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Chromosome-centric approaches in crop genomics: Focus on Mendel's pea plant

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Abstract: Gregor Mendel laid foundations of genetics after his experiments in pea plant hybridization. The choice of pea (*Pisum sativum* L.) and its seven morphological characters as a model system was fortuitous and enabled the fundamental discoveries. Nevertheless, other model organisms were chosen by his followers who aimed at discovering the nature of hereditary information. This remained so until the era of molecular biology and genomics, largely due to the huge size of the pea plant genome. However, the introduction of methods for dissecting the genome to single chromosomes by flow cytometric sorting simplified physical mapping and sequencing the pea genome and the analysis of its evolution. An unexplored potential of chromosome flow sorting in pea includes gene cloning and also the analysis of the molecular organization of condensed mitotic chromosomes. In line with the advances in various omics techniques and a variety of physiological and morphological characters, this makes the pea plant an attractive candidate for a new plant model.

Keywords: chromosome sorting; flow cytogenetics; gene cloning; genome mapping; genome sequence; *Pisum sativum* L.

Since the development of agriculture, humankind relied on domesticated plants and animals to produce food and products for various uses. Domesticated plants and animals were subjected to selection and the forms satisfying human needs were propagated. Uninformed selection practiced by early farmers was gradually replaced by thoughtful activities, and by the end of the 18th century and during the 19th century, we see the first individuals who improved considerably the yield and quality of field crops and farm animals by cross breeding (Wood 1973; Janick 2015). Without knowing the principles of inheritance, they succeeded by intuitive selection of parents to produce progenies with improved characters.

It was Moravian monk Gregor Mendel living in Augustinian Abbey in the town Brno (now in the Czech Republic) who discovered the secrets of inheritance (Mendel 1866). Together with De Vries (1900), Correns (1900) and Tschermak (1900), who at the beginning of 20th century rediscovered Mendel's findings, they provided the much-needed knowledge to perform informed breeding. Although the nature of hereditary material was not known by that time, it was clear that it resides in cell nuclei, focusing the attention on nuclear chromatin and its changes during cell division, when condensed chromosomes were observed. It was during this same time that Walter Sutton (Sutton 1903) realized the parallelism between Mendel's factors and the behaviour of chromosomes during meiosis. His work underlined the role of chromosomes in the transmission of hereditary information and generating variation as observed in the progenies.

The twentieth century witnessed major discoveries on the nature of hereditary material, its structure and function (Williams 2019). The advent of molecular

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biology and subsequent rise of genomics pushed chromosomes slightly out of the focus of research community. Main efforts concentrated on determining the order of nucleotides in DNA, which represents the nuclear genome. Only recently it became clear that genome function depends on chromatin organization in the three-dimensional space of nuclei, including the arrangement of chromosome domains (Jost et al. 2014). This, together with other discoveries and advances turned the attention back to chromosomes as basic units of genome organization and function.

Mitotic chromosomes have been studied by numerous methods, at various degrees of spatial resolution, and focusing on their constituent DNA, RNA and proteins. Out of the numerous approaches employed to analyse condensed mitotic chromosomes, a distinct one relies on their isolation by flow cytometric sorting. The ability to separate chromosomes from cell or tissue homogenates greatly facilitated studies on their organization by various methods, including electron microscopy to analyse their ultrastructure, biochemical techniques, including proteomics, to examine their molecular composition, and to prepare chromosome-specific DNA for downstream applications. The latter process offers attractive opportunities to analyse nuclear genome, in particular when chromosome in a karyotype can be separated from each other. The availability of chromosome-specific DNA greatly simplifies genome sequencing, gene mapping and cloning (Zwyrtková et al. 2021).

This paper outlines the contribution of chromosome flow sorting to the study of nuclear genome of pea (*Pisum sativum* L.) – the experimental object on which Mendel made his seminal discoveries. This information is provided in the context of using this approach in other crops. Interestingly, pea was the second plant species after faba bean (Doležel et al. 1992) for which a high-yield protocol was developed to prepare chromosome samples from root tips (Gualberti et al. 1996). The samples prepared this way where suitable for flow cytometry and made it possible to apply chromosome-centric approaches in pea genome analysis. The pioneering work on faba bean and pea paved the way for the application of chromosome genomics in other important crops.

Chromosome analysis and sorting using flow cytometry

The application of flow cytometry to analyze and sort mitotic metaphase chromosomes is collectively termed flow cytogenetics. While the analysis of chromosomes by flow cytometry, or flow karyotyping, has been surpassed by other cytogenetic methods and has not been used widely, chromosome sorting played a major role in genomic research. The ability to isolate chromosomes, be it the entire complement or a specific chromosome type, offers attractive opportunities in genomics, allows simplification of downstream analyses and significantly reduces cost of genomic projects (Zwyrtková et al. 2021).

Primal reasoning behind chromosome flow sorting was a lossless reduction of the complex plant genomes prior to analysis and sequencing. Other techniques offering genome simplification such as Cot-based fractionation (Paterson et al. 2002), methylation filtration (Palmer et al. 2003) and exome capture (Albert et al. 2007) suffer from a massive loss of genetic information. Main advantage of chromosome flow sorting over these methods is that it allows dissecting the genome into its natural subunits without losing a single base pair of the organism's DNA. Mitotic chromosomes may be isolated also using laser microdissection (Matsunaga et al. 1999). However, the throughput is thousand-fold lower compared to flow cytometry and the quality of DNA thus obtained is compromised.

First flow sorting of plant chromosomes was reported in *Haplopappus gracilis* (De Laat & Blaas 1984), which was selected for its low chromosome number ($2n = 4$), and suitability for *in vitro* culturing and cell cycle synchronization. Thus, suspensions of mitotic chromosomes suitable for flow cytometry were prepared from synchronized suspension cells. A second attempt was performed in *Petunia hybrida* (Conia et al. 1987), where leaf mesophyll protoplasts were the donor tissue of mitotic chromosomes. Both of these preparatory methods were found unsatisfactory as they couldn't be used in a wider range of plant species and alternative strategies were sought. A solution was to use meristem root tips from which intact chromosomes could be isolated in large quantities by mechanical homogenization after mild formaldehyde fixation (Figure 1). This method was originally developed for *Vicia faba* (Doležel et al. 1992) and to date it has been optimized and used for 35 plant species (Doležel et al. 2021). The chromosomes isolated this way are resistant to mechanical shearing forces during flow cytometric sorting and their DNA remains intact. Thus, they proved to be suitable templates for a vast and still expanding range of cytogenetic and molecular biology techniques (Zwyrtková et al. 2021).

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Isolation of pea chromosomes using flow cytometry

Pea is an important source of plant-based dietary proteins in human and animal nutrition. According to the Food and Agriculture Organization (http://fao.org.faostat), pea is the second most grown and consumed legume in the world. Its importance as protein source and hence also its production is expected to increase in relation to the growing popularity of plant-based meat substitutes (Tziva et al. 2020). Apart from its significance in food production, it also delivers important services to the ecosystem. The symbiosis with nitrogen-fixing soil bacteria reduces the requirement for application of industrial fertilizers (Crews & Peoples 2004), making pea a crop suitable for environmentally sustainable agriculture.

Given the importance of pea as a crop with positive environmental impact, its genetic information has been studied by many researchers and using different approaches following the experiments of Gregor Mendel. One of them has been chromosome sorting using flow cytometry. The method makes it possible to prepare samples of pure mitotic metaphase chromosomes. Depending on the type of a project, either a particular chromosome, or all chromosomes from a karyotype, are isolated. This approach provides unique materials that simplify gene mapping and cloning, support genome sequencing and facilitate the analysis of molecular composition of mitotic chromosomes and their ultrastructure.

Preparation of samples for flow cytometry and chromosome sorting. As noted by Doležel et al. (2021), a protocol for preparing intact chromosome suspensions must be optimized for each species separately. The foundational work of Gualberti et al. (1996) described a high-yield protocol for preparation of suspensions of intact mitotic metaphase chromosomes from root tips of young seedlings. Like in the protocol for chromosome isolation in faba bean (Doležel et al. 1992), cycling root tip cells were synchronized using hydroxyurea and accumulated in metaphase stage of mitosis using amiprophos-methyl (APM). After a mild formaldehyde fixation of root tips, chromosomes were released into an isolation buffer by mechanical homogenization (Gualberti et al. 1996).

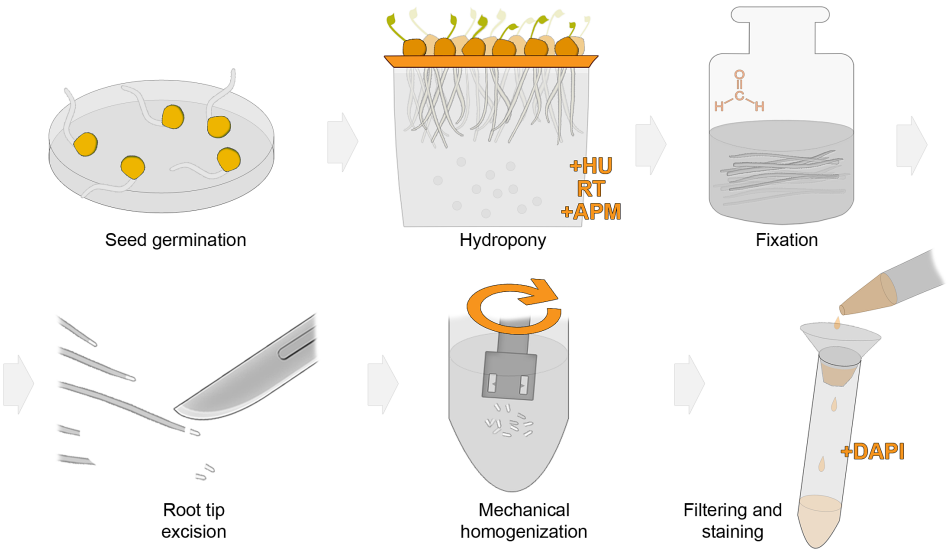

Figure 1. Preparation of chromosome suspensions for flow cytometry; seeds are germinated on moistened paper tissue in Petri dish until the roots reach ~2 cm; young seedlings are positioned onto a plastic cover lid and placed onto a tray so that roots are fully immersed in aerated nutrient solution; cycling meristem tip cells are accumulated at G1/S interphase by a treatment with hydroxyurea (HU); during a recovery time (RT), which follows the removal of HU, the cells resume DNA synthesis and traverse S and G2 phases of cell cycle; upon reaching mitosis, the cells are accumulated at metaphase by a treatment with amiprophos methyl (APM); immediately afterwards the roots are fixed by formaldehyde, meristem root tips are excised and mechanically homogenized to release chromosomes into a solution; prior to flow cytometric analysis, crude chromosome suspension is filtered through a nylon mesh and stained by 4',6-diamidine-2'-phenylindole (DAPI); for more details see Doležel et al. (2021)

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Similar to the preparation of chromosome samples, also flow cytometric chromosome analysis and sorting needs to be optimized for each species and the readers are referred to Vrána et al. (2016) for more details. Briefly, flow cytometry classifies chromosomes according to their light scatter and fluorescence properties, and in their work, Gualberti et al. (1996) used fluorescence as the main parameter to classify pea chromosomes that were stained by DNA fluorochrome 4',6-diamidine-2'-phenylindole (DAPI). The authors analyzed the samples at rates of 200 chromosomes/s and obtained histograms of relative DAPI fluorescence intensity (flow karyotypes) comprising four composite peaks, with three peaks representing two chromosomes each, and one peak representing one chromosome. The same approach to analyze pea chromosomes using flow cytometry was used in all subsequent studies (Neumann et al. 1998, 2002; Kreplak et al. 2019). In these studies, pea chromosomes were sorted using electrostatic droplet sorters when the droplets carrying chromosomes of interest are charged and deflected by passage between charged plates and collected in an appropriate receptacle (Robinson & Grégori 2007).

Physical mapping using flow-sorted chromosomes

Physical mapping unique genomic loci by PCR was the very first application of flow-sorted chromosomes in genomics. Given the sensitivity of PCR, the number of flow-sorted chromosomes per single reaction is low and five hundred to one thousand chromosomes, representing roughly half nanogram amount of DNA can be sorted in the order of minutes. The first successfully mapped were the *vicilin* genes in *Vicia faba* (Macas et al. 1993). Other successful applications included mapping of genes to sex chromosomes in *Silene latifolia* (Kejnovský et al. 2001) and to chromosomes in chickpea (Vláčilová et al. 2002) to integrate cytogenetic and genetic maps or inferring marker positions towards cloning the gene conferring resistance to Russian aphid in wheat (Šimková et al. 2011). Sub-chromosomal resolution was reached when using wheat-barley ditelosomic addition lines 7HL and 7HS to verify mapping of SSR markers to the long arm of barley chromosome 7 (Cseh et al. 2011). Powdery mildew resistance gene was positionally cloned from chromosomal arm 4AL in wheat exploiting chromosome flow sorting as well (Jakobson et al. 2012) and later the integration site of barley sucrose transported *HvSUT1* was mapped to its respective chromosomes in three different wheat transgenic lines (Cápal et al. 2016). Mapping of COS (conserved orthologous set) markers was used to infer the degree of homology between wheat and its wild relatives *Aegilops markgrafii*, *Ae. triuncialis* and *Ae. cylindrica* to support introgression breeding programs (Molnár et al. 2015). The same approach was used to detect chromosomal rearrangements relative to wheat karyotype in *Aegilops* subgenomes (Molnár et al. 2016).

Chromosomes flow-sorted onto microscopic slides proved to be suitable templates for fluorescence *in situ* hybridization (FISH) almost immediately after the protocol for sample preparation was established (Lucretti et al. 1993). This provided an opportunity to use FISH with repetitive DNA probes to identify flow-sorted chromosomes and to estimate the purity in the sorted chromosome fractions (Kubaláková et al. 2003). Flow-sorted chromosomes also convey additional advantage over conventional preparatory methods (squashes or dripping methods) as they land on microscopic slide without cell wall debris and cytoplasmic remnants, leading to significantly reduced background. This fact facilitated the location and ordering of short 2kb-long low-copy bacterial artificial chromosome (BAC) subclones onto flow-sorted short arm of wheat chromosome 1B (Janda et al. 2006), while mutual positions of cDNA clones specific to long or short chromosome arms of barley were inferred using FISH on flow-sorted chromosomes (Karafiátová et al. 2013). Although the protocol was not used in further studies, an attractive approach was developed for stretching flow sorted chromosomes directly on microscopic slide with up to 100fold longitudinal expansion of the original chromosome size, greatly improving the spatial resolution to locate and order neighboring loci (Valárik et al. 2004).

Anchoring pea genetic linkage groups (LGs) to chromosomes

In their work, Gualberti et al. (1996) demonstrated the suitability of chromosomes sorted onto microscopic slides for physical mapping DNA sequences using primed *in situ* DNA labelling (PRINS). However, the histograms of relative fluorescence intensity (flow karyotypes) obtained by analyzing fluorescence of pea chromosomes stained by DAPI comprised only four instead of the expected seven peaks. With the exception of one peak, which represented chromosome IV, each of the remaining three peaks represented two different chromosomes with similar fluorescence

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intensity. The inability to dissect the genome to single chromosomes would greatly limit the potential of chromosome sorting in pea genomics.

As shown earlier in faba bean (Lucretti et al. 1993), one way to increase the number of chromosomes that can be purified individually is to prepare samples from chromosome translocation lines with altered chromosome size and hence different DNA content. Neumann et al. (2002) tested this approach in pea by analysing three lines JI145, JI146 and JI148, each with one reciprocal chromosome translocation. To assign peaks observed on flow-karyotypes to particular chromosomes, they sorted chromosomes from each peak onto a microscope slide and used them as templates for FISH. The identity of chromosomes was determined using probes for 5S rDNA and PisTR-B DNA repeat. As shown earlier by Neumann et al. (2001), FISH with these probes allows identification of all chromosomes of pea. The analysis of chromosomes from pea cv. Ctirad resulted in a flow karyotype on which chromosomes 5 and 7 formed well-discriminated peaks and thus could be sorted individually. The number of chromosomes sortable from the translocation lines was higher and ranged from three in line JI145 to four in lines JI 146 and JI 148. Chromosomes isolated from line JI148 were used to assign until then unassigned pea genetic linkage groups IV and VII to particular chromosomes. To do this, translocation chromosomes 2^7 and 7^2 and a pool of the remaining chromosomes were flow sorted and their DNA used as template for PCR using markers genetically mapped by Gilpin et al. (1997).

The study of Neumann et al. (2002) marked the completion of the efforts to unambiguously assign all pea genetic linkage groups to individual chromosomes. It also showed the utility of chromosome translocation lines for physical mapping of DNA sequences to sub-chromosomal regions. As larger quantities of seeds of translocation lines may not be readily available and some of such lines may be difficult to propagate, Neumann et al. (1998) developed an alternative system for chromosome isolation. They showed that the so-called hairy root cultures, which can be obtained by transformation of plants by *Agrobacterium rhizogenes*, can be maintained *in vitro* for long periods and easily propagated.

Next generation sequencing of isolated chromosomes

Continuous improvement of genome assembling algorithms compatible with the short-read sequencing technologies made individual chromosome sequencing feasible, and the ability to increase chromosomal DNA *via* multiple displacement amplification significantly lowered the requirements for number of sorted chromosomes (Šimková et al. 2008). This strategy has been extensively used and the first experiment involved barley chromosome 1H. A comparison of chromosome-derived shotgun sequence reads with the sequenced genomes of rice and *Brachypodium* made it possible to construct virtual gene order along chromosome 1H – a so called genome zipper (Mayer et al. 2009). The same group finished the task for the remaining six barley chromosomes and the entire barley genome had its genome zipper completed only two years later (Mayer et al. 2011). Wicker et al. (2011) compared genic sequences in homoeologous group 1 of wheat chromosomes and confirmed collinearity of wheat genes with rice and *Brachypodium* genomes and identified non-functional pseudogenes on these chromosomes, while Akhunov et al. (2013) shed light onto chromosomal rearrangements and pseudogenization process on chromosome 3A. In a similar way, virtual gene orders were established for all chromosomes of rye (Martis et el. 2013). The complete assembly of the entire gene space of chromosomal arm was accomplished for wheat chromosome arm 7DS (Berkman et al. 2011) by sequencing flow-sorted chromosome arm with 30× coverage by Illumina technology. Later the same team assembled the remaining short arms of wheat chromosome group 7 (Berkman et al. 2012, 2013).

The most extensive use of chromosome-centric approach to sequence crop genomes so far was demonstrated in generation of draft sequence of hexaploid wheat (IWGSC 2014). Chromosome arms were flow-sorted from ditelosomic lines, sequenced by Illumina and used to annotate more than 75 000 genes and to position them along the chromosomes, anchor more than 3.6 million markers, describe the relationship of the three homoeologous genomes and to uncover the phylogeny of this important crop. In addition to *de novo* sequencing, sequencing flow-sorted chromosomes enables validation of already assembled genomes. This approach was found beneficial in chickpea (Ruperao et al. 2014) where numerous misassemblies were found in desi and kabuli cultivars. Doležel et al. (2014) and Zwyrtková et al. (2021) provided comprehensive lists of sequencing projects that relied on flow-sorted chromosomes.

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Pea genome sequence

Given the importance of pea as a crop and the potential impact of the availability of its genome sequence on pea improvement, it was no surprise that in 2014, International Pea Genome Sequencing Consortium was established. The consortium was coordinated by Judith Burstin (INRA, France) and due to vast genomic resources developed for the French inbred pea cultivar Caméor, this genotype was selected for sequencing. However, the effort to deliver a draft genome sequence was hampered by the genome complexity. Flow cytometric analysis of intact cell nuclei gave genome size estimate as 4.45 Gb (Doležel et al. 1998), placing pea among plants with large genomes (Michael 2014). The large genome size is the result of both, whole genome evolutionary duplications and propagation of DNA repeats, which comprise a major part of the genome (Macas et al. 2007). This redundancy made the sequencing project a challenging endeavour.

To obtain the pea genome sequence, several sequencing and assembly strategies were combined (Kreplak et al. 2019) and flow-sorted chromosomes were used in several phases of the project. After assembling whole genome illumina short read sequences into contigs and joining them into scaffolds using long-read PacBio sequences, sequence reads obtained from flow-sorted chromosomes were used to verify the location of sequence contigs to individual chromosomes and to identify inter-chromosomal chimeric scaffolds. As it is not possible to discriminate each of the seven pea chromosomes by flow cytometry, and as it was critical to avoid any cross contamination in the sorted chromosome fractions, single copies of chromosomes were sequenced. To increase a probability of collecting a similar number of copies of each of the seven pea chromosomes, several sort windows were set on a flow karyotype corresponding to different chromosome subpopulations (Figure 2A–C). Chromosomes were sorted into PCR

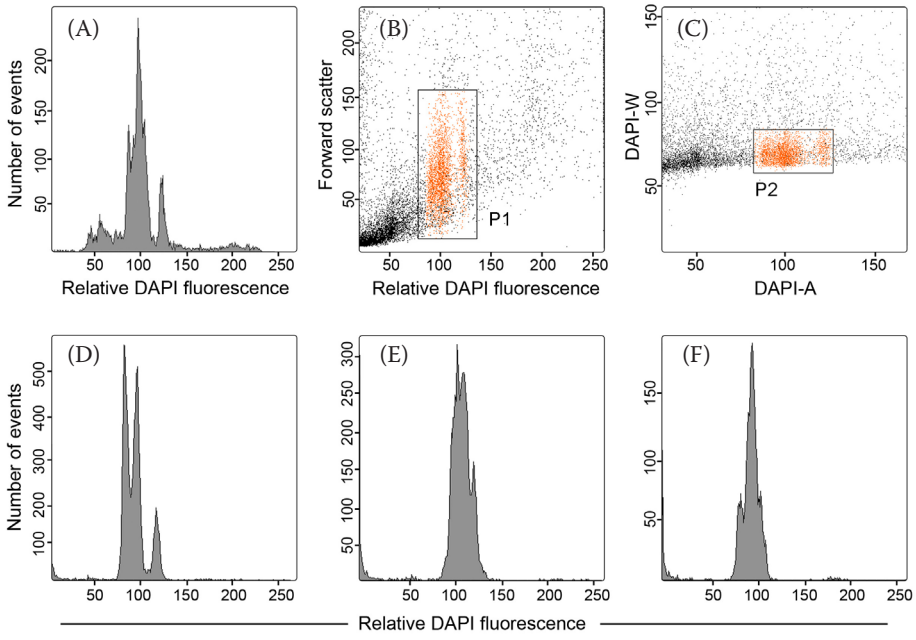

Figure 2. Flow cytometric analysis (flow karyotyping) and sorting of pea chromosomes; prior to flow cytometric analyses, a suspension of intact mitotic chromosomes is stained with a DNA fluorochrome 4',6-diamidine-2'-phenylindole (DAPI); thus, chromosomes are classified according to relative DAPI fluorescence, or relative DNA content: histogram of DAPI fluorescence intensity (flow karyotype) of pea cv. Caméor, with a major composite peak on channel 100, which represents six chromosome types and one minor peak on channel 130 representing chromosome 5 (A), for chromosome sorting, initial gate P1 was set on scatter plot of chromosome forward scatter vs relative DAPI fluorescence (B), dependent final sorting gate P2 was drawn around chromosome population on scatter plot of DAPI fluorescence pulse area (DAPI-A) vs DAPI fluorescence pulse width (DAPI-W) to discriminate single chromosomes from chromosome doublets (C), flow karyotypes of *P. fulvum*, *P. humile* and *P. elatius*, respectively (D–F); differences in peak height and position reflect karyotype differences between the three wild species and pea cv. Caméor

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tubes (one chromosome per tube) and their DNA amplified using isothermal multiple displacement amplification following a protocol of Cápal et al. (2015). Samples with the highest amount of amplified DNA and representing all seven chromosomes were selected for sequencing by illumina technology. Mapping sequence reads to the genome sequence scaffolds identified scaffolds containing contigs from different chromosomes, which were then split into smaller scaffolds. Following this step, the sequence scaffolds were joined into super-scaffolds after aligning them onto BioNano optical maps (Chen et al. 2017).

Optical genome mapping. Optical mapping is an elegant approach to bridge genome scaffolds by comparing assembled sequence contigs with map of short sequence motifs tagged by labelling enzyme (Lam et al. 2012). As a rule, whole genome optical maps are developed using high molecular weight DNA prepared from cell nuclei. However, Staňková et al. (2016) demonstrated that it is possible to construct optical maps from individual chromosomes. The authors constructed optical map for the short arm of wheat chromosome 7D (7DS) and identified until then unassembled arrays of tandem repeats on the arm. The use of flow-sorted chromosome arm, which represented only a fraction of the whole wheat genome, greatly simplified the assembly process. Moreover, the map was of high quality as the DNA of flow-sorted chromosomes has high molecular weight. The same optical map was later used for anchoring BAC-based physical map onto 7DS arm (Tulpová et al. 2019a). International wheat genome sequencing consortium used optical maps of the homoeologous chromosome group 7 (i.e., chromosomes 7A, 7B and 7D) when producing wheat reference sequence (IWGSC 2018).

Optical maps of the pea genome were constructed both using nuclear DNA prepared from isolated nuclei and DNA prepared from flow-sorted chromosomes. To purify all chromosomes for the construction of optical map, the sort window was set on a flow karyotype to include all populations representing the seven pea chromosomes. In total, 5.6 million pea chromosomes, corresponding to about seven micrograms DNA, were flow-sorted and embedded in eight agarose miniplugs, each comprising 700 000 chromosomes. Optical map was constructed using 2.7 μg of purified chromosomal DNA following the protocol of Staňková et al. (2016). The final pea genome assembly consists of seven pseudomolecules of the seven pea chromosomes and its size is 3.92 Gbp (Kreplak et al. 2019), representing 88% of the estimated 4.45 Gbp size (Doležel et al. 1998).

Mendel's genes

In his work on pea, Mendel studied inheritance of seven qualitative characters: seed shape, stem length, cotyledon colour, seed coat colour flower colour, pod colour, pod form and flower position. Out of them, four have been cloned and their mutations identified (cf. Reid & Ross 2011; Ellis et al. 2011). The first was the gene controlling seed shape (round vs wrinkled, *R* vs *r*), which encodes starch branching enzyme I (SBEI), with the mutant allele containing inserted transposon (Bhattacharya et al. 1990). The gene controlling stem length (tall vs short, *Le* vs *le*) encodes gibberellin 3-oxidase and the mutant allele contains a single nucleotide substitution (Lester et al. 1997; Martin et al. 1999). The third gene that was cloned controls cotyledon colour (yellow vs green, *I* vs *i*) and encodes a stay green protein (SGR) which encodes a positive regulator of the chlorophyll-degrading pathway (Armstead et al. 2007; Sato et al. 2007). Mutant allele of the gene differs by a six-nucleotide insertion (Sato et al. 2007). Seed coat and also flower colour (coloured vs clear/colourless testa, coloured vs white flowers, *A* vs *a*) is controlled by a gene encoding basic helix-loop-helix (bHLH) transcription factor. Mutant allele *a* harbours a nucleotide transition at donor splice site. In their effort to clone the fifth gene, Shirasawa et al. (2021) identified a genome locus that controls pod colour (green vs yellow, *Gp* vs *gp*). Two members of the 3'exoribonuclease gene family were found in the locus as candidate genes controlling pod colour. However, functional analysis of the candidates is yet to be done as well as the identification of sequence differences between wildtype and mutant alleles. Using pea genome assembly of Kreplak et al. (2019) and web version of URGI BLAST tool (https://urgi.versailles.inrae.fr/blast/) we have identified genomic sequences of all five genes, their genomic coordinates on respective pseudomolecules/linkage groups are specified in Table 1.

Identification of the two remaining genes has been hampered, among other, by the fact that there are two different candidate loci on two different linkage groups for each of them (Ellis et al. 2011). Thus, the gene controlling pod form (inflated vs constricted) could be located on LG 3 (*V* vs *v*) or LG 6 (*P* vs *p*) and the gene for the position of flowers (axial vs terminal) could be located on LG 3 (*Fas* vs *fas*) or LG 4 (*Fa* vs *fa*). According to Ellis et al. (2011), NAC domain transcription factors, which act as master switches

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Table 1. Genomic localization of Mendel's genes; the five genes that have been previously identified and assigned to respective linkage groups are listed, together with their exact genomic coordinates in the current version of the pea genome assembly

Gene	Full name	Trait	Pseudomolecule/ linkage group	Start position	End position
SBE1	starch branching enzyme 1	seed shape	Chr3 LG5	71671821	71671686
GA3Ox1	gibberellin 3-oxidase 1	stem length	Chr5/LG3	567366063	567367491
SGR1	stay-green protein 1	cotyledon colour	Chr2/LG1	419922790	419922617
bHLH	basic helix-loop-helix TF	flower colour	Chr6/LG2	403314697	403314599
XRN3	3' exoribonuclease	pod colour	Chr3/LG5	54423519	54423781

for secondary cell wall thickening, are candidates for *P* or *V* in pea. Sinjushin and Gostimskii (2007), Ellis et al. (2011) and Reid and Ross (2011), consider homologs of *Arabidopsis thaliana* gene *CLAVATA* (*CLV*) as candidates for *Fa*, and *FASCIATA* (*FAS*) and *BRCA2* gene homologs as candidates for *Fas*. By searching the pea genome assembly (Kreplak et al. 2019) we have identified possible loci of the above-mentioned genes. Table 2 shows BLAST hits for the respective *Arabidopsis thaliana* gene analogues (Araport11 genome release, www.arabidopsis.org).

Evolution of the pea genome organization

In addition to using chromosome-derived DNA sequences to support the assembly of the pea genome, Kreplak et al. (2019) utilized flow-sorted chromosomes to elucidate genomic events that shaped the evolution of the legume clade and the pea genome, in particular. Earlier studies identified chromosome translocations in genus *Pisum* (Sansome 1937; Lamm & Miravalle 1959). However, chromosomes involved in the rearrangements were not known, nor the positions of chromosome breaks. In order to clarify this issue, Kreplak et al. (2019) sequenced chromosomes isolated by flow sorting from three wild pea relatives, *P. elatius*, *P. humile* and *P. fulvum*. According to Ben-Ze'ev and Zohary (1973), hybrids of these species with *P. sativum* had low fertility and chromosome rings in meiosis, indicating the presence of chromosome translocations, which accompanied speciation within the genus.

To identify and characterize chromosome translocations, 84 copies of single chromosomes were isolated by flow sorting from each of the three wild relatives of cultivated pea (Figure 2D–F). Theoretically, twelve copies were isolated for each of the seven chromosomes. To increase a probability of collecting the same number of copies from each chromosome, two or three different sort windows were used. DNA from single chromosomes was individually amplified according to Cápal et al. (2015) and sequenced using illumina technology. Sequence reads thus obtained were mapped onto the pseudomolecules of the pea reference genome. All chromosomes from the three wild relatives mapped unambiguously to one of the pea pseudomolecules, except for chromosome 5, where the reads mapped only to a region 0 – 465 Mb on the pseudomolecule (Figure 3). The remaining sequence reads from the corresponding chromosomes of *P. eliatus* and *P. humile* mapped to pea chromosome 1, identifying a translocation between chromosomes 5 and 1 in *P. eliatus* and *P. humile,* while the sequence reads from the corresponding chromosome of *P. fulvum* aligned to the distal part of pea chromosome 3 (Figure 3), indicating a translocation between chromosomes 5 and 3.

Table 2. Genomic localization of to date uncloned Mendel's genes; the table shows two candidate loci for each gene, with respect to putative localization described by Ellis et al. (2011) and Reid and Ross (2011)

Gene	Full name	Trait	Pseudomolecule/ linkage group	Start position	End position
Fa	FASCIATA	flower position	Chr5/LG3	390756333	390755930
Fas	CLAVATA		Chr4/LG4	134878438	134878039
P	NO APICAL MERISTEM	pod form	Chr5/LG3	491538559	491538857
V	NO APICAL MERISTEM		Chr1/LG6	21854502	21854294

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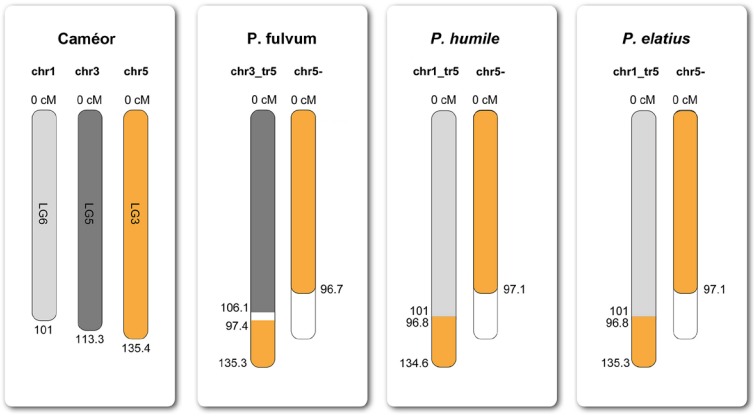

Figure 3. Structure of translocated chromosomes in wild relatives of pea: *P. fulvum*, *P. humile* and *P. elatius*; chromosome translocations in three wild relatives of pea could be delimited precisely by mapping of sequencing reads obtained from flow-sorted single chromosomes onto the Caméor pseudomolecules (Kreplak et al. 2019); in all the three accessions, the largest pea chromosome 5 was truncated at the same position and its fragment was translocated onto chromosome 3 in case of *P. fulvum* and onto chromosome 1 in case of *P. humile* and *P. elatius*; this finding was further supported by mapping DNA markers from available genetic map onto the assembled contigs from single chromosomes sequencing reads (only the terminal marker positions in cM are shown)

The chromosome-centric approach not only allowed to delimit the translocation event with almost a base-pair resolution, but also permitted the investigation of synteny in legume clade and revealed the exact processes that led chromosomal evolution in *Pisum* genus (Kreplak et al. 2019). Clearly, these valuable insights into the pea genome composition and evolution would be much more difficult to obtain without the ability to isolate chromosomes by flow cytometric sorting and the so-called chromosome genomics.

The potential of chromosome genomics in pea

Almost exactly 150 years after Mendel published his work on pea hybrids, a genome sequence of his main experimental model was released (Kreplak et al. 2019). The availability of this unique genomic resource will make it possible to apply new breeding techniques to develop cultivars with improved quality, suitable for sustainable agriculture and adapted to climate change. Moreover, the dramatic improvement in the knowledge of the pea genome and the availability of new omics tools make pea a hot candidate for a new plant model. Although it has much larger genome than *Medicago truncatula* and *Lotus japonica*, until recently popular models for legume research, comparatively smaller number of genes and a variety of physiological and morphological characters make it a very attractive alternative (Cesarino et al. 2020). Given that previous results confirmed a feasibility of flow cytometric chromosome sorting in pea, chromosome genomics could play a role in these efforts. To date, DNA of flow-sorted chromosomes was used to map and sequence the genome of pea. However, the ability to purify mitotic metaphase chromosomes and, if needed, dissect nuclear genome to its functional units – chromosomes, offers much broader range of important applications (Figure 4). The following text gives examples of successful uses in other crops as an inspiration for possible future work in pea.

Targeted development of DNA markers

DNA markers are an important molecular tool in plant sciences and especially in breeding. They have been used to develop genetic maps, integrate genetic and physical maps, clone genes and perform marker-assisted selection (Powell et al. 1996). Dissecting the genome into chromosomes allows targeted and cost-effective development of DNA markers from particular genome regions. Thus, short-insert chromosome-specific DNA libraries were used to isolate microsatellite clones for development of SSR markers (Macas et al. 1996). Sequencing ends of BAC clones from rye 1RS-specific chromosome BAC library enabled to design insertion site-based polymorphism (ISBP) markers specific to the rye chromosomal arm (Bartoš et al. 2008). Chromosome-specific transposable element junction markers were developed also for barley, where more than 400 000 unique markers

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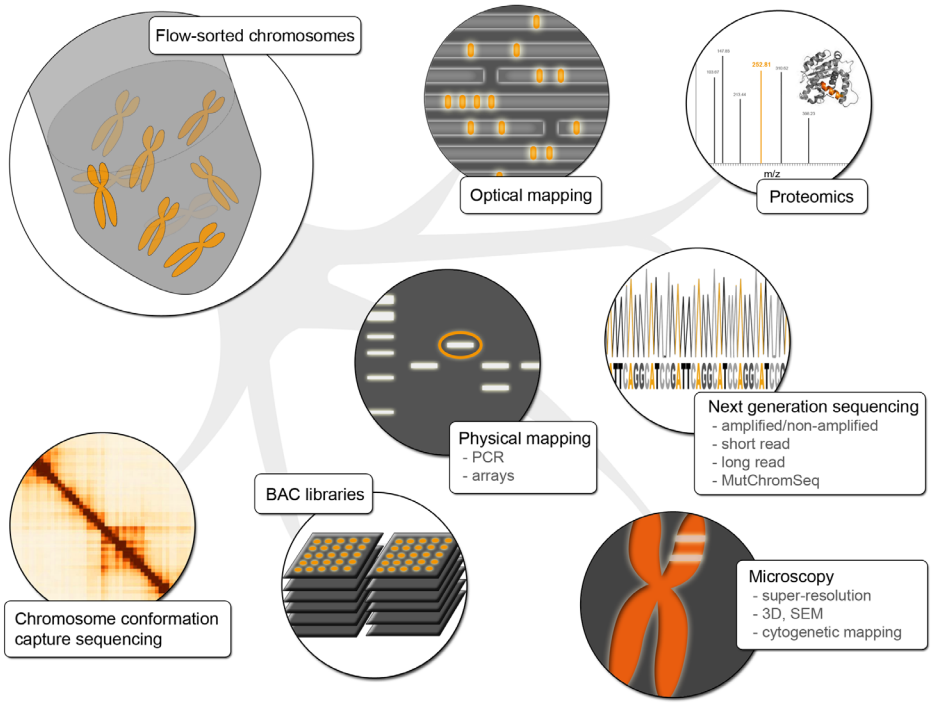

Figure 4. Diverse applications of flow-sorted chromosomes in plant genomics; the diagram shows the most important current applications of flow-sorted chromosomes in plant genomics; flow-sorted chromosomes proved to be suitable template for a plethora of downstream applications, while opening new avenues of research by providing the unique input material

were used as a valuable resource supporting the assembly of the barley genome sequence (Mayer et al. 2011). Single nucleotide polymorphism (SNP) markers are indispensable tool in genomics due to their abundance and low cost per data point. To construct genotype-specific genetic maps, Shatalina et al. (2013) developed SNP markers after low coverage illumina sequencing of chromosome 3B purified from two cultivars of wheat, Arina and Forno. A single SNP marker tagging a region carrying resistance genes against leaf rust (*Lr57*) and stripe rust (*Yr40*) was identified among markers obtained by sequencing chromosome arm 5MgS, which was flow-sorted from wheat-*Aegilops geniculata* substitution line (Tiwari et al. 2015). Eleven SNP markers associated with a gene responsible for resistance to Russian wheat aphid were mined from sequences generated from isolated wheat chromosome arm 7DS (Staňková et al. 2015). A stepping stone towards cloning leaf rust resistance gene locus *Lr49* was the sequencing and SNP mining of chromosome 4B from two parental lines of recombinant inbred line population contrasted in the susceptibility to this fungal disease (Nsabiyera et al. 2020).

Gene cloning

Identification and characterization of genes responsible for agronomically important traits and genes for resistances against biotic and abiotic stresses is a holy grail of crop genomics. Chromosome genomics has been instrumental to reach this goal in a number of studies. An *in silico* method named RICh (rearrangement identification and characterization) was utilized to perform sequence comparison of long arms of wheat chromosome 4A flow-sorted from standard susceptible cultivar and a cultivar with introgression from *Triticum militinae* conferring resistance against powdery mildew. The analysis narrowed down the region carrying the *QPm-tut-4A* resistance gene to a locus with 169 putative genes (Abrouk et al. 2017). An analogous approach was employed to identify candidate genes responsible for leaf rust (*Lr76*) and stripe rust (*Yr70*) resistance. In these projects, DNA sequence comparison was performed of flow-sorted chromosomes 5D from susceptible reference cultivar, homoeologous chromosome 5U from *Aegilops umbellulata* and a translocation chromosome 5D from resistant wheat-*Aegilops* introgression line.

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This effort brought new markers for the resistances and delineation of introgressed chromatin carrying resistance genes to 9.47 Mbp-long region (Bansal et al. 2020). Shotgun sequence assemblies and optical maps of chromosome arms 7DS from Chinese Spring and line CI2401, contrasted in resistance against Russian wheat aphid, led to identification of *Epoxide hydrolase 2* as the most probable candidate gene (Tulpová et al. 2019b). Fifteen years after a method for high molecular weight DNA isolation from flow-sorted chromosomes was established (Šimková et al. 2003), it found its new use in gene cloning. A protocol developed by Thind et al. (2018) called TArgeted Chromosome-based Cloning via long-range Assembly (TACCA), uses high molecular weight DNA for long-range scaffolding based on proximity ligation of in vitro–reconstituted chromatin (Putnam et al. 2016). Thind et al. (2018) used TACCA to clone leaf rust resistance gene *Lr22a* after generating a continuous assembly of chromosome 2D from resistant wheat cultivar Campala. Subsequently, Xing et al. (2018) used TACCA to clone *Pm21* gene by sequencing and assembling translocated wheat chromosome 6VS.6AL. The gene is responsible for powdery mildew resistance and was introduced to bread wheat from *Haynaldia villosa* (Xing et al. 2018). As compared to positional gene cloning, TACCA permits gene cloning in regions with low rates of recombination, including pericentromeric regions and regions with introgressed alien chromatin.

Cloning major genes using induced mutations. A rapid gene cloning strategy that relies exclusively on low-coverage sequencing of flow sorted chromosomes was developed by Sanchéz-Martín et al. (2016). The method was termed Mutant Chromosome Sequencing (MutChromSeq) and the prerequisites for its application are that the investigated trait provides a strong discernible phenotype, is underlined by single gene (a so called "mendelian" trait), the chromosome carrying such a locus is known and that chromosome can be discriminated and sorted from the rest of the karyotype. When these prerequisites are met, the method offers a rapid and cost-effective way for gene cloning. The principle lies in sequencing flow-sorted chromosomes from a wild-type plant as well as from several (ideally six or more) mutants derived from it that are contrasted in target trait. The sequences are assembled in short contigs and compared to find mutational overlap, or in other words, a contig that shows mutations in all the mutants, but not in the wild type (Figure 5). The gene responsible for desired trait is located on this contig

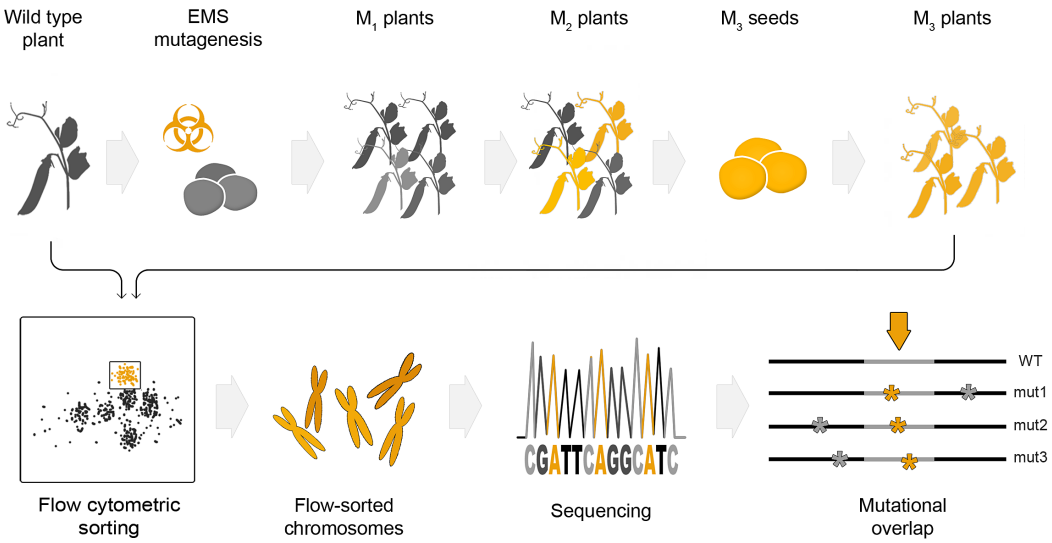

Figure 5. Gene cloning using the MutChromSeq strategy; at the core of the method stands flow-sorting of chromosome carrying the gene of interest, both from wild type (WT) and from independent mutants derived from it; the WT seeds are mutagenized, plants in M2 population are screened for mutations and their progeny is tested in M3 generation; the seeds of confirmed mutated plants are donor material for chromosome sorting; DNA is isolated from flow-sorted chromosomes, sequenced (at ~100× coverage for WT and ~30× for all the mutants) and WT chromosome is assembled into contigs; sequencing reads from mutants are aligned against wild type chromosome and variants are called; the candidate gene is identified by searching of mutational overlaps between the mutants

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and the only remaining task is to identify it by gene searching strategies.

The potential of MutChromSeq was confirmed by re-cloning *Eceriferum-q* gene in barley, while in the same study, the powdery mildew resistance gene *Pm2* was cloned in wheat (Sanchéz-Martín et al. 2016). Later was MutChromSeq used to clone the semi-dwarfism gene in wheat, where the mutations in locus located on chromosome 6A identified increased expression of *gibberellin 2-oxidase*, leading to semi-dwarf phenotype (Ford et al. 2018). By sequencing wild type chromosome 2H from barley cv. Sudan and mutants derived from it, Dracatos et al. (2019) successfully cloned *Rph1* gene encoding coiled-coil nucleotide binding site leucine-rich repeat receptor protein, conferring leaf rust resistance in barley. Gene *SuSr-D1* suppressing resistance to stem rust in bread wheat was identified by sequencing flow-sorted chromosome 7D from susceptible cultivar and from resistant mutant lines (Hiebert et al. 2020). Sequence comparison of barley chromosome 5H isolated from wild type and from mutants with elevated lysine content allowed to identify *LYS3* gene encoding PBF transcription factor to be responsible for this trait, promising to improve barley varieties with high lysine content for animal feed (Orman-Ligeza et al. 2020).

Mendel (1866) analyzed inheritance of traits using seven lines of pea carrying different spontaneous mutations affecting plant development. However, the number of induced mutant lines affecting other traits is much higher and more can be induced (Blixt 1972; Sinjushin 2013). Thus, MutChromSeq represents an attractive approach to clone the two remaining Mendel's genes and perhaps many other underlying important agronomic traits.

Other attractive uses of flow-sorted plant chromosomes

Purified mitotic metaphase chromosomes have been shown a unique source of chromosome-specific DNA to support gene mapping, gene cloning and genome sequencing in plants. However, samples of isolated chromosomes could also help to solve fundamental questions of molecular organization of condensed plant mitotic chromosomes. Despite the critical role mitotic chromosomes have in transmission of hereditary information, and in contrast to the progress in the analysis of genome organization in interphase nuclei (Jerkovic & Cavalli 2021), this remains largely unexplored area. Thus, Perutka et al. (2021) used mass spectrometry to analyze peptides from flow-sorted barley chromosomes to identify almost 900 proteins – the largest set to date of proteins associated with plant mitotic chromosome. This advance provided a launching pad to characterize molecular function of proteins associated with mitotic chromosomes, some of them known to localize at the perichromosomal layer. Flow-sorted chromosomes could also clarify the issue of DNA transcription during mitosis (Palozola et al. 2017) and retention of transcription factors on mitotic chromosomes (Raccaud & Suter 2018). Coupling the environmental scanning electron microscopy (ESEM), which enables observation of uncoated biological objects in a native state with flow cytometric chromosome purification should clarify chromosome topology at nanometer scale and avoid artefacts inherent to standard scanning electron microscopy. Due to its large chromosomes, pea is an attractive model to study all these endpoints, including the organization of meta-polycentric centromere (Neumann et al. 2012, 2016).

To conclude, the ability to prepare fractions of purified mitotic metaphase chromosomes of pea, the availability of which simplifies genome mapping, sequencing and gene cloning and which are suitable for a range of applications in molecular biology provides unsurpassed research opportunities. Together with a number of physiological and morphological characters of pea, these opportunities could bring Mendel's pea plant back into the spotlight and support its use as a new experimental model.

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