analyses of changes in genes expression.

The study of the three genes: *CBF4*, *COR47* and *F3H*, isolated from two strawberry cultivars, 'Elsanta' and 'Selvik', which differ in cold tolerance, showed their participation in plant tolerance increase to frost according to the model accepted for *A. thaliana*. The difference in the response of the compared genotypes is an effect of a multilevel and complicated mechanism of cold hardiness, including G (genotype) × E (environment) interactions.

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