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COMPARISON OF FLORAL ONTOGENY BETWEEN WILD-TYPE *XANTHOCERAS SORBIFOLIA* BUNGE AND ITS DOUBLE-FLOWERED MUTANT

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Abstract

A comparative study of floral ontogeny in single (wild-type) and double (mutant) flower phenotypes of yellow-horn floral development was categorized into six phases. In the wild-type, sexual differences arose after phase 6. Male flowers formed after the later abortive development of female tissues, while in female flowers the stamen selectively arrested its development. Stamen and carpel development initiated similarly in the mutant compared with the wild-type during the early stages; however, stamens fused to small inner petals, and the stigma later became petaloid. As a result, the mutant was completely sterile. In the mutant, stamens were generally replaced by petaloid or intermediate appendages, but these were not rigid having 1 : 1 substitutions. Some small and curved petals developed between the outer petals and the inner appendages. The mutant represented a case of phenotypic variation that blended the features of both homo- and neoheterotopy.

Introduction

Considering the crises of fossil fuel depletion and the increased concern for the environment in recent years, oil-rich seed plants that can be used to produce renewable and environmentally friendly biodiesel have received much attention. Yellow-horn (*Xanthoceras sorbifolia* Bunge, Sapindaceae) adapts well to drought, low temperature, salt, alkali, and marginal lands (Shao *et al*. 2008). Its seeds have a large amount of oil (55 - 66% in the seed kernel). Moreover, the seed oil has been recognized as a high-quality raw material for biodiesel production based on its physiochemical properties (Zhang *et al*. 2010, Van Gerpen 2005). Yellow-horn can produce over 800 gallons of oil per acre of cultivation. As a result, it is considered as one of the most promising plant species for use as renewable energy source. In addition, yellow-horn tree can assist in eliminating desertification and erosion. Also, it is grown as an ornamental tree and used as a source of edible oil.

Yellow-horn has two distinct floral morphologies: single flowers (wild-type) versus double flowers (mutant) with additional modified petals or petaloid organs with stamen features (Ao 2010). Because stamens and pistils are petaloid, the mutant plant is unfruitful, yet it has ornamental value.

Double-flowered varieties have been evident for a long time. Formation of a normal organ in the location where a different type of homologous organ typically originates was identified as homeosis (Rudall *et al*. 2002). Homeosis can result in the formation of double flowers (Ronse *et al*. 2003). Another type of phenotypic variation is neoheterotopy (Rudall *et al*. 2004). In neoheterotopy, the feature under scrutiny is generated in a novel location on the body plan of the organism (Rudall *et al*. 2003, Chatelet *et al*. 2007), whereas in homoheterotopy, the feature has a spatial location previously occupied by a contrasting feature, with or without concomitant transfer of any function(s) originally fulfilled by that feature.

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There is much speculation concerning the type of heterotopy of yellow-horn double-flowers. The mutant was first described by Yu (1985), but the initiation and development of the double flower has not been documented in detail before the present study. The aim of this study was to compare the organogenic processes of floral organ initiation and the stages of floral development in detail between the double-flower mutant and wild-type yellow-horn and to discuss whether the mutant represents a particular type of homo- or neoheterotopy.

Materials and Methods

Wild-type and mutant flowers of 40-year-old yellow-horns were found in natural populations from a hill in Chengde, Hebei Province, China. All flowers are single in the wild-type and double in the mutant. The flower characteristics of each type were stable every year. Three trees of each type were used for sampling. More than ten flower buds were collected periodically at various developmental stages from one-year-old branches. Floral organogenesis and morphogenesis in both types were investigated by dissection and observation using an Olympus SZX16 stereo microscope (Tokyo, Japan).

At each sampling point, floral tissues were immediately fixed in formalin/acetic acid/ethanol (FAA) at a ratio of 5 : 5 : 90. After 24 hrs, the samples were then dehydrated using an ethanol series (50 - 100%), infiltrated with toluene and paraffin, and supplemented with paraffin (48 hrs, 58 - 60°C). After solidification of the paraffin, the samples were cut into 8 µm thick sections. Paraffin slides were stained with safranin O and fast green and then observed and photographed using an Olympus DP72 light microscope (Tokyo, Japan).

For scanning electron microscopy, the samples were fixed overnight in 2% glutaraldehyde in phosphate buffer (pH 7.2 - 7.4) (Wang *et al*. 2006), dehydrated in an ascending series of tertiary-butanol, freeze-dried, and mounted on metal stubs before coating with platinum. The samples were examined using a Hitachi TM-1000 scanning electron microscope (Tokyo, Japan).

Results and Discussion

To identify structural differences between wild-type and mutant flowers in yellow-horn, clarification of the structural features of mature flowers was necessary. The plant produces flowers on a raceme with the number of flowers per raceme varying from approximately 13 to 46 in both the wild-type and mutant.

Typically, mature flowers of the wild-type are unisexual. The corolla of both male and female flowers consists of three bracts, five sepals, five petals, eight stamens, five yellow filamentous appendages, and one pistil (Fig. 1A, B). The female flowers are located at apical buds, while male flowers flourish at lateral buds. These characteristics of male and female flowers led to allogamy in the wild-type. In male flowers, a degenerated pistil and normal stamens are formed (Fig. 1B). The sterile carpels are shorter than the stamen filaments (Fig. 1C). Anthers crack to disseminate pollen when they become mature (Fig. 1D). Ovary growth is arrested very early with no further ovule development (Fig. 1E). In female flowers, the pistil is well developed (Fig. 1F). The ovary is covered with villi (Fig. 1F, G), and after fertilization, ovules grow within it and develop into seeds (Fig. 1G). The stamens are composed of indehiscent anthers and relatively short filaments (Fig. 1F). The anthers are filled with immature pollen (Fig. 1H).

In the mutant, there are supernumerary petals in several additional whorls. As a result, the mutant has a larger corolla than that of the wild-type. The double flower is formed by approximately 17 - 25 petals and petal-like appendages, three bracts, five sepals, deformed stamens, and a deformed pistil (Fig. 2A, C). Additionally, the inner modified petals or appendages, approximately 4 - 7 mm long and 1 - 4 mm wide, are slender and smaller than the

outer petals. They are distributed asymmetrically around the floral axis. The morphology ranges from reduced and curved petals to petals with attached yellow sacs, blending the features of both petals and stamens (Fig. 2E). There is a great deal of variation in the number and morphology of petal-like appendages, not only among plants but even among flowers of the same plant.

Fig. 1. Characteristics of the wild-type *Xanthoceras sorbifolia* flower. A: Female flower. B: Male flower. C: Stamens and an ovary from a male flower. D: Vertical section of a male flower anther. E: Vertical section of a male flower ovary. F: Stamens and an ovary from a female flower. G: Vertical section of a female flower ovary. H: Vertical section of a female flower anther. Se - sepal; Pe - petal; S - stamen; Y - yellow filamentous appendages; Ova, ovary; Ovu, ovule; A - anther; Po - pollen; F - filament; St - style. Bars: A, B, E, and F = 1 mm; C, G and H = 200 μm; D = 500 μm.

Although the mutant is sterile, present author found that its flowers can still be classified as male and female (Fig. 2A and B). As mentioned above, the stamens of male and female flowers are similar structurally and mutate into small inner petals with yellow sacs (Fig. 2C). The mutant was reported previously to lack stamens. However, rudimentary pollen grains were seen after cutting open the sacs (Fig. 2D), which demonstrates that the small inner petals with yellow sacs arose from mutations in the stamen. The most obvious difference between male and female flowers lies in the pistils. The ovary of the female flower is larger than that of the male. Only rudimentary ovules are formed in the female flower (Fig. 2E), and no ovule initiates in the male flower (Fig. 2F). The pistils of both male and female flowers are covered with villi, and the stigma is petaloid. As a result, the pistils cannot be pollinated. Because of the petaloid pistil and stamens, the mutant is completely sterile and can only be propagated by asexual methods.

When yellow-horn plants grow into a flower-matured state, their inflorescence meristem will differentiate under the appropriate environmental conditions. To analyze the structural characteristics of the flowers, the development of flowers of both the wild-type and mutant was divided into six distinct phases.

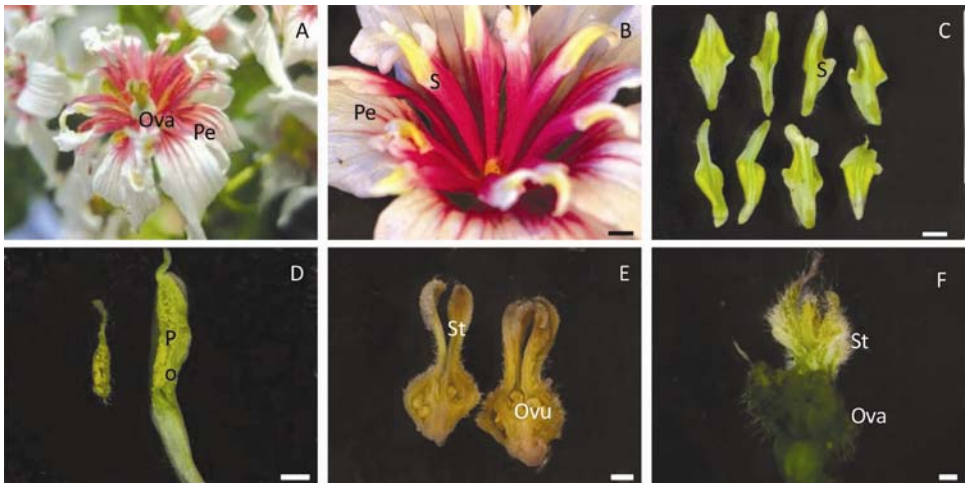

Fig. 2. Characteristics of the mutant *Xanthoceras sorbifolia* flower. A: Female flower. B: Male flower. C: Petaloid stamens of male and female mutants. D: Vertical sections of petaloid stamens of male and female mutants. E: Vertical sections of female flower ovaries. F: Vertical section of a male flower ovary. Se - sepal; Pe - petal; S - stamen; Ova, ovary; Ovu, ovule; Po - pollen; St - style. Bars: C and E = 1 mm; D = 200 μm; B and F = 500 μm.

For the wild-type, the meristem at the top of the branch forms a rounded protrusion, indicating the beginning of phase 1, and plants begin to shift from vegetative to reproductive growth. The meristem further enlarges to form the inflorescence primordia (Fig. 3A). Further differentiation and development of inflorescence primordia then proceed. Phase 2 begins with the emergence of the abaxial bract, which grows faster than the other two bracts and protects the structure of the inner whorl. A spherical protrusion is formed on the axilla of the bract, indicating the emergence of the first flower primordium (Fig. 3B, C). With the development of the inflorescence rachis, flower primordia appear continuously, forming a racemose inflorescence (Fig. 3D). The initiation of sepal primordia occurs during phase 3 (Fig. 3E, F, G). At later stages, the five sepals start to elongate centripetally. Formation of the primordia of five petals is referred to as phase 4 (Fig. 3H). Petal primordia appear as semicircular protuberances. Phase 5 is defined as the initiation of stamens. Eight spherical androecial primordia are seen within the whorl (Fig. 3I). During this phase, the five petal primordia may demonstrate slower growth than the stamen primordia. Here, the elongated stamens appear cylinder-shaped (Fig. 3J, K). This process is followed by formation of anther and filament primordia (Fig. 3L). The stamens of male flowers are structurally similar to those of female flowers, albeit longer and more slender. As the stamens develop, phase 6 begins with the emergence of the pistil primordium. Carpel tissues become differentiated and form from the top protrusion of the meristem (Fig. 3M). Simultaneously, five yellow filamentous appendages appear in an intercalary position relative to the petal primordia. Carpel primordia then close and differentiate into a cylinder-shaped ovary (Fig. 3N, O). Next, carpel tissues undergo enlargement and elongation, and the growth of the ovary wall subsequently closes up the ovarian cavity. Finally, the ovules, style, and stigma are formed (Fig. 3P, Q).

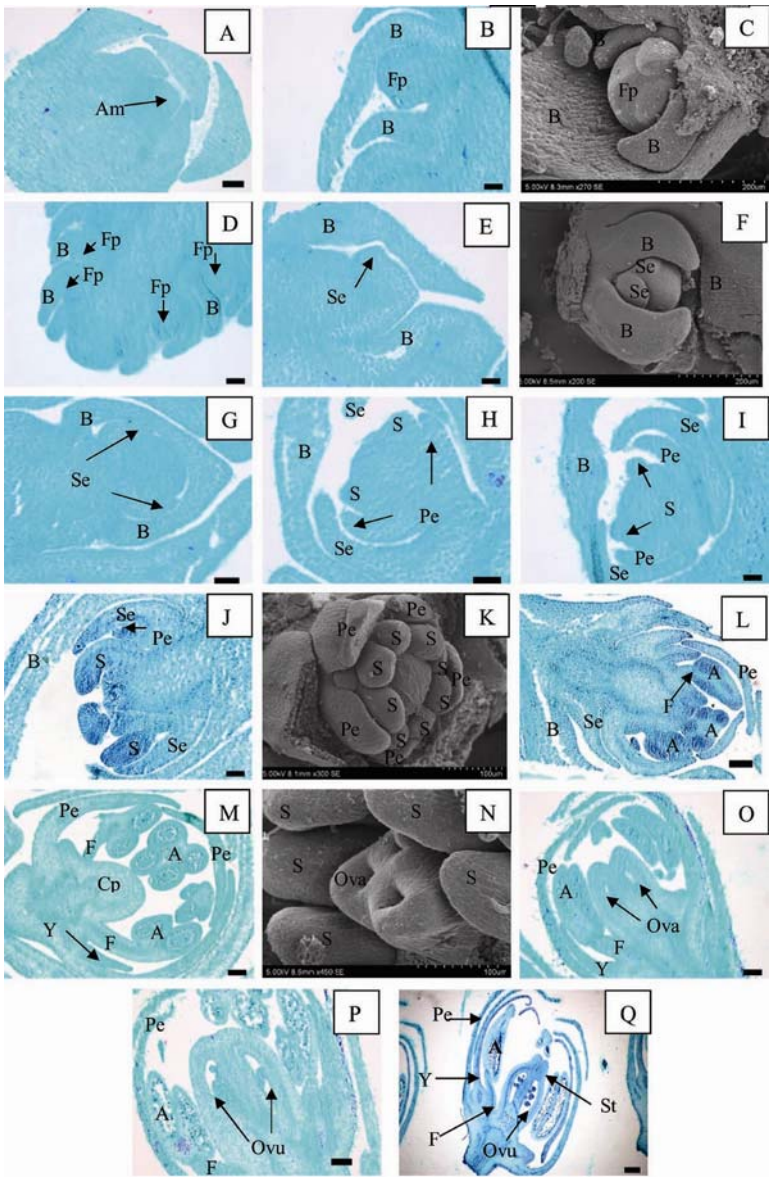

Fig. 3. Floral ontogeny of the wild-type *Xanthoceras sorbifolia.* A: Initiation of the apical meristem. B and C: Bract and flower primordium formation. D: Appearance of the racemose inflorescence. E: Emergence of the abaxial sepal. F and G: Emergence of other sepals. H: Occurrence of petal primordia. I: Stamen initiation. J and K: Elongation of the stamen primordia. L: Anther and filament primordium formation. M: Emergence of the carpel primordia and yellow filamentous appendages. N and O: The carpel primordia close and differentiate into a cylindrical ovary. P and Q: Formation of the ovules, the style, and the stigma. Am - apical meristem; B - bract; Fp - flower primordium; Se - sepal; Pe - petal; S - stamen; F - filament; A - anther; Cp - carpellary primordia; Y - yellow filamentous appendages; Ova, ovary; Ovu - ovule; St - style. Bars: A, L, M, O, and P = 100 μm; B, D, E, G, H, I, and J = 50 μm; Q = 200 μm.

The differentiation of functionally male and female yellow-horn flowers takes place after organogenesis of the primary floral parts. Male and female flowers can be structurally distinguished only after phase 6. After that stage, stamen primordia continue to develop in male flowers. Their filaments elongate and most pollen grains are round in shape. The pistillode of male flowers is smaller than that of female flowers. Although the loculi are well developed, only rudimentary ovules are formed. If the stigma develops and matures and the filaments do not elongate, then female flowers are formed. In female flowers, pollen grain development is aborted, and the pistil is characterized by a globose ovary, an elongated style, and a prominent stigma. After this stage, male and female flowers can be distinguished with the naked eye based on the different dimensions of their pistils and stamens. Not long after this period, gametophytes reach maturity and flowers blossom.

In the wild-type, the modes of unisexual flower formation are in accordance with other unisexual plants. The selective developmental arrest of preformed organ primordia is the most common method for generating unisexual flowers (Wu *et al*. 2011). Present author propose factors that regulate sexual differentiation in yellow-horn, which may selectively affect sex organ primordium occurrence.

In the mutant, the process of floral ontogeny is similar to that of wild-type flowers, with exception of the initiation of several corolla whorls. No obvious structural differences are observed before phase 4, as similar tissues and organs such as bracts and sepals are formed. The first floral organs to be produced are the bracts (Fig. 4A, B). Subsequently, five sepal primordia appear within the second whorl (Fig. 4C, D). Following sepal initiation, petal primordia arise within the subsequent whorl (Fig. 4E). From this point on, developmental patterns begin to diverge between the two types. In the mutant, more petal primordia are formed and produce several whorls randomly. There is no orderly, repeated pattern of primordium initiation on the ring, even among flowers on the same plant. The primordia cannot be classified precisely as either petals or stamens (Fig. 4F). They apparently occur in positions in which only stamens appear in single flowers, but these are not necessarily 1 : 1 substitutions. The primordia differentiate later; there are stamens in the middle of the top meristem (Fig. 4F, G). The transitional morphology of the intermediates indicates developmental blending of two organ categories, an example of homeosis in the broad sense. Petals enlarge slowly compared with stamens. As they elongate, stamens adopt a cylindrical shape (Fig. 4H), forming anthers and filaments. However, during a later period, stamens demonstrate petaloidy. The yellow sacs of the small inner petals are anther traces containing pollen (Fig. 4I, J). The pistil is visible following initiation of the stamens, and its formation begins with that of carpel primordia. Though the mutant is sterile, the flowers can still be categorized as male and female flowers, and the structural differences arise during pistil development. In the later period of phase 6, flowers whose carpel primordia undergo abortion or form extremely short styles without further differentiation or development develop into male flowers (Fig. 4J, K). Ovules do not initiate in the male flower. If the protuberance of the apical meristem undergoes further differentiation and forms an ovary with rudimentary ovules, then female flowers form (Fig. 4L, M, N). The ovary of the female flower is larger than that of the male. The styles and stigma are petaloid in both male and female flowers.

Many double flower varieties undergo conversion of stamens into petals, which should be regarded as a type of homeosis referred to as petaloidy. In homoheterotopy, a particular ecological function carried out at one position is transferred to another spatial location, and stamens are generally replaced by petals (Kellogg 2000, De Craene 2003). Homeotic mutations in flowers can be divided into two classes. In one class, replacement of one organ type with another results in a 1 : 1 substitution. In the other class, the positional interchange is not a 1 : 1 relationship. In neoheterotopy, a feature is created at a new location not previously occupied by a distinct organ

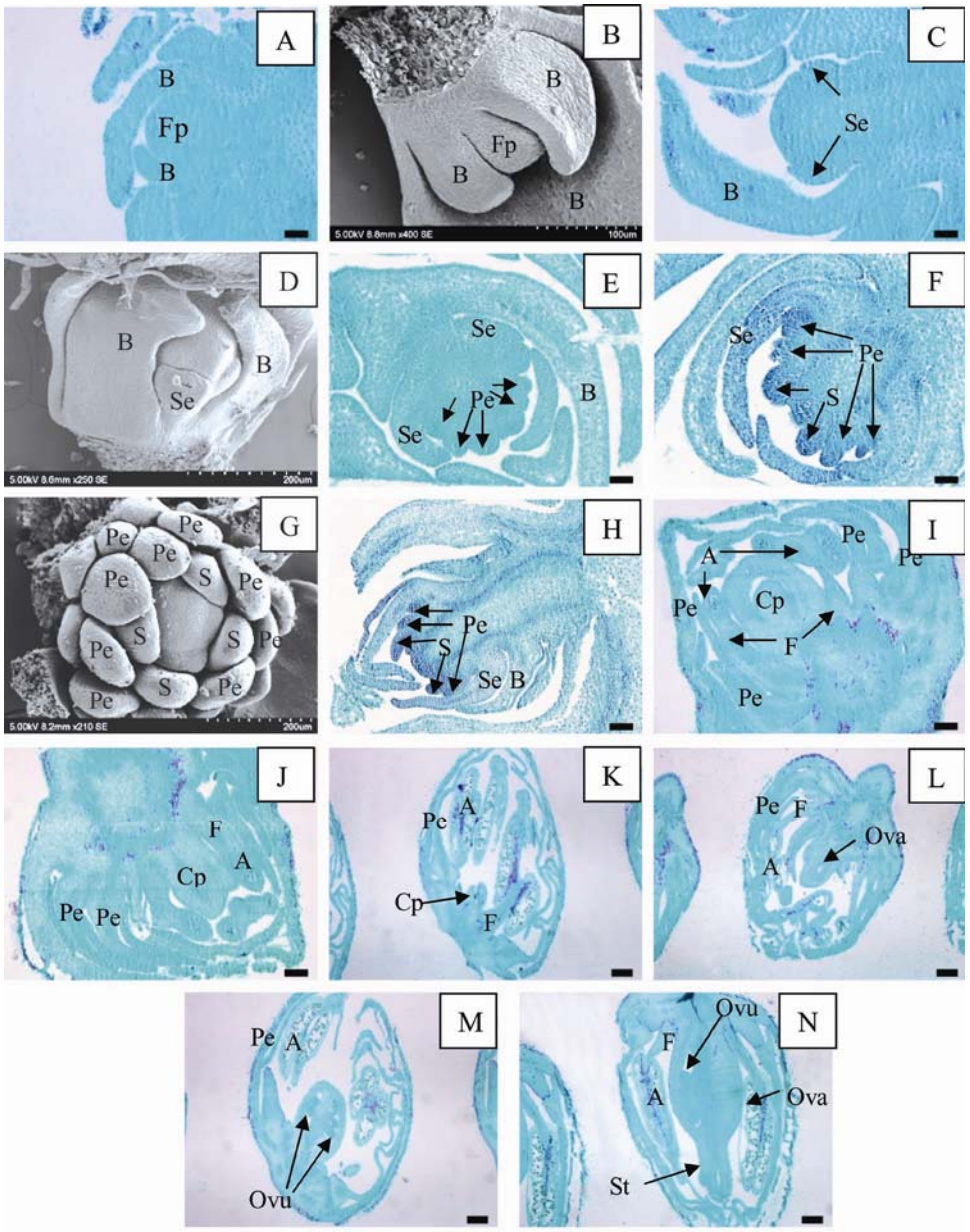

Fig. 4. Floral ontogeny of the mutant *Xanthoceras sorbifolia.* A and B: Bract and flower primordium formation. C and D: Sepal appearance. E: Emergence of petal primordia. F and G: Stamen initiation. H: Elongation of the stamen primordia. I: Anther and filament primordium emergence. J and K: The carpel of the male flower undergoes abortion. L, M, and N: The carpel of the female flower differentiates further, and an ovary with ovules and the style are formed. The style is petaloid. B - bract; Fp - flower primordium; Se - sepal; Pe - petal; S - stamen; F - filament; A - anther; Cp - carpellary primordia; Ova - ovary; Ovu - ovule; St - style. Bars: A, C, E, and F = 50 µm; H, I, and J =100 µm; K, L, M, and N = 200 µm.

The molecular mechanisms may lead to a better understanding of the increased number of perianth whorls. In the ABC model, class A genes specify sepals in whorl 1; the combined activity of class A and B genes specifies petals in whorl 2; B and C genes specify stamens in whorl 3; and C gene alone specify carpels in whorl 4 (Coen *et al*. 1991). Based on the features of the double flower, it is inferred that the expression of class B and C gene is abnormal or suppressed in the mutant. In addition, it was suggested that the genes expressed in the petal of an ancestor came to be expressed in a position previously occupied by a stamen. However, further research is required to clarify these points.

According to previous research, some miRNAs expressed differentially between simple- and double flowers such as miR164, miR166, and miR319 (Ao *et al*. 2012). These miRNAs are associated with the numbers of petal, sepal, pistils and carpels, and the size and shape of floral organs (Laufs *et al*. 2004, Jung *et al*. 2007, Nag *et al*. 2009). Nevertheless, whether these miRNAs are involved in the regulation of class B and C gene expression domains and the relationship between miRNA expression and the ABC model remain to be clarified.

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