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HUMAN LACTOFERRIN CHANGES LEAF MORPHOLOGY AND PATHOGEN RESISTANCE OF *MEDICAGO SATIVA* L.

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Abstract

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Human lactoferrin (hLf) is an iron-binding glycoprotein having antimicrobial activity, which is known to be involved in iron absorption and cell growth and proliferation. This study aimed investigation of the effect of hLf expression on leaf epidermal cell morphology of transgenic alfalfa plants (*Medicago sativa* L.), which exhibited enhanced resistance to bacterial pathogens. Leaf epidermal parameters were measured and a clear tendency of increasing size and decreasing number of pavement cells was found, which seemed to be related to hLf-cell cycle inhibition and compensatory cell enlargement. Furthermore, stomatal density was lower on both leaf surfaces of transgenic plants, probably due to Lf-induced inhibition of stomatal development as well. In addition, transgenic plants exhibited some characteristics, such as significant elongation of leaf epidermal cells, reduction in overall leaf size, and occasionally visible reduction of leaf chlorophyll content, which are usually related to a condition of iron deficiency, and in our case might due to iron chelation properties of hLf. Based on our observations, we assume that hLf expression changed leaf morphology, which partially contributed to the improved pathogen resistance of alfalfa in addition to the direct antimicrobial effect of the recombinant protein.

Key words: human lactoferrin, alfalfa, expression, leaf morphology

Abbreviations: hLf – human lactoferrin

Introduction

Molecular farming is a promising strategy for production of valuable pharmaceutical and commercial proteins in plants. Transgenic plants expressing different pharmaceuticals, monoclonal antibodies and antigens for edible vaccines are increasingly being produced and tested for their ability to synthesize safe recombinant proteins with high authenticity and biological activity.

Human lactoferrin (hLf) is a major iron-binding glycoprotein of milk and other epithelial secretions, known to have various biological functions including role in iron metabolism, cell proliferation and differentiation, modulation of immunity and defense against pathogens. A characteristic of significant importance to molecular farming is the capacity of hLf to act as a natural anti-bacterial, antiviral, anti-fungal and anti-parasitic agent. It has bacteriostatic activity due to ability to bind free iron, essential for the microbial growth (Arnold et al., 1980), as well as direct bactericidal effect involving N-terminal antimicrobial domain (lactoferricin), which damages bacterial cell membranes (Yamauchi et al., 1993).

Human Lf has been already produced in different plant systems including tobacco (Salmon et al., 1998; Zhang et al., 1998), potato (Chong and Langridge, 2000), tomato (Anzai et al., 2000; Lee et al., 2002), rice (Anzai et al., 2000; Nandi et al., 2002; Takase et al., 2005), maize (Samyn-Petit et al., 2001), barley (Kamenarova et al., 2007) and ginseng (Kwon et al., 2003). We have previously reported that expression of hLf cDNA in transgenic alfalfa plants (*Medicago sativa* L.) confers resistance against some bacterial pathogens and influences phenotype of the plants as well (Stefanova et al., 2013). In this paper we describe effect of recombinant hLf expression in transgenic alfalfa plants on their leaf epidermal morphology in order to determine cellular basis of the leaf phenotype variation and its eventual relation to plant pathogen resistance.

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The epidermis is the outer layer of cells covering the leaf that protects plants from environmental damages. It has several functions essential for plant survival including protection against water loss by transpiration, regulation of gaseous exchange, secretion of metabolic compounds and absorption of water. Furthermore, it has been shown that epidermis has a crucial developmental role serving as a key regulator of leaf growth and expansion interacting with internal tissues (Becraft, 1999; Marcotrigiano, 2010). Usually upper (adaxial) and lower (abaxial) leaf surfaces have different construction and are supposed to serve different functions. The epidermis includes several different cell types: epidermal ("pavement") cells, most numerous, largest and least specialised; guard cells, forming small pores called stomata, responsible for regulation of gas exchange and water vapor between the plant tissue and atmosphere; and trichomes, highly specialized cells, providing defense against insects and pathogens.

This paper analyses the hLf-induced change in organisation of alfalfa leaf epidermal cells, as a plausible candidate for the observed leaf phenotype variation and higher plant resistance against bacterial pathogens.

Materials and Methods

Plant growth conditions

Transgenic alfalfa plants (*Medicago sativa* L.) were obtained by transformation with *Agrobacterium tumefaciens* harboring the expression vector pCB-LF, which contained hLf cDNA under the control of the constitutive *cauliflower mosaic virus* (CaMV) *35S* promoter as described previously (Stefanova et al., 2013).

Well-developed *in vitro* regenerants, for which incorporation of hLf cDNA into plant genome and its expression were proven, were transferred and grown in greenhouse conditions under 16/8 h light/dark photoperiod at 24°C, relative humidity between 50% and 60%, and 700 μmol m^{-2} s^{-1} PAR. Non-transformed alfalfa plant grown under the same conditions was used as a control in the performed analyses.

Microscopic observations and morphometric analysis

For analysis of leaf morphology, upper recently matured **leaves** (generally the 3rd-5th **leaf** from the **top)** from each experimental line were **collected** and pictures were taken with Canon Powershot A610 digital camera (Canon Inc., Japan). The leaflet **area** and **index** of **circularity** were determined by analysing pictures with ImageJ software (National Institutes of Health, Bethesda, USA).

For the study of leaf epidermal features, at least 5 **central leaflets** were **randomly** selected and stored in absolute ethanol. The clearing procedure was performed as described previously (Vassileva et al., 2010). Microphotographs were taken using upright microscope Olympus BX51 coupled to a XC50 digital microscope camera and Cell B software (Olympus, Germany). Digital images were processed and analysed by ImageJ software. The size, perimeter and circularity index of pavement cells as well as density of stomata were measured for both the upper and lower epidermal surfaces.

Statistics

Size, perimeter and circularity index of 10 cells per image were measured and two images per plant were analysed by ImageJ software. Counting of the stomata was performed in all images available. Data were analysed using bifactorial ANOVA analysis for repeated measures in order to determine **epidermal** cell morphological **variations as dependent** on the location on the leaf surface and transgenic line (Sokal and Rohlf, 1981). The **results** in each graph **represent** the **mean** ± **standard error** of the **mean.** All the statistical analyses were performed using Microsoft Excel 2010 software.

Results

In this study, the attention is focused on hLf's effects on epidermal cell morphology of transgenic plants showing increased pathogen resistance. Among the obtained alfalfa transgenic plants for which expression of recombinant hLF was proven, one clone (53) with obviously different phenotype was selected. Plants from this clone were prevailing multifoliate with elongated leaflets compared to the control plants, which possessed trifoliate leaves (Figure 1A-C). To monitor the effect of hLf expression on the growth and development of *M. sativa*, leaflet size and shape were quantified in the leaves of Lf transformant lines. The mean **size and circularity index** of the **leaflets** was significantly reduced in all of the analysed transgenic plants, compared to the control (Figure 1D, E).

We performed a detailed characterization and morphometric analysis of leaf epidermal cells in order to determine the pattern of variation in pavement and stomatal guard cells in plants expressing h**Lf** protein and control non-transformed plants. **Apparent structural differences between the** typical pavement cells of **these two plant groups were noted** (Figure 2A-F). The cells from transgenic plants had elongated irregular shape and many deep lobes (Figure 2A-D), compared to the cells from control plants which were less elongated and had fewer and shallower lobes (Figure 2E, F). In general, epidermal cells from plants, expressing hLf had a tendency of being larger (measured as average pavement cells area, μm^2) and displayed cell perimeter values (μm) higher than controls as a result from the deeper undulation of their cell walls (Figure 3A, B).

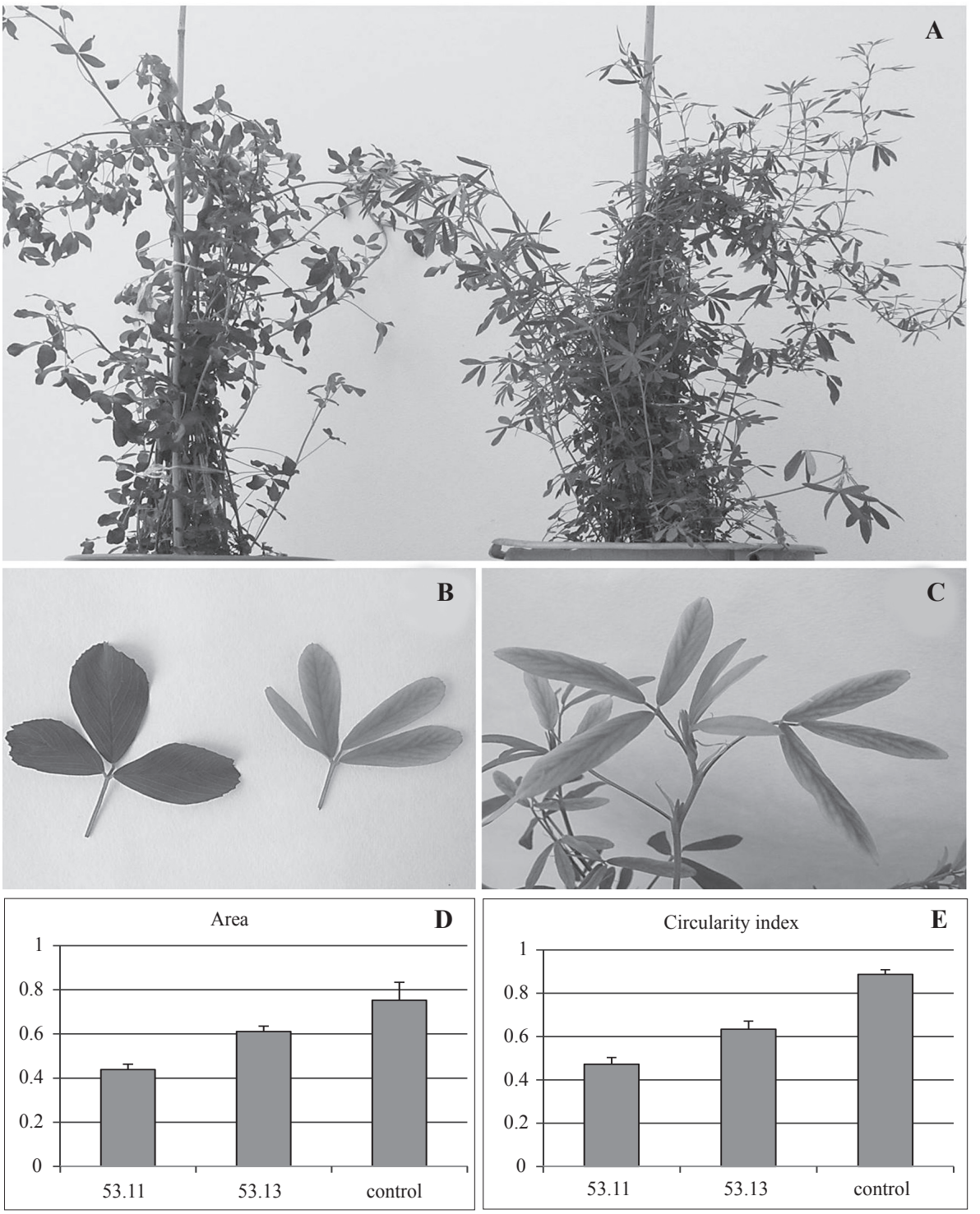

Fig. 1. Transgenic alfalfa plant expressing hLf – clone 53 (right) and non-transformed control plant (left) (A); Leaves from transgenic plants differed significantly by their size, shape and number of leaflets from the control leaves (B, C); Average size (cm^2) and circularity index of leaflets from transgenic and control plants (D, E). Bars represent standard error of the means

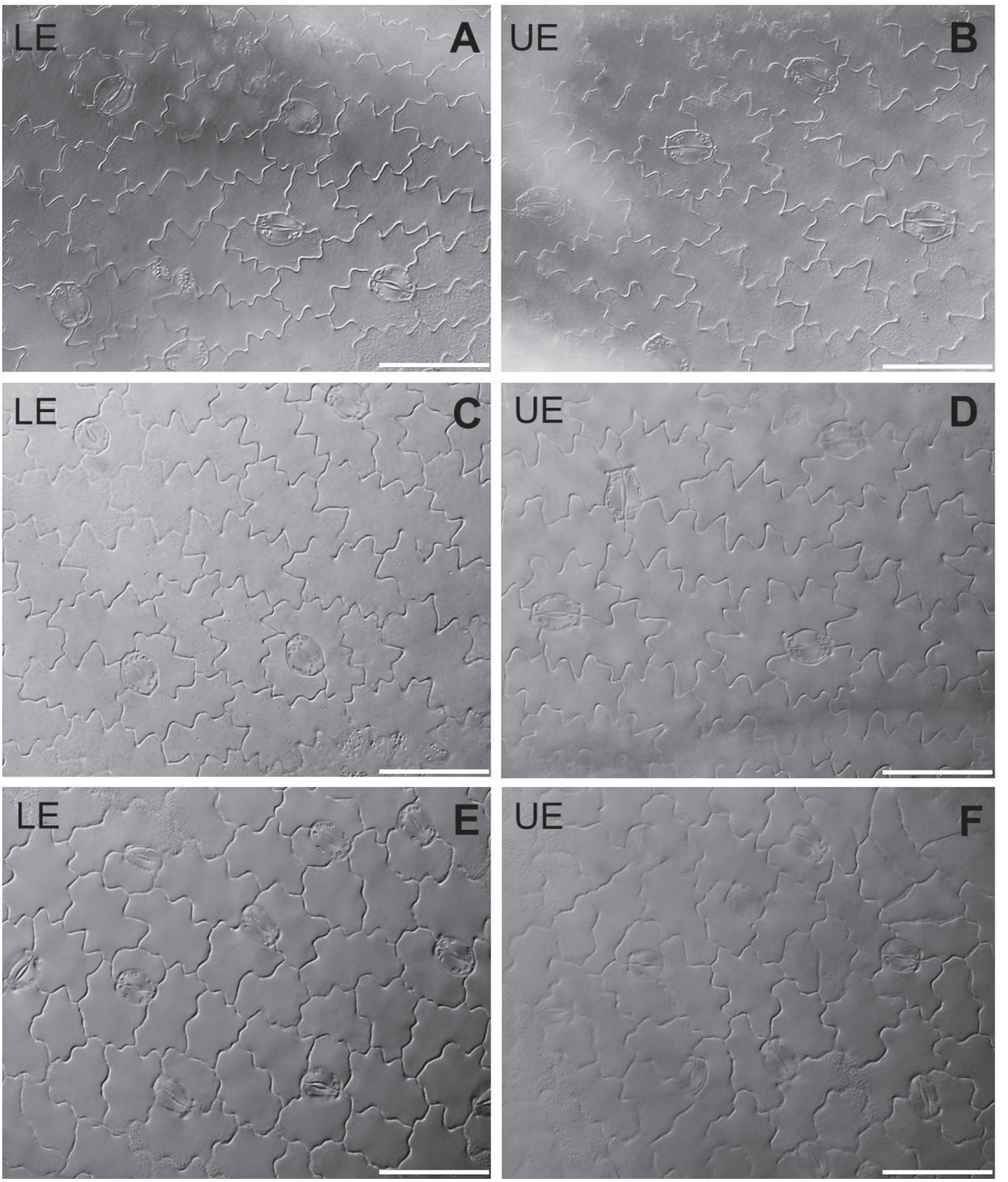

Fig. 2. Representative images of leaf epidermal surfaces of two transgenic alfalfa plants from clone 53: 1 (A,B) and 2 (C, D) and control non-transformed plant (E, F)
Noted: **LE – lower epidermis; UE – upper epidermis; Scale bar, 50 µm**

In order to demonstrate differences in pavement cells shape, we used ImageJ based generated value, called circularity (Sorek et al., 2011). Circularity index is defined by the correlation between cell area and perimeter, 4π (area)/(perimeter)^2, and reveals to similarity of an object with perfect circle (Schwartz, 1980). For a given cell, circularity index would have a value between 0 and 1. If cells are rectangular without lobes, their circularity index would be closer to 1, while for cells with many deep and narrow lobes it would have a value closer to 0 (Sorek et al., 2011). We found that pavement cells of all transgenic plants analysed had an average value of circularity index significantly lower than that of control cells, which confirmed the visible from microphotographs epidermal cell elongation in the leaves of hLf expressing plants (Figure 3C).

To determine whether the increased size and polarity of the pavement cells correlates with the final size and shape of the leaves, we calculated the average area and circularity index of leaflets from transgenic and non-transformed plants (Figure 1D, E). The comparison of these two parameters showed a negative correlation between leaflet area and epidermal cell size and highly significant positive correlation between circularity index of cells and whole leaflets.

We also found a variation in stomatal density in leaves from the transgenic plants compared to the control leaves. In general stomata were distributed on both the abaxial and adaxial epidermis but their number per unit leaf area was lower in leaves from hLf-expressing plants (Figure 3D).

Discussion

In this report, we summarize leaf morphological characteristics of transgenic alfalfa plants, expressing recombinant hLf protein. Our observations showed that size and shape of epidermal cells in all analyzed transgenic plants differed significantly from those of epidermal cells in control non-transformed plants.

The final leaf size is primary attributed to the total number of cells and the average cell size. Leaf development of dicotyledonous species is characterized by a cell proliferation phase with actively dividing cells, followed by a post-mitotic cell expansion phase, when cells grow and differentiate. After expansion, cells mature and the final leaf size is achieved (Beemster et al., 2005). The dynamics of these processes is still poorly understood, partially because the reduced cell proliferation is often compensated by an increase in cell size and vice versa (Tsukaya, 2002).

Generally, leaf area enlargement is strongly correlated with an increase in the size of pavement cells (Asl et al., 2011). However, in our study the size of leaves and pave-

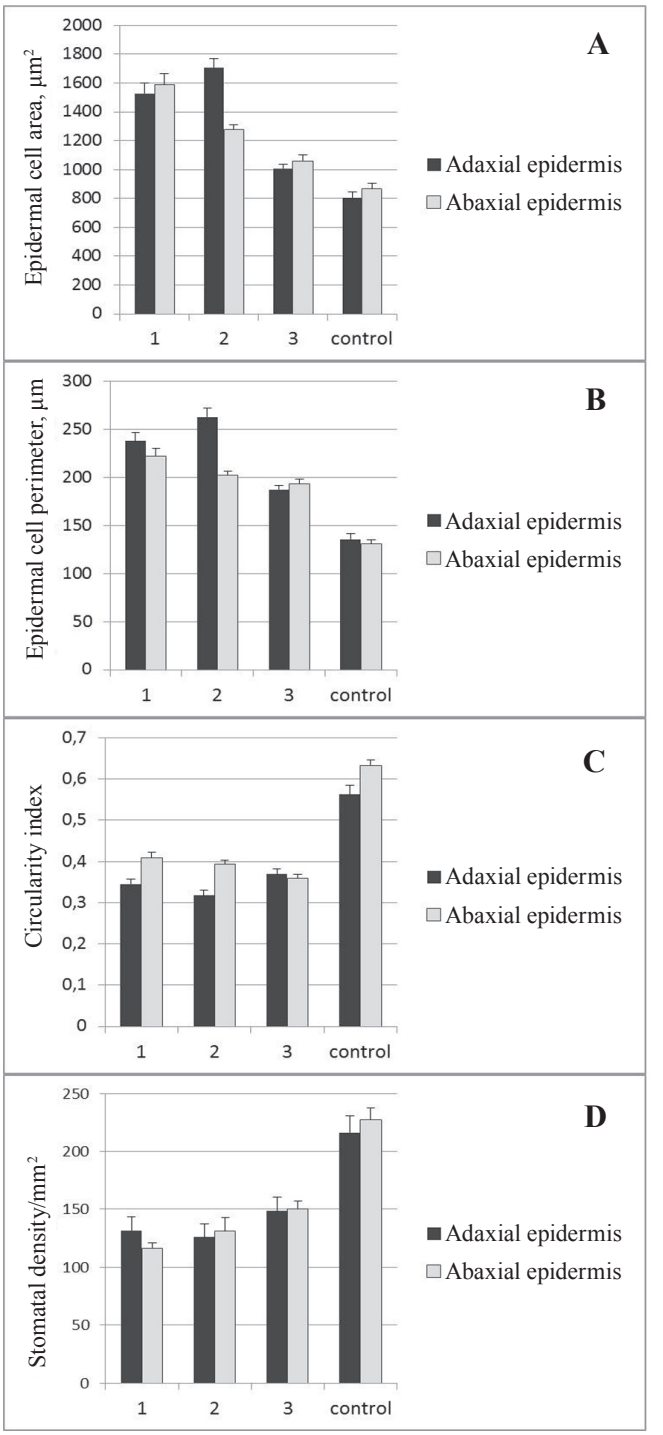

Fig. 3. Average size (µm²) (A), perimeter (B) and circularity index (C) of epidermal cells and stomatal density (number of stomata per mm²) (D) on the upper and lower leaf surfaces of hLf- expressing plants compared to non-transformed control plant. Bars represent standard error of the means

ment cells showed a negative relationship. In the smaller size leaves of plants expressing hLf, larger pavement cells were observed, compared to control leaves. Thus, the reduced leaf size cannot be explained by the decreased size of pavement cells. It can be rather related to the reduced pavement cell division and increased cell expansion, which could lead to the observed leaf morphology. Many previous studies have reported that Lf gene expression correlates with cell growth and proliferation (Teng, 2006). Treatment of human cancer cells with Lf causes cell cycle inhibition, resulting from increased levels of specific cyclin-dependent kinase inhibitors (Damiens et al., 1999; Xiao et al., 2004). Our findings are consistent with these observations, indicating a clear trend of an **increased size** and **decreased number** of leaf pavement cells in Lf **transformant** lines. This implies that Lf gene expression has affected epidermal cell proliferation and growth in alfalfa leaves.

It is known, that the number of cells produced during the cell division phase of leaf development determine the final leaf size (Gonzales et al., 2010). When cell proliferation is reduced because of certain mutations, the reduction in the final leaf area is compensated by an increase in the size of individual leaf cells. In *Arabidopsis* leaves, a decrease in cell number below a certain threshold triggers an increase in mature cell size, a phenomenon that has been termed "compensation" (Tsukaya, 2002; 2008). Examination of the mutants exhibiting a compensation phenotype has shown that cell enlargement does not occur via the uncoupling of cell division and cell growth, rather, it is sustained by the specific upregulation of cell expansion (Ferjani et al., 2007). Cell expansion is often associated with DNA endoreplication, a modified cell cycle in which DNA successively duplicates without the subsequent completion of mitosis. Given that significant cell enlargement occurs during compensation and an increase in ploidy level is associated with an increase of cell size in specialized cell types, such as pavement cells and trichomes (Melaragno et al., 1993), endoreplication could participate in compensation-induced cell enlargement. Although there are reports demonstrating that ploidy level is not always correlated with cell size (De Veylder et al., 2001; Beemster et al., 2005), it is tempting to suggest that in our study the increased pavement cell size in Lf lines is related to the Lf-induced cell cycle inhibition and compensatory cell expansion (Damiens *et al.* 1999; Xiao et al. 2004).

In addition to the observed enlargement of the pavement cells, we found a significant reduction of their circularity index in transgenic plants. As an iron-binding glycoprotein hLf takes part in iron transport, cellular iron delivery and control of the free iron in external secretion in animals (Ward and Connelly, 2004). It is well known that iron deficiency affects the morphology and physiology of plants (Briat, 2007). Furthermore, it has been recently shown that hLf expression alters iron homeostasis and leads to iron deficiency in transgenic tobacco (Kumar et al., 2012). Decreased iron content due to expression of hLf in transgenic alfalfa plants could be a possible explanation for the elongation of leaf epidermal cells. Iron deficiency leads to increased epidermal cell length and decreased final leaf size in peach and pear (Fernández et al., 2008). Our results from circularity index calculation were consistent with these findings and confirmed the visible from microphotographs trend of elongation of pavement cells. In addition, one of the transgenic plants analysed displayed a visible reduction of chlorophyll content (as seen in Figure 1B, C) - a first sight symptom of iron deficiency, which might due to hLf binding to iron.

Differences were also observed in stomatal density of transgenic and control non-transformed plants. Microscopic analysis revealed that leaves of Lf lines displayed a considerable reduction (35%-45%) in number of stomata. Stomata are specialised epidermal structures consisted of two guard cells surrounding a pore, through which plants absorb CO_2 and release O_2. The rest of the epidermal surface is covered with an impermeable layer of wax that reduces water loss by transpiration, which make stomata critical for plant survival and productivity. The development of stomata requires careful control over cell division and differentiation. Several reports have demonstrated that the up- or downregulation of cell cycle-related genes is essential for mitotic division and stomata formation in leaves (Menges et al., 2006; Kono et al., 2007). The decreased stomatal density found on both epidermal layers of Lf leaves suggests a Lf-induced inhibition of cell division during stomatal development as well.

Furthermore, altered epidermal morphology of plants has the potential to affect plant-pathogen interactions, as many pathogens utilise the epidermal features of leaves to facilitate infection. For instance, stomatal pores provide a natural entry point for the infection and facilitate its spread (Underwood et al., 2007). Therefore, both number and size of stomata may affect plant resistance to pathogen attack. In contrast, Stenglein et al. (2005) have shown an inverse relationship of leaf susceptibility to infection with altitudinal range; stomatal numbers may be affected by decreasing CO_2 partial pressure, thus contributing to pathogen resistance.

In our study, the reduced stomatal density of **both** adaxial and abaxial surfaces in the leaves of Lf lines (Figure 3D) correlated well with the decreased level of infection, as compared with the control (Stefanova et al., 2013). On the other hand, pathogen challenge alone has the capacity to change the stomatal characteristics of newly emergent leaves and these changes are CO_2-dependent. The adaxial epidermis

shows a significant increase in stomatal numbers following infection of mature leaves under ambient CO_2, which reach even a higher level under CO_2 enrichment (Lake and Wade, 2009). Whether altered stomatal density is a consequence or a cause of the observed pathogen resistance of the Lf lines remains a moot point. It could be suggested that the presence of less stomata in Lf lines could underpin their higher pathogen resistance, providing less entry points for the infection.

In conclusion, this study demonstrated that expression of human Lf in alfalfa led to changed leaf structure and guard cell morphology, partially alleviating plant pathogen resistance. The obtained results represent a promising **basis for further investigations** of the mechanisms underlying Lf-associated changes in leaf morphology and pathogen resistance at cellular and molecular levels.

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