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Morphological, histochemical and ultrastructural indicators of maize and barley leaf senescence

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Abstract

In this study we report on morphological and histochemical indicators of maize and barley leaf senescence. We determined how the traits such as distribution of stomata and hairs, presence of epicuticular wax, staining of tissues with toluidine blue, change with leaf age and within the leaf blade. We identified regions of young, non-mature leaves as exhibiting juvenile phase, regions with features typical for mature and fully differentiated leaves - as an adult phase and regions with traits of age damage as a senescing phase. Ultrastructural analysis of these regions of leaves gives a clear picture of the time development of the senescence process. Appearance of morphological and histological indicators of senescence in certain regions of leaf is correlated with ultrastructural changes of mesophyll cells of the same regions. We have thus found a relatively simple method of estimating the stage of senescence both in maize and barley.

Additional key words: adult phase, *Hordeum vulgare*, juvenile phase, programmed cell death, *Zea mays.*

Introduction

Leaf senescence is a genetically regulated stage in the plant life cycle leading to death (Yen and Yang 1998, Simeonova *et al.* 2000, Yao *et al.* 2001, Simeonova *et al.* 2004). Full understanding of leaf senescence requires analysis of this process in different regions of leaves of different age.

Leaf development and cellular differentiation in monocotyledons begins at the tip of the leaf and then gradually progresses towards the leaf base. Cells at the base of an immature leaf are likely to be much less mature than cells at the tip (Rascio *et al.* 1980, Sylvester *et al.* 1990). Therefore a monocotyledon leaf appears to be a very good model of leaf differentiation.

It was reported that morphological and histological features of monocotyledon leaves can be indicators of phase change from juvenile to adult (Kerstetter and Poethig 1998, Orkwiszewski and Poethig 2000, Sylvester *et al.* 2001). Some phase-specific traits of leaf identity have been determined (Lawson and Poethig 1995, Orkwiszewski and Poethig 2000, Sylvester *et al.* 2001). Traits that are typical for the juvenile phase, such as production of epicuticular wax and bulbous epidermal cells, lack of epidermal hairs and cell walls staining purple with toluidine blue (Poethig 1990, Freeling and Lane 1994, Sylvester *et al.* 2001) are indicators of development process while those typical of the adult phase are indicators of maturity.

Strong correlations between different tyrosine phosphorylated proteins (considered as phase-related and age-related proteins) and plant development were shown in cultured juvenile, adult and rejuvenated *Sequoia sempervirens* shoots (Huang *et al.* 2003/4).

Genes that regulate vegetative development in maize have been identified by screening for mutations that affect one or more phase-specific traits. Many maize mutations required for the expression of juvenile traits and appearance of adult traits have been identified (Evans *et al.* 1994, Moose and Sisco 1994, Evans and Poethig 1995, 1997, Poethig 1997, Vega *et al.* 2002) as well as mutations of leaf morphogenesis altering cell division orientations, size and shape of leaf tissue (Becraft *et al.* 1990, Sylvester *et al.* 1990, Smith *et al.* 1996, Larkin *et al.* 1997, Reynolds *et al.* 1998, Gallager and Smith, 1999, Croxdale 2000). Fewer studies on barley mutants have been performed (Döring *et al.* 1999).

In our study we report on morphological and histochemical indicators of different stages of leaf ontogenesis. We determined how the distribution of stomata and hairs, presence of epicuticular wax and staining of tissues with toluidine blue change with age of the leaf and within the leaf blade. We performed ultrastructural analysis of particular regions of the leaf.

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We correlated these morphological and histological features of particular regions of leaves with their ultrastructure. We identified regions of leaves with traits typical for young, non-mature stage as exhibiting juvenile phase, regions with features typical of a mature and fully differentiated stage - as an adult phase and regions with traits of age damage - as a senescing phase. These results provide a simple and straightforward method of estimating the stage of leaf ontogenesis.

We used maize (*Zea mays* L.), a well known model for evaluating plant progress from juvenile to adult phase. We compared leaf senescence process in maize with the corresponding process in barley (*Hordeum vulgare* L.). We showed that the distribution of morphological indicators in barley and maize leaves is very similar although both species differ in the dynamics of growth.

Material and methods

Plants and growth conditions: Seeds of *Zea mays* L. cv. Oleńka and *Hordeum vulgare* L. cv. Rataj were soaked for 24 h in water and placed in wet filter paper in darkness at room temperature. The next day germinated seeds were sown in soil in pots in a growth chamber (16-h photoperiod, irradiance of 45 $W\,m^{-2}$, day/night temperature 24/22 °C, relative air humidity 50 %). Second leaves were taken for analysis.
Blade length was measured from the ligule to the tip of the leaf. For microscopic observations, basal, middle and apical parts (10 mm of tissue) of second leaves of 10- or 20-d-old seedlings and basal regions only of leaves of 30-d-old seedlings were analyzed.

Light microscopy: Fragments of leaves were fixed in solution containing three parts of ethanol to two parts of acetic acid. Lower and upper epidermes were removed and the number of stomata was counted on 30 squares (36 μm^2) in a *Malassez* chamber. For cross sections, fragments of leaves were fixed in chrome:acetic acid:formalin (CrAF; 0.5:1:20), dehydrated, embedded in paraffin and cut on a microtome. 12 μm-thick cross sections were stained with 0.05 % toluidine blue at pH 4.4 in 0.1 M phosphate-citric buffer. Metachromatic cytochemical reaction revealed purple staining in the case of cellulose cell walls and blue staining in the case of cell walls, which changed their composition, probably due to lignification. Sections were analyzed under *Zeiss Amplival* (*Carl Zeiss*, Jena, Germany) light microscope and photographs were taken using a *Nikon FDX-35* camera (Tokyo, Japan).

Scanning electron microscopy was used to determine the distribution of leaf hairs and epicuticular wax both on adaxial and abaxial leaf surfaces. Samples at each development stage were dehydrated in a graded ethanol series, then in a mixture of absolute ethanol and acetone (1:1), next in pure acetone and finally dried in a *Critical Point Dry System* (CPDS). CPDS involves heating in a specialized pressure chamber in which the specimen is immersed in liquid CO_2 for 1 h, so that the liquid around the specimen is converted to a supersaturated vapour (after Williams and Sylvester 1994). Subsequently the specimen was gold coated in *JEOL JEE-4X* (Tokyo, Japan), and examined with a *JEOL JSM S1* microscopes. Photographs were taken on *FOTOPAN* 100 ISO 100/21 type 120 films.

Transmission electron microscopy: Samples, about 1 - 4 mm^2 in area, were cut from each part of a leaf. The material was fixed in 1.6 % glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2 for 2 h, washed in buffer and placed in 1 % OsO_4 at 4 ºC in 20 μM cacodylate buffer for about 12 h. The specimens, dehydrated in a graded acetone series, were embedded in a low viscosity epoxy resin (Spurr 1969) and cut on a *Leica UCT* (Wien, Austria) ultramicrotome. Sections stained with uranyl acetate and lead citrate (Reynolds 1963) were examined with a *JEM 1200EX* (Tokyo, Japan) electron microscope. All experiments were repeated three times.

Results

Morphological and histochemical observations: Second leaves of 10- and 20-d-old maize (*Zea mays* L.) and barley (*Hordeum vulgare* L.) are fully developed and green from their base to the top. The basal regions of second leaves of 30-d-old seedlings of both plant species are yellowish and the other regions are completely withered.

Second leaves of 10-d-old and also of 30-d-old seedlings of maize and barley have similar length: 12 and 28.5 cm on average, respectively. However, the dynamics of leaf growth is different; maize leaves grow faster than barley leaves between the age of 10 and 20 d, barley leaves grow faster than maize leaves between the age of 20 and 30 d.

The number of stomata on abaxial and adaxial leaf sides is the lowest in the basal part of leaves both in maize and barley, in the range of 5 - 15 mm^{-2}. In middle and apical parts of the same leaf it amounts to 30 - 50 mm^{-2} in maize and 30- 40 mm^{-2} in barley. In maize leaves stomata are more numerous in the abaxial than in adaxial epidermis.

Leaves of young, 10-d-old maize plants have different types of hairs; some of them are sharp, emerging from the blade margins, typical for this plant species

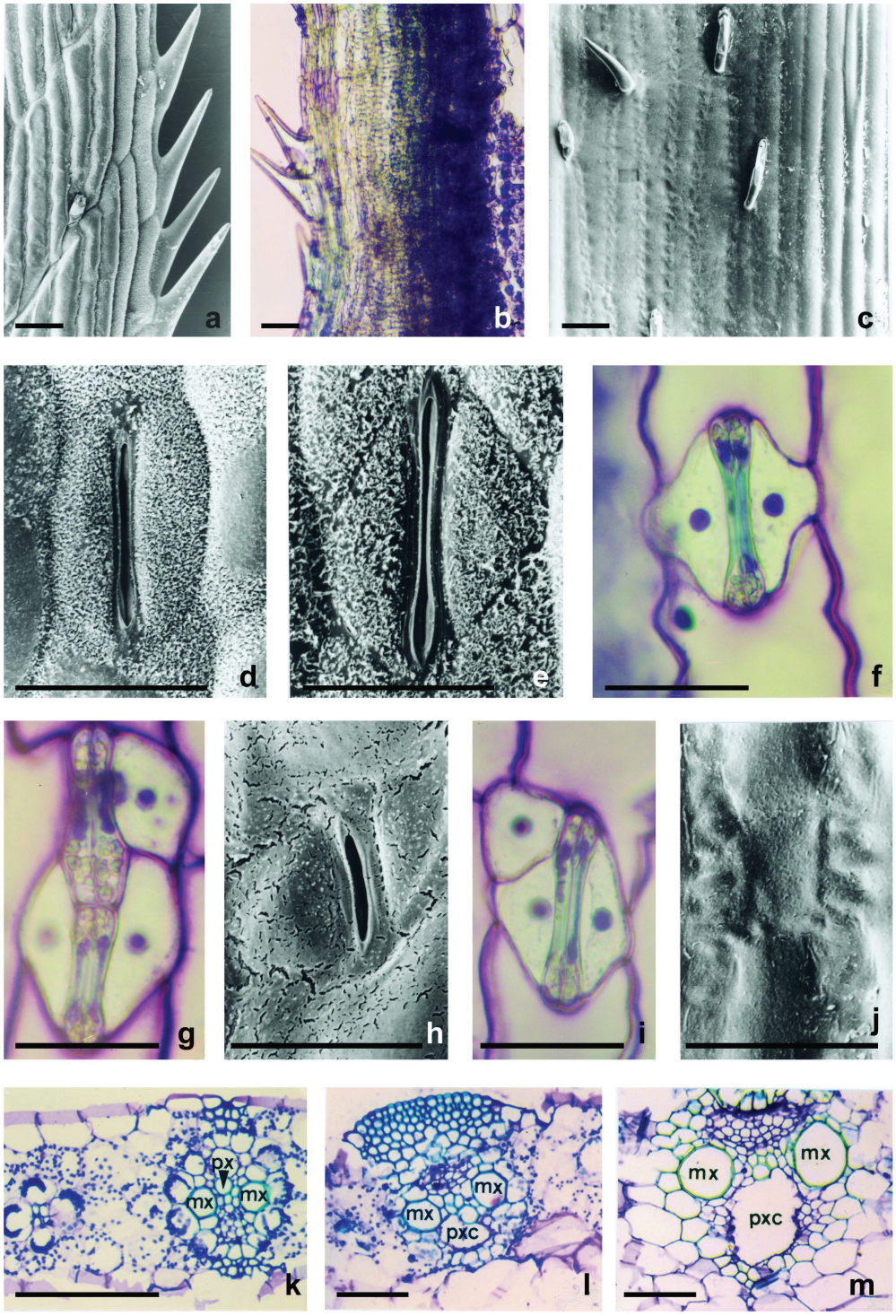

Fig. 1. *a - c*: Epicuticular wax and hairs in apical parts of leaves of 10-d-old maize seedlings (*a*,*b*) and of leaf of apical part of 30-d-old maize seedlings (*c*); *d - f*: Epicuticular wax and stomata in apical part of leaf of 10-d-old seedlings (*d*), in basal part of leaf of 20-d-old seedlings (*e*) and of apical part of leaf of 20-d-old seedlings. (*f*); *g - j*: Stomata in apical part of 20-d-old maize seedlings; *k - m*: Cross sections of middle part of second leaf of 10-d-old maize seedlings (*k*) of middle part of second leaf of 20-d-old maize seedlings (*l*) and basal part of second leaf of 30-d-old maize seedlings (*m*), mx - metaxylem, px - protoxylem, pxc - protoxylem canal; *a*,*c*,*d*, *e*,*h*,*j* - pictures from scanning electron microscope; *bar* = 200 μm. *b*,*f*,*g*,*i*,*k.l*,*m* - pictures from light microscope, metachromatic reaction with toluidine blue; *bar* = 200 μm.

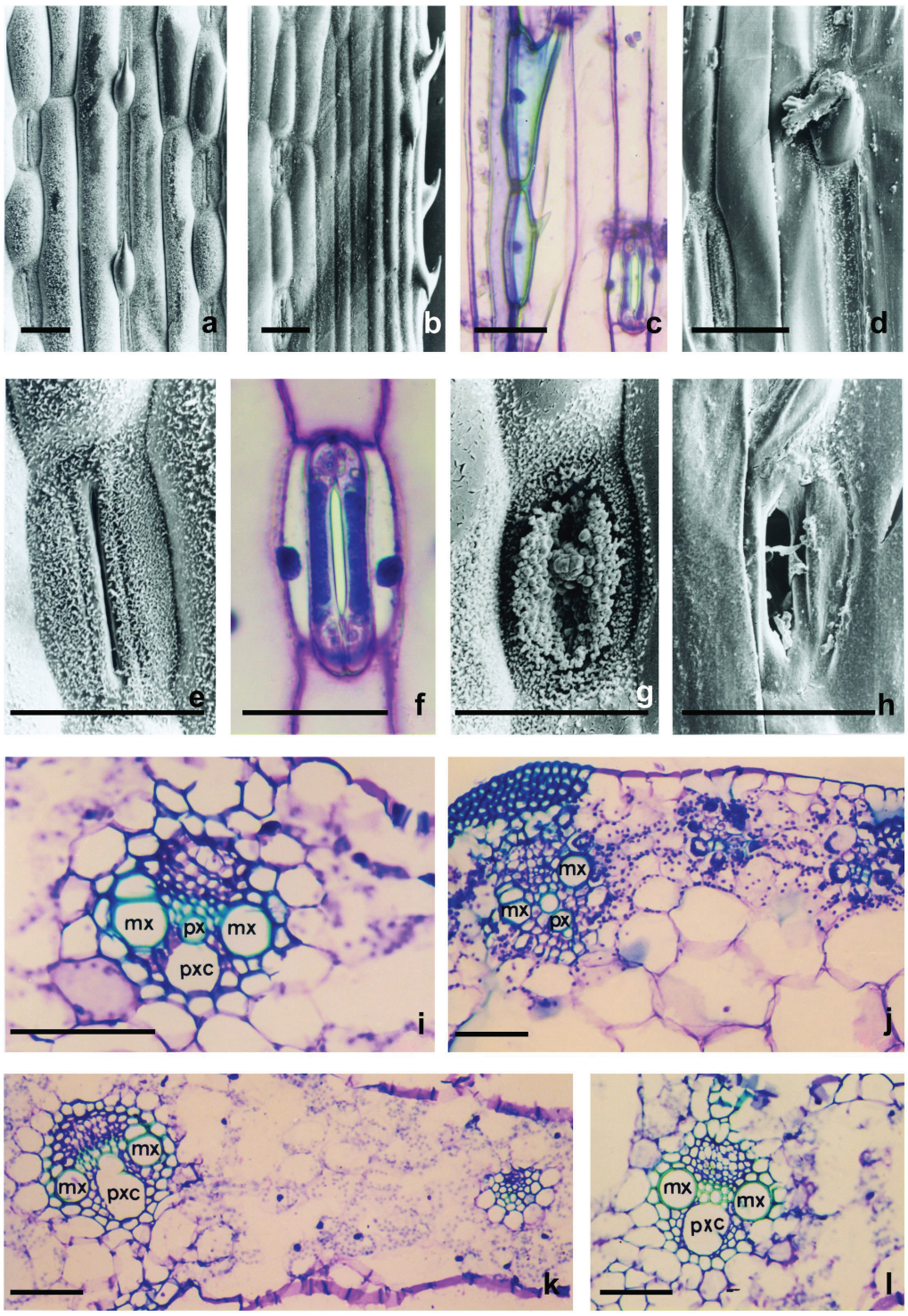

Fig. 2. *a* - *d*: Epicuticular wax and hairs in middle parts of second leaves of 10-d-old barley seedlings (*a*,*b*), in apical parts of second leaves of 20-d-old barley seedlings (*c*,*d*); *e* - *h*: Epicuticular wax and stomata in middle (*e*) and apical (*f*) parts of second leaves of 10-d-old barley seedlings, basal part of second leaf of 20-d-old barley seedlings (*g*), basal part of leaf of 30-d-old barley seedlings (h); *i* - *l*: Cross sections of basal (i) and middle (j) parts of second leaves of 10-d-old barley seedlings, of middle part of second leaf of 20-d-old barley seedlings (*k*) and of basal part of second leaf of 30-d-old barley seedlings (*l*); mx - metaxylem, px - protoxylem, pxc - protoxylem canal. *a*,*b*,*d*,*e*,*g*,*h* - pictures from scanning electron microscope, *bar* = 200 μm, *c*,*f*,*i*,*j*,*k*,*l* - pictures from light microscope, metachromatic reaction with toluidine blue, *bar* = 200 μm.

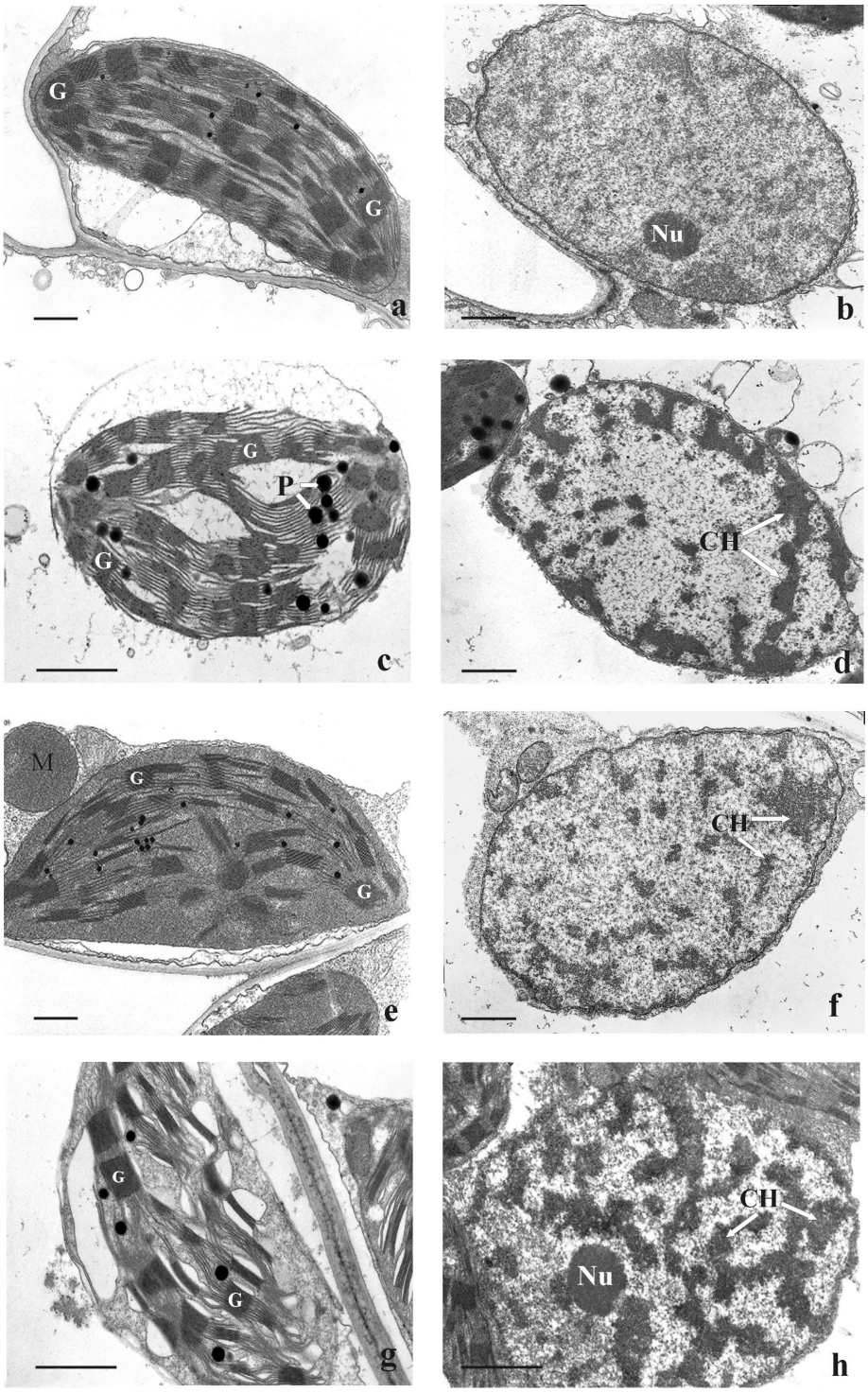

Fig. 3. *a* - *d*: Ultrastructure of mesophyll cells of maize second leaves. Differentiated chloroplast (*a*) and nucleus with dispersed chromatin (*b*) in mesophyll cell from middle part of leaf of 10-d-old plant. Chloroplast with disoriented and disrupted thylakoid membranes, with numerous plastoglobules and with broken envelope (*c*) and nucleus with many regions of condensed chromatin (*d*) in mesophyll cell from basal part of leaf of 30-d-old plant. *e* - *h*: Ultrastructure of mesophyll cells of barley second leaves. Differentiated chloroplast (*e*) and nucleus with dispersed chromatin (*f*) in mesophyll cell from middle part of leaf of 10-d-old plant. Chloroplasts with swollen and disrupted thylakoid membranes and with damaged chloroplast envelope contain more and larger plastoglobules (*g*) and nucleus with advanced chromatin condensation (*h*). CH - condensed chromatin, G - granum, M - mitochondrion, P - plastoglobules; *bar* = 1 μm.

(Fig. 1*A*,*B*). Hairs have cellulose cell walls that stain purple after the metachromatic reaction with toluidine blue (Fig. 1*B*). Only in apical parts of leaves of older, 20-d-old seedlings (Fig. 1*C*), hairs become damaged and rarely visible. Leaves of 10-d-old maize seedlings leaves are coated with epicuticular wax along the whole length (Fig. 1*A*,*D*), while leaves of 20-d-old maize seedlings are coated with wax in the basal parts only. Fig. 1*E* shows the basal part of a 20-d-old maize leaf covered with wax on stomata and in its neighbourhood. Maize stomatal apparatus consists of two elongated guard cells accompanied by two subsidiary cells. Stomatal subsidiary cells in the leaves of young, 10-d-old maize have ellipsoidal shape, their surface is coated with wax (Fig. 1*D*), whereas those in 20-d-old plants have mainly a triangular shape (Fig. 1*E*,*F*). With age, stomatal cell walls change their composition and stain blue in apical parts of leaves of 20-d-old maize seedlings (Fig. 1*F*,*G*,*I*). At that age stomata become deformed and their shapes differ a lot (Fig. 1*G*-*J*). Sometimes the deformation is so profound that guard and subsidiary cells are hardly visible (Fig. 1*J*). Only the largest metaxylem vessels stained blue after metachromatic cytochemical reaction with toluidine blue on the cross sections of leaves of 10-d-old maize seedlings (Fig. 1*K*). This proves change of cell wall composition already at this age of leaves (Fig. 1*K*). Nearly whole vascular tissue together with sclerenchyma cells stained blue (Fig. 1*L*,*M*) in older seedlings. In the basal parts of 30-d-old maize leaves, large rhexigenic areas emerge due to damage of leaves upon senescence (Fig. 1*M*).

Similarly to maize, barley leaves of 10-d-old seedlings have sharp hairs distributed in rows on epidermis. The shape and dimensions of barley hairs are typical for this plant species and different from maize hairs (Fig. 2*A*,*B*). Hairs last for a long time during leaf ontogenesis; their cell walls stained blue after metachromatic reaction with toluidine in apical parts of leaves of 20-d-old barley seedlings (Fig. 2*C*). As in maize, hairs in apical parts of leaves of 20-d-old seedlings become damaged and rarely visible (Fig. 2*D*). Epicuticular wax is preserved on the adaxial surface of leaves. As in maize, leaves of 10-d-old barley seedlings are coated with epicuticular wax along the whole leaf length (Fig. 2*A*,*B*,*E*). Older, 20-d-old barley leaves are coated with wax in the basal parts only. Young, 10-d-old barley leaves have narrow stomatal guard and subsidiary cells of ellipsoidal shape and their surfaces are coated with wax (Fig. 2*E*). In apical parts of leaves of this age, stomatal cell walls change their composition and stain blue after metachromatic reaction with toluidine blue (Fig. 2*F*). In 20-d-old barley, stomata are covered with wax in basal parts of leaves only (Fig. 2*G*). Stomata often become strongly damaged and have collapsed cells in basal parts of 30-d-old leaves (Fig. 2*H*). Cross sections of basal parts of leaves of 10-d-old barley seedlings revealed purple staining of cells except for large metaxylem vessels which are stained blue (Fig. 2*I*). Sclerenchyma cells also stain blue in the middle part of leaves of the same age (Fig. 2*J*). Cross sections of the middle parts of leaves of 20-d-old barley seedlings show blue staining of nearly the whole vascular tissue (Fig. 2*K*). Rhexigenic areas are found already at this age of barley seedlings. In the basal parts of leaves of 30-d-old barley the whole vascular tissue stain blue (Fig. 2*L*).

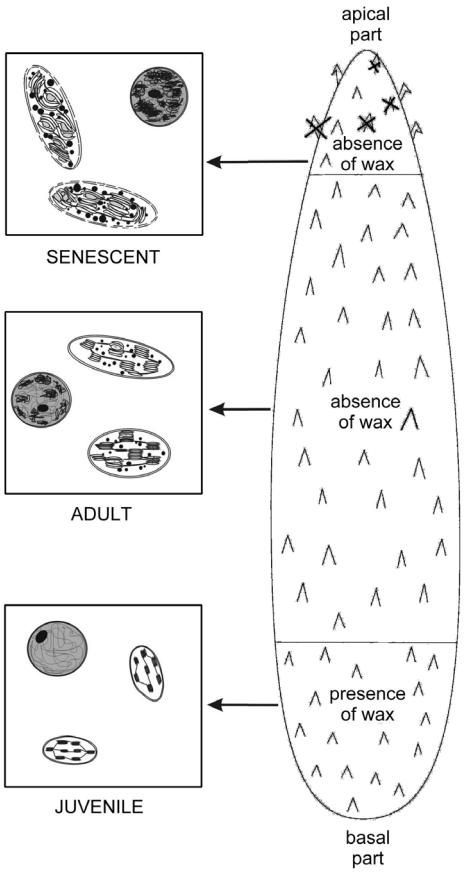

Fig. 4. Model of senescing leaf of maize/barley. Correlations between morphological traits and ultrastructural changes in the same regions of the leaves.

Ultrastructure of mesophyll cells: Chloroplasts of mesophyll cells of the basal, middle and apical regions of the fully green second leaves of 10-d-old maize are fully differentiated with few small plastoglobules dispersed in chloroplasts (Fig. 3*A*). These cells contain nuclei with dispersed chromatin (Fig. 3*B*). Gradual changes in chloroplast and nuclei structure can be observed with age of leaves. In the final stage chloroplasts in mesophyll cells of basal regions of 30-d-old maize seedlings have more plastoglobules than the younger cells, evidently disrupted thylakoid membranes and damaged chloroplast envelope (Fig. 3*C*). In this stage nuclei contain many regions of condensed chromatin (Fig. 3*D*).

Similarly to maize, chloroplasts of mesophyll cells of the basal, middle and apical regions of the green second leaves of 10-d-old barley are fully differentiated and contain small plastoglobules dispersed in chloroplasts (Fig. 3*E*). Mesophyll cells in the basal regions of leaves

contain nuclei with dispersed chromatin, however in middle and apical parts of leaves nuclei condensation of chromatin already began (Fig. 3*F*). The structure of chloroplasts and nuclei gradually change with the age of leaves. Eventually ultrastructure of the mesophyll cells of the basal regions of leaves of 30-d-old barley seedlings exhibits many characteristics of senescence. Chloroplasts, often with disrupted thylakoid membranes and with damaged chloroplast envelope, contain more and larger plastoglobules (Fig. 3*G*) as compared to previous stages. Advanced chromatin condensation in nuclei of mesophyll cells is observed (Fig. 3*H*). Concluding, barley mesophyll cells of the basal regions of leaves of 30-d-old plants, are more damaged than in maize of the same age.

Discussion

Development and differentiation of leaves can be divided into the juvenile and adult phases (Poethig 1997). This division is based on different processes and features of this organ. The transition from the juvenile to the adult phase of leaf growth has been studied in many species, mainly monocotyledons (Poethig 1988), but also in conifers (Greenwood 1995). This transition process involves coordinated changes in many morphological and physiological traits. It is possible that the juvenile, adult and senescent leaf identities are regulated by genes that mutually repress each other's expression and act by modifying a more fundamental program of leaf morphogenesis (Poethig 1997). Development of leaves of maize plants that formed from an excised, cultured shoot apex resemble very closely normal differentiation and phase change from juvenile to adult is indistinguishable from phase change in leaves of seed-derived plants (Poethig 1990, Irish and Jegla 1997). It is known that environmental conditions that retard the growth, such as poor mineral nutrition or water stress prolong the juvenile phase. In contrast, conditions that speed up the growth, such as various gibberellins, accelerate the transition to the adult phase (Poethig 1990). Although factors that influence phase change have been studied for many years mechanisms of their action is still unclear.

In this paper we assessed morphological and histochemical indicators of leaf ontogenesis stages, senescence in particular. We determined how the distribution pattern of these indicators change in time during ontogenesis and within leaf blades. According to our results the distribution of morphological indicators in the corresponding parts of barley and maize leaves of the same age is similar although the dynamics of leaf growth is different in both species. Density of stomata is lower in the basal than in the middle and the apical parts of leaves, except for leaves showing symptoms of senescence. This means that the density of stomata might be an indicator of the developmental phase. Fewer stomata are visible in senescing parts of leaves than in adult parts. In senescing parts of leaves, stomatal apparatus becomes deformed with age, closed with wax and finally hardly visible. Lack of visibility of stomata is typical for the senescing phase. Deformations of stomata, described in different mutants (Gallagher *et al.* 1999, Reynolds *et al.* 1998, Larkin *et al.* 1997, Becraft 1990, Smith *et al.* 1996, Vega *et al.* 2002), might be independent of age.

Distribution of leaf hairs is typical for each plant species. According to our results, the distribution remains similar along the whole length of the leaf during the senescence process. However, in apical parts of leaves of older, 20-d-old seedlings hairs become damaged and hardly visible, stained blue after metachromatic reaction with toluidine blue. Our results, as well as those of Freeling and Lane (1994), Bongard-Pierce *et al.* (1996) and Orkwiszewski and Poethig (2000), show that hairs last longer than wax. Existence of hairs is a typical trait for the adult phase (Orkwiszewski and Poethig 2000), however, according to our results, damaged hairs indicate advanced senescence.

According to our results, which are consistent with that of Orkwiszewski and Poethig (2000) and Sylvester *et al.* (2001) the appearance of epicuticular wax is a typical trait for the juvenile phase. Lack of wax is an indicator of the adult phase. If epicuticular wax is observed only in basal parts of the second leaf of barley and maize, then most probably it is a leaf of a 20-d-old plant. The presence of wax is one of the best diagnostic indicators of the phase of leaf ontogenesis, but only in plants that have wax-coated leaves.

We showed that metachromatic cytochemical reaction with toluidine blue reveales purple and blue staining on the cross sections of maize and barley leaves. Each tissue of young and mature parts of leaves stains purple except of subsidiary cells, metaxylem vessels and sclerenchyma cells, which stain blue probably because of lignifications of their cell walls. Blue staining appears in early stages of leaf ontogenesis (Sylvester *et al.* 2001) meaning that lignification starts already during leaf differentiation. Our results are consistent with those of Sylvester *et al.* (2001); regions of the leaf with cell walls that stain mainly purple with toluidine blue are in the juvenile phases and regions that stain purple and blue are in adult phases. According to our results, blue staining expands to some neighbouring cells during senescence. In the oldest analyzed stage many cell walls still remain purple. We could not find significant differences between staining of adult and senescent tissues. Therefore, we claim that the metachromatic cytochemical reaction with toluidine blue is not a good indicator of the phase of leaf ontogenesis.

Based on the observations described above we consider regions of the leaf coated with wax, with lower density of stomata than in other parts of the leaf and with cell walls that stain mainly purple with toluidine blue as exhibiting a juvenile phase. We consider regions of the leaf without epicuticular wax, larger density of stomata

than in the juvenile phase and with cell walls that stain purple and blue with toluidine blue as exhibiting adult phase. Finally we consider regions with damaged or broken hairs with mainly lignified cell walls and strongly damaged stomata as exhibiting senescing phase.

We correlated the obtained results of morphological and histochemical indicators with ultrastructural changes. Ultrastructural aspects of leaf senescence were analyzed previously (Rascio *et al.* 1980, Simeonova *et al*. 2000). It is known that changes in chloroplast ultrastructure correspond to a change in functioning and of age of leaves and other organs (Bondada and Oosterhuis 2003/4).

In the model leaf (Fig. 4), the basal part is coated with wax, middle and apical parts are wax free; leaf hairs are distributed similarly in the basal and middle parts, but leaf hairs are damaged in the apical part. The correlations are summarized in the three points: *1*. Leaves of 10-d-old plants of both analyzed species and also the basal region of 20-d-old plants, exhibit juvenile phase; their ultrastructure is characterized by fully differentiated chloroplasts and by nuclei usually with dispersed chromatin. One cannot be certain if the apical part of a 10-d-old leaf represents the juvenile phase since the process of chromatin condensation in barley mesophyll can sometimes, but rarely, be noticed. *2*. Middle parts of leaves of 20-d-old plants and basal parts of leaves of 30-d-old plants of both species exhibit adult phase; their ultrastructure is characterized by fully differentiated chloroplasts and by nuclei in which chromatin begins to condense. *3*. Apical parts of leaves of 20-d-old plants of both species exhibit senescing phase; their ultrastructure is characterized by swollen and damaged membranes of chloroplasts and nuclei with advanced chromatin condensation.

These results provide a relatively simple method of estimating the stage of senescence.

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