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Cell biology

Black seed color of the spring common vetch (*Vicia sativa* L.) cultivar Obskaya 16 is caused by blue anthocyanins accumulating in macrosclereids

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Abstract: In legumes, the color of seed coat is an important agronomic trait that affects dormancy, germination rate, and resistance to pathogens. In the current study, the black-coated seeds of spring common vetch cultivar Obskaya 16 were analyzed by qualitative tests to define the nature of pigmentation and were studied by microscopy to trace pigmentation development. It was shown that the black color of seeds in this cultivar is caused by blue anthocyanins starting to accumulate in the macrosclereids (epidermal cells) at the yellow pod developmental stage. Observed dark dots on the seed surface at this stage correspond to clusters of macrosclereids with blue pigment inside, while at the brown pod developmental stage, totally black seeds have all the macrosclereids with blue pigment. The chlorophyll and PsbA fluorescent signals which are characteristics of chloroplasts did not colocalize with blue pigment in macrosclereids at any developmental stages. Moreover, there was no correlation between blue pigment accumulation and plastid development and their functional activity. The data implies that chloroplasts do not involve in the blue pigment synthesis.
Key words: anthocyanins; light microscopy; macrosclereids; plastids; PsbA.

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Клеточная биология

Черная окраска семян сорта яровой вики посевной (*Vicia sativa* L.) Обская 16 обусловлена накоплением синих антоцианов в макросклеридах

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Аннотация: У бобовых окраска оболочки семян служит важным сельскохозяйственным признаком: влияет на период покоя, скорость прорастания и устойчивость к патогенам. В данной работе семена ярового сорта вики обыкновенной Обская 16 проанализированы с помощью качественных тестов для определения природы черной пигментации и изучены с помощью микроскопии в динамике развития. Показано, что черный цвет семян этого сорта обусловлен синими антоцианами, которые начинают накапливаться в макросклеридах (эпидермальных клетках) на молочно-восковой стадии развития боба, когда он приобретает желтую окраску. Наблюдаемые темные точки на поверхности семян на данной стадии соответствуют скоплениям макросклерид с синими пигментами внутри. На стадии зрелого боба, когда семена имеют плотную черную окраску, все макро-склериды несут синие пигменты. Ни на одной из проанализированных стадий флуоресцентные сигналы хлорофилла и белка

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PsbA, которые характерны для хлоропластов, не локализовались совместно с синим пигментом в макросклеридах. Более того, не выявлено корреляции между накоплением синего пигмента и развитием пластид и их функциональной активностью. Полученные данные свидетельствуют о том, что хлоропласты не участвуют в синтезе синего пигмента.
Ключевые слова: антоцианы; световая микроскопия; макросклериды; пластиды; PsbA.

Introduction

Spring common vetch (*Vicia sativa* L.) is an important annual forage legume. It is mainly cultivated as a cover crop, green manure, pasture, and for silage and hay production, but since γ-glutamyl-β-cyano-alanine toxins accumulating in the seeds it does not use for feeding purposes (Huang et al., 2017). Among the *Vicia* plants, it is the widely cultivated species in the world with high genetics and phenotypic variability including color variations of the seed coat (Dong et al., 2016; Tiryaki et al., 2016). The pigmentation of vetch seeds may vary from light (grey to slightly green and bright to slightly yellow) to strong (bright brown, brown, dark brown, or black) with a different level of tint intensity (Grela et al., 2021).

In legumes, pigmentation of the seed coat is an important agronomic trait. It is associated with physical dormancy often called as hardseededness which involves the development of a water-impermeable seed coat, caused by the presence of phenolics- and suberin-impregnated layers of palisade cells of the epidermis (Smýkal et al., 2014). In many species, unpigmented seeds were shown to deteriorate more rapidly and to be more susceptible to imbibition damage in comparison to pigmented ones. For example, black-coated soybean seeds are characterized by slower initial imbibition rates, higher resistance to filed deterioration, thicker and tougher testas, higher lignin content and fungicidal properties in comparison with non-black seed coated cultivars (Souza, Marcos-Filho, 2001). Besides dormancy, the pigmentation affects resistance to pathogens. For example, white beans, which lack any coloration, are more susceptible to root rots and other infections (Smýkal et al., 2014).

The seed coat color of legume species was shown to be determined mainly by flavonoid glycosides, anthocyanins and proanthocyanidins, known either as condensed tannins, which can be accumulated in epidermal cells called as macrosclereids (Smýkal et al., 2014). The content of condensed tannins and anthocyanins can vary significantly between different bean genotypes and some relationship between the level of these components and seed color was found (Díaz et al., 2010). It was demonstrated that dark seed coats have higher concentration of anthocyanins and proanthocyanidins than lighter colored white seed coats (Nurzyńska-Wierdak et al., 2019). Association of tannin content with imbibition rate was described (Kantar et al., 1996).

In common vetch, the varieties with the black color are known, but the pigments were not extracted and development of the black pigmentation by microscopy has not been studied yet. In the current study, we tested chemically the pigments in black-coated seeds of common vetch variety Obskaya 16 bred for Siberian region and analyzed by microscopy the pigmentation development and plastid activity in these seeds.

Material and methods

Plant material

The cultivar of spring common vetch (*Vica sativa* L.) Obskaya 16 released in 2019 by Siberian Research Institute of Plant Production and Breeding – Branch of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences (Krasnoobsk, Novosibirsk region, Russia) (Goncharova, 2020) was used to study seeds pigmentation development. The cultivar produced 92% of seeds having black velvet color and 8% – brown color. The seeds are characterized by smooth surface and low gloss.

Cytological analysis

All the steps of cytological analysis, including cryosections preparation, assessment of the visible pigments, immunostaining and detection of the fluorescent signals, were performed according to the earlier developed protocols with the identical equipment and with no modifications (Shoeva et al., 2020; Mursalimov et al., 2021).

Qualitative chemical analysis

To test the pigments accumulating in the seeds coat of common vetch the qualitative chemical tests were performed. The crashed seeds were soaked in 800 μL of alkaline and acidic solvents, such as 1 N NaOH and 1% HCl/methanol, respectively (Downie et al., 2003; Castañeda-Ovando et al., 2009).

Results

The seed coat anatomy of cultivar Obskaya 16 at different developmental stages was studied by light microscopy on cryosections. Four distinct cell layers were observed outside of cotyledon: aleurone (endosperm remains), macro-, osteosclereids and parenchyma (Fig. 1). The distribution of visible pigments as well as plastid development were assessed in all the mentioned cell layers. Visible pigments were observed by common light microscopy. Immunosignal of PsbA protein and autofluorescence of chlorophyll were analyzed by fluorescent microscopy.

It was shown that at the early stage of seed development (*mid-full seed*) the seeds were bright green inside and outside and had no any dark pigments (see Fig. 1, *a*). Individual cell layers of the seed coat were easily distinguishable at this stage. The biggest amount of visible green pigment was observable in osteosclereids. In the same time, strong chlorophyll autofluorescence as well as PsbA immunosignal were detectable in all the layers including cotyledon. At the following *full seed* stage the seeds became dark green inside and outside (see Fig. 1, *b*). Still no dark pigments were observed. Chlorophyll autofluorescence and PsbA immunosignal were in the same level as at previous stage in all the cell layers. At the *yellow pod* stage the seeds be-

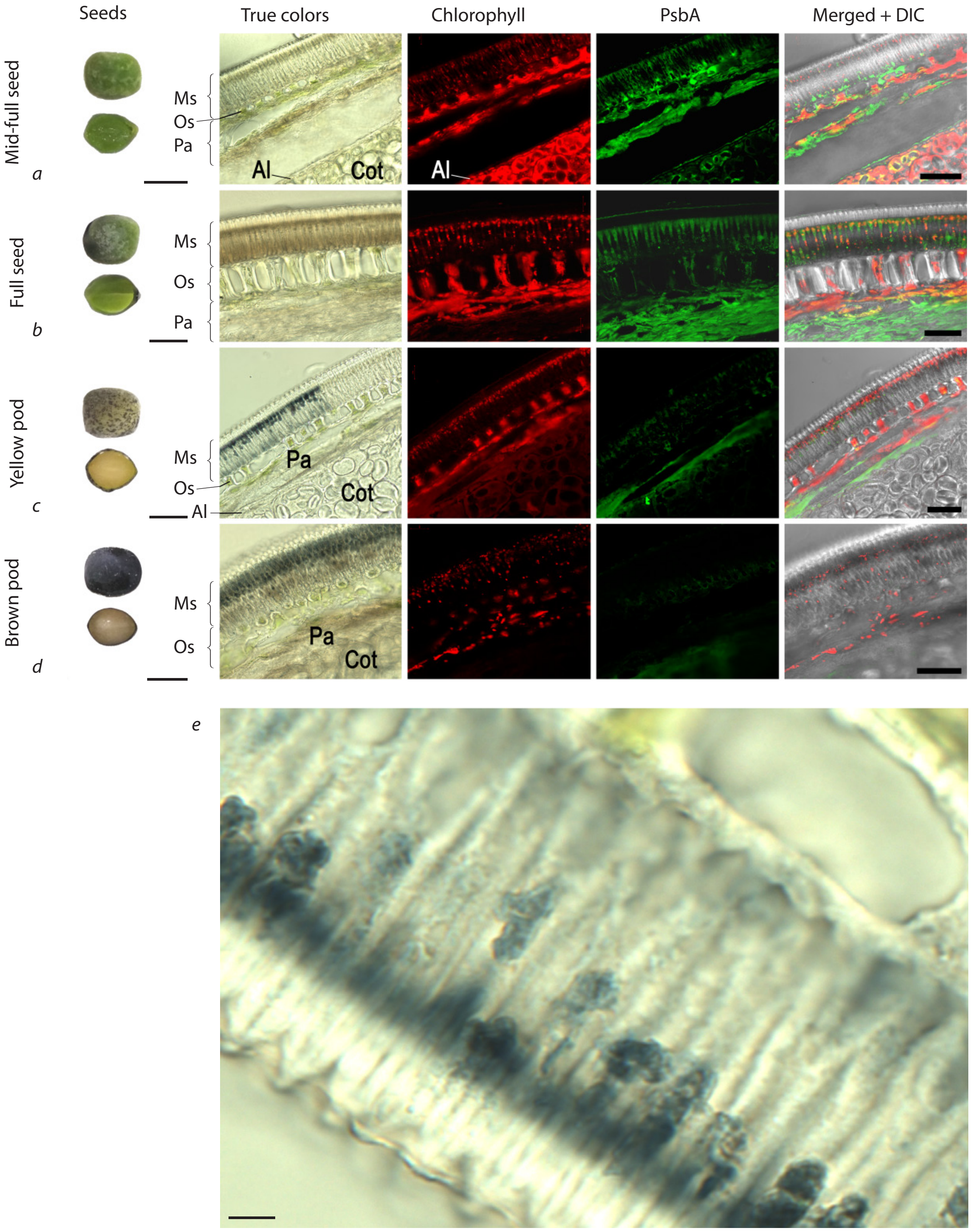

Fig. 1. The seeds and cross-sections of the seeds of common vetch cultivar Obskaya 16 at different developmental stages (*a*–*d*) and magnified macrosclereids with the blue pigment inside (*e*)

Al: aleurone, Cot: cotyledon, Ms: macrosclereids, Os: osteosclereids, Pa: parenchyma. Merged section combines chlorophyll autofluorescence (red), PsbA immunosignal (green) and DIC (differential interference contrast microscopy) tissue images. Scale bars for the seeds are 5 mm, 50 μm for the micrographs *a*–*d* and 5 μm for *e*

came brownish-green inside and outside (see Fig. 1, *c*). Dots of dark pigment were presented on the seed surface at this stage. On the cryosections the presence of blue pigment was observed in some of macrosclereids (see Fig. 1, *c, e*). Macrosclereids with the blue pigment were not distributed uniformly but clusters of them were formed. The green pigment was distinctly seen in osteosclereids at this stage. The intensity of the chlorophyll autofluorescence and PsbA immunosignal decreased in all the cell layers. The green pigment as well as both of the fluorescent signals did not colocalize with blue pigment in macrosclereids. Blue pigment inside of individual macrosclereids did not form any types of crystals and was localized in vacuole-like struc-

Fig. 2. Qualitative testing vetch seed pigments by soaking crashed seeds in basic (left tube) and acidic (right tube) solutions

tures with no regular shape (see Fig. 1, *e*). At *brown pod* stage the seeds were brown inside and black outside (see Fig. 1, *d*). All the macrosclereids had blue pigment at this stage. The green pigment still was observed in osteosclereids. In the same time, the chlorophyll autofluorescence decreased significantly at this stage and only small amount of individual plastids were detected in macro-, osteosclereids and parenchyma. PsbA immunosignal was barely detectable in all the seed coat layers; however it was still noticeable in cotyledon cells.

Qualitative chemical test was performed to determine the nature of the pigments accumulating in the vetch seed coat. The basic solution was stained brown and the acidic solution – pink (Fig. 2).

Discussion

Previously in the seed coat of common vetch only three cell layers were identified on the epoxy resin embedded material (Büyükkartal et al., 2013). Using cryosections we observed one more cell layer that was identified as aleurone. The red chlorophyll autofluorescence made aleurone visible. Thus, cryosections provide more information than resin embedded section. Cryosectioning also allows to preserve native pigment profile and does not demand additional staining. Using this approach we observed two visible pigments in the developing seeds of common vetch. Expectedly, we identified the green pigment as chlorophyll owing to its colocalization with red autofluorescence characteristic for chlorophyll (Krause, Weis, 1991). It was shown that black color of the seeds of cultivar Obskaya 16 forms by blue pigment accumulating in macrosclereids.

As seeds extract in the alkaline solution was stained brown, while it in the acidic solution – pink, the pigments in the vetch seeds coat were identified as anthocyanins unlike melanins, that keeps the acidic solution transparent and stains the alkaline solution brown (Downie et al., 2003; Castañeda-Ovando et al., 2009). Previously, anthocyanins delphinidin 3-glucoside, petunidin 3-glucoside, and malvidin 3-glucoside were shown to be responsible for black color of beans (Takeoka et al., 1997).

The involvement of macrosclereids in flavonoid biosynthesis was revealed in *Medicago truncatula* by high performance liquid chromatography-mass spectrometry and microarray assays (Fu et al., 2017). In the present work, we directly observed vacuole-like structures with the blue pigment in macrosclereids. We found that the pigment accumulation in macrosclereids is not uniform process and clusters of the pigmented macrosclereids were formed initially. It fits well with the observed dot-like distribution of the dark pigment on the seed surface. No blue pigment were detectable in any cells expect of macrosclereids at any stages of seed development. It proves the role of macrosclereids as the defense barrier (Fu et al., 2017). There were not detected any correlation with blue pigment accumulation and plastid development and their functional activity.

In terms of plastid activity and development of their internal structure that were determined by presence of chlorophyll and a thylakoid membrane marker PsbA, surprisingly the lowest amount of chlorophyll and poorly developed internal membranes were detected in macrosclereids which are exposed to the light the most. In the same time, the biggest amount of chlorophyll and the most developed plastid internal structure were detected in cotyledon cells which are hided from the light by seed coat. During seed development, chlorophyll signal drastically decreases in all the cell types in the same time PsbA signal presented in cotyledon even in the *brown pod* stage. It allowed to conclude that plastids in cotyledon cells preserve their well-developed internal membrane structure in the absence of chlorophyll and could be transformed in chloroplasts quite fast.

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