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Commentary

Controlled lavender field fragrance: the biotechnological "Provence" of the future provided by antisense oligoilators and olinscides

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Abstract: Successful management of the synthesis of secondary metabolites of essential oil plants is the basis for the economic growth of the essential oil industry. Against the backdrop of a growing global population and a decrease in land available for cultivation, simple and effective ways to increase the content of certain components in essential oils are becoming increasingly important. Selection is no longer keeping pace with market needs, which stimulates the search for faster methods to control the biosynthesis of secondary metabolites. In this article, using the genus *Lavandula* as an example, we will consider the prospects for use of antisense oligonucleotides (ASO), oligoilators, to rapidly increase the concentration of valuable components in essential oil. This article discusses the use of unmodified ASOs as regulators of plant secondary metabolism to increase the synthesis of individual valuable components, presenting a completely new way to increase the yield of valuable substances based on unique nucleotide sequences. The proposed approach is effective, affordable, safe, and also significantly reduces the time needed to obtain plants that synthesize the required concentrations of target substances. Oligoilators can the used along with oligonucleotide insecticides in complex formulations used for green agriculture. Further investigation is needed to determine maximum economic efficiency of this approach.

Keywords: essential oil plants; antisense oligoilators; secondary metabolites; oligonucleotide insecticides; green agriculture

1. Introduction

Secondary metabolites produced by essential oil plants are widely used in various fields of human activity. Today, a fairly large amount of research is aimed at improving the existing methods of cultivation of essential oil plants to increase their overall productivity and manage their metabolic pathways to obtain a higher content of certain valuable secondary metabolites. Most of the existing approaches – selection [1], methods of genetic modification [2], and genome editing [3] – require a significant investment of time and energy. Nevertheless, people will continue to use essential oils for their healing properties and pleasant aromas, which drives the search for intensified agriculture designed to meet human needs. The reduction in sown areas coupled with increased population pits agriculture against two competing forces and exacerbates the problem of intensifying agriculture designed to meet human needs.

2. Kingdom of scents in kingdom of plants

Essential oil plants are valued primarily for their secondary metabolites. Generally, the synthesis of secondary metabolites is based on a few precursors: 5-6 amino acids for alkaloids, phenylalanine or tyrosine for phenolic compounds, and mevalonic acid or 5-hydroxyxylulose for isoprenoids. The precursors of essential oils in plant cells are lin-

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ear prenyl pyrophosphates, which are synthesized into the more than 55,000 terpenes (isoprenoids) that have been found in various plants [4].

In plants, terpenes are derived from two universal five-carbon precursors, isopentynyl pyrophosphate and C5-dimethylallylpyrophosphate [5]. Starting with C5-dimethylallylpyrophosphate, head-to-tail condensation reactions with one to three molecules of C5-isopentynyl pyrophosphate generate C10-geranylpyrophosphate, C15-farnesylpyrophosphate, and C20-geranylgeranylpyrophosphate. These products are generated by reactions involving the corresponding enzymes: C10-geranylpyrophosphate synthase, C15-farnesylpyrophosphate synthase, and C20-geranylgeranylpyrophosphate synthase. These synthases are the key intermediates for the biosynthesis of diverse terpenes [6,7].

Because the lengths of lavandulyl diphosphate synthases (LPPs) determine their distinct physiological roles, the production of LPPs is precisely regulated by their respective prenyltransferases, groups of highly conserved enzymes in the cells [4]. Prenyltransferases can be further classified into cis- and trans-types on the basis of the type of double bond formed during C5-isopentynyl pyrophosphate condensation [8].

To date, about 300 industrially significant essential oils are widely used. The most popular of these are mint, lavender, citrus, and coniferous oils. In addition, some terpenes are commercially significant for use in drugs, such as taxol (anti-cancer) [9] and artemisin (antimalarial) [10]; as flavors, including menthol and linalool [11]; and as nutraceuticals, particularly carotenoids [12].

Essential oils have well-established antibacterial, antibiotic, and antiviral properties, and are also believed to be beneficial for use in treating Alzheimer’s disease, cardiovascular disease, cancer, migraine [13], and pain during pregnancy [14,15]. The use of essential oils as antioxidants in the preservation of various foods is gaining in popularity [16]. Essential oils are particularly valuable for their varied traditional uses among different cultures worldwide who have used plants and their derivatives from time immemorial [17,18].

The global production of essential oils as of 2020 totaled 370,000,000 kg, valued at more than $10B USD. Despite their high value for use in perfumery and medicine, the largest consumer of essential oils is currently the food industry. The production of most non-alcoholic beverages and some alcoholic, confectionery, and semi-finished products is not complete without essential oils or other secondary plant metabolites. The largest producers of essential oils are Asian countries: China and India produce the most, followed by Indonesia, Sri Lanka, and Vietnam. Brazil and China are confirmed leaders in the production of orange; India and China, corn mint and eucalyptus; Argentina and Spain, lemon; and France and Spain, lavender oil. According to the European Federation of Essential Oils, the global production of essential oils covers about 600,000 hectares of the 1.6 billion hectares under agricultural production. Some oils are produced in small quantities due to the way the plant is harvested; for example, patchouli, ylang-ylang, clove, and nutmeg are still harvested from forests or grown on small farms [19].

The rise in the standard of living and purchasing power of populations worldwide has contributed to the rapid increase in the production of essential oils. For example, over the past 10 years, production in Europe has increased by 41%. In Germany, which produces 46% (19,000,000 kg) of European oils, production has increased by more than 50%. France, although producing fewer kilograms over the same period, increased its sales by 90% due to the cost of oil. By 2016, world exports had already reached $4.54B USD [20]. Analysis of the next decade projects a clear increase in this indicator, due not only to the natural tendency to increase the scale of production, but also to the coronavirus pandemic, as a consequence of the increased demand for natural antibacterial and antiviral agents in the prevention of respiratory diseases [21].

3. Biosynthesis of essential oils: far, far away in nucleotide land

Like all cell components, essential oils are synthesized with the participation of enzymes encoded by genes. A large number of studies are aimed at identifying the genes involved in the synthesis of essential oils. Developments in the field of RNA-Seq have made it possible for detailed transcriptomic studies of species without a reference genome [22]. *De novo* transcriptome assembly has been widely used to investigate. This has proved to be particularly useful in the study and identification of genes vital to the biosynthesis of secondary metabolites in aromatic plants, such as *Artemisia annua*, *Mentha spicate*, *Cinnamomum camphora*, *Salvia miltiorrhiza*, *Lindera glauca*, and *Huperzia serrata* [23]. Transcriptomes of different lavender species (*Lavandula* spp.) are currently being actively studied. The results of these studies are aimed at identifying patterns between genes, the chemical composition of the oil, and its aromatic properties [24]. Of particular interest is the observation that the genome *L. angustifolia* contains a high copy number (14 and 7, respectively) of 1-deoxyxylulose-5-phosphate synthase and 4-hydroxy-3-methylbut-2-enyl diphosphate reductase genes, encoding the two known regulatory steps in the plastidial isoprenoid biosynthetic pathway. This fact confirms that the genome of most essential oil plants is strongly duplicated, which in turn allows the plant to produce a large amount of essential oil [25]. The accumulating information concerning genome sequences of essential oil plants encourages the use of this knowledge as a tool to obtain practical results and economic benefits.

One of the most promising and simplest ways to regulate gene expression is the use of antisense oligonucleotides (ASO), oligoilators, which has found wide application in both medicine and agriculture [26-29]. This simple alternative technology effectively replaces more complex and expensive methods such as RNA interference [30] or classical selection [31]. It should be noted that short unmodified ASO as oligonucleotide insecticides have already proven themselves as effective preparations for insect pest control [32,33]. In our research, we drew attention to the possible joint use of oligonucleotide insecticides with preparations that can affect the plant cell in order to trigger integrated increase of crop yields.

In theory, use of ASO during gene expression can disrupt transcription, splicing, and translation. They employ four main mechanisms of action in eukaryotic cells. The first mechanism is steric blocking of protein synthesis. The second mechanism is based on the action of RNAse-H, which mediates the degradation of the mRNA-ASO complex. The third mechanism of action relies on the ability of ASO to form a triple helix by binding to DNA strands, which leads to inhibition of transcription initiation. The fourth mechanism is associated with the formation of the ASO-mRNA complex, which can affect splicing. Using antisense technologies, oligonucleotides enter plant cells and inhibit the expression of target genes [34]. There have been many successful attempts to implement this approach for a large number of plants, including *Nicotiana tabacum* [35], *Arabidopsis thaliana*, *Triticum aestvium* [36], and *Linum usitatissimum* [37-39].

The first report concerning gene regulation by synthetic ASOs in plant cells dates back to 1992, when Tsutsumi and co-workers demonstrated that a specific ASO against barley α-amylase can block in vitro mRNA translation and decrease α-amylase activity in barley aleurone layers [40]. Initially, this ASO was used to study gene function in seeds and pollen tubes [34]. Almost immediately after Tsutsumi et al. published their results, this approach began to be used by other researchers, since the high permeability of the cell wall of pollen tubes makes them a convenient model. One of the first of these studies was carried out in 1994 by Estruchet al. [41]. The next breakthrough step in the application of ASO was the use of ASO sequences in green tissue of barley [42] and then further for the whole plant [43]. Currently, more and more research is aimed at obtaining economic benefits when using non-transgenic methods of plant modification [44] and protection [27,28,45]. However, with respect to the essential oils of plants, no one has yet attempted to modify their composition with the help of ASO.

Over the past 30 years, since the beginning of the use of ASO on plants, research has focused primarily on testing whether this technology works effectively. The alpha-amylase gene, calcium-dependent calmodulin-independent protein kinase, nuclear-encoded phytoenedesaturase, chlorophyll a/b-binding protein, chloroplast-encoded light-harvesting protein, and transcription factor SUSIBA2 were among those used. The research mainly involved genes whose function does not lead to the accumulation of valuable components of plant cells that can lead to economic benefits, such as secondary metabolites.

Thus, the ASO approach has been proven to be a simple and effective way to influence the expression of plant genes. This technology is suitable for both dicotyledonous and monocotyledonous plants, especially in cases where it is difficult to obtain transgenic plants. In addition, it should be noted that this approach is more accessible than the usual methods of plant breeding and has more flexibility in the case of the undesirable mutations that constantly occur.

The availability of this approach for large scale application is largely determined by the price of oligonucleotide synthesis. For the synthesis of oligonucleotides, the most widely used method is automatic solid-phase phosphoramidite synthesis, which makes it possible to obtain the desired oligonucleotide sequences relatively quickly, with high yield and purity [46,47], to solve a wide range of tasks. However, solid-phase synthesis is not particularly efficient, consuming on average several times more than the required amounts of reagents to create oligonucleotide strands. In addition, expensive automated nucleic acid synthesizers and large amounts of controlled-pore glass, which is the most expensive component of solid-phase synthesis, are used in solid-phase synthesis.

A rational alternative to this technology is liquid-phase synthesis, which can significantly reduce the cost of the process by removing the need to use expensive controlled-pore glass [48], reduced waste of phosphoramidites, and transference of synthesis to chemical reactors, which obviates the need for automatic synthesizers [49-51]. According to approximate calculations, liquid-phase synthesis will reduce the cost of oligonucleotides by a factor of 100. While the Achilles' heel of liquid-phase synthesis is its lack of automation, it is clearly more competitive compared to solid-phase synthesis for obtaining short oligonucleotides, such as DNA insecticides [52] and DNA preparations [53,54]. This includes the DNA regulators of secondary metabolism of essential oil plants (antisense oligoilators), which will allow large volumes of targeted short nucleic acid sequences to be produced at a low cost.

It should be noted that unmodified oligonucleotides, particularly oligoilators, have a zero (or minimal) carbon footprint. This feature gives oligoilators a competitive edge in the effort to reduce global warming in the industrial era [55]. Studies show that neither the substances necessary for synthesis, nor the phosphoramidite synthesis of DNA itself, leads to any noticeable release of greenhouse gases (under publication).

Although data on plant genomes are growing exponentially, to date, the complete genomes of fewer than 400 higher plants have been sequenced [56], among which most are crops of great economic importance, along with a number of species that serve as model objects in biology. Nevertheless, today the sequencing of a new generation is flourishing and genome data will accumulate exponentially.

4. Field of lavender dreams: a magic wand made of an oligonucleotide strand

Provence is an authentic and unique region in France that has become a symbol of lavender cultivation, a brand known to the whole world. Climatic, soil, and landscape factors helped to determine the specialization of this region into one of agricultural production. However, current trends require up-to-date approaches to production: the rapidly growing demand for essential oils and extracts forces farmers to look for new breakthrough biotechnologies that can rightfully become a worthy alternative to traditional breeding or expensive methods of genetic modification.

Our pioneer developments [26,57] are the basis for investigations of the idea of a biotechnological 'Provence' of the future, where it will be possible to control the aromatic components of essential oils with the help of antisense oligonucleotides. In particular, successful management of the synthesis of secondary metabolites by plants was demonstrated in peppermint (*Mentha piperita*) using an unmodified antisense oligonucleotide of oligoMEP-11 (5'-ACACTCTTTTG-3'), which is complementary to the mRNA of the menthon reductase that catalyzes the formation of menthol from menthon. Peppermint

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leaves were treated with antisense oligonucleotides at a concentration of 50 pmol/cm^2. Analysis of the composition of the essential oil was carried out 4 days after the treatment. The menthol content of the oligoMEP-11 group was 2.03 times less than content measured in the control group (13.12±1.64% vs. 6.47±1.04%, respectively; $p<0.05$). The decrease in menthol content was accompanied by a significant increase in menthon content compared with control (61.2±1.31% vs. 53.5±1.74%, respectively; $p<0.05$). The control oligoYM-11 fragment (5'-CGTA-CGTA-CGT-3') did not affect the accumulation of menthol or menthon ($p>0.05$). This biotechnology may allow manufacturers of essential oils to be faster and more flexible in their work, without harming nature. To demonstrate the viability and flexibility of this approach, we applied ASO in our experiments on lavender to increase the concentration of linalyl acetate in the oil [26].

The existing developments in this area, namely the method of increasing the content of linalyl acetate in the essential oil of narrow-leaved lavender plants using unmodified ASOs, confirms the viability of the approach [26,29]. Work on the impact on the biosynthetic apparatus of lavender cells includes the use of DNA-based preparations that selectively act on the expression of the linalool synthase gene. By treating plants with aqueous solutions of the antisense oligoilator LAVAN-11 (5'-GCAACAATCC-3') from a cold fog generator with a drop size of 10-20 microns (concentration 0.15 g/L) at a consumption rate of 100 mL of solution per bush in the full flowering phase with a diameter of 1 m 4-5 days before harvesting inflorescences, it was possible to achieve an increase in the content of linalyl acetate by 8.64±1.45% and a decrease in linalool of 6.13±0.49%. Significant differences ($p<0.05$) were found between the control groups and LAVAN-11 on the fourth day after treatment. The concentrations of linalool and linalyl acetate in the controls were 43.52±0.50% and 21.91±0.73% ($p<0.05$), respectively, and in bushes treated with LAVAN-11, 37.39±0.99% and 30.55±2.18% ($p<0.05$), respectively. The control (random) fragment C-11 (5'-CCCCCCCCCCC-3') did not show a significant effect on the percentage composition of essential oil components and its total yield [57,58].

Our results demonstrate how the use of preparations based on antisense DNA oligoilators acted selectively on the expression of the linalool synthase gene. A schematic diagram of the use of antisense oligoilators to enhance valuable components in plant essential oils is shown in Figure 1.

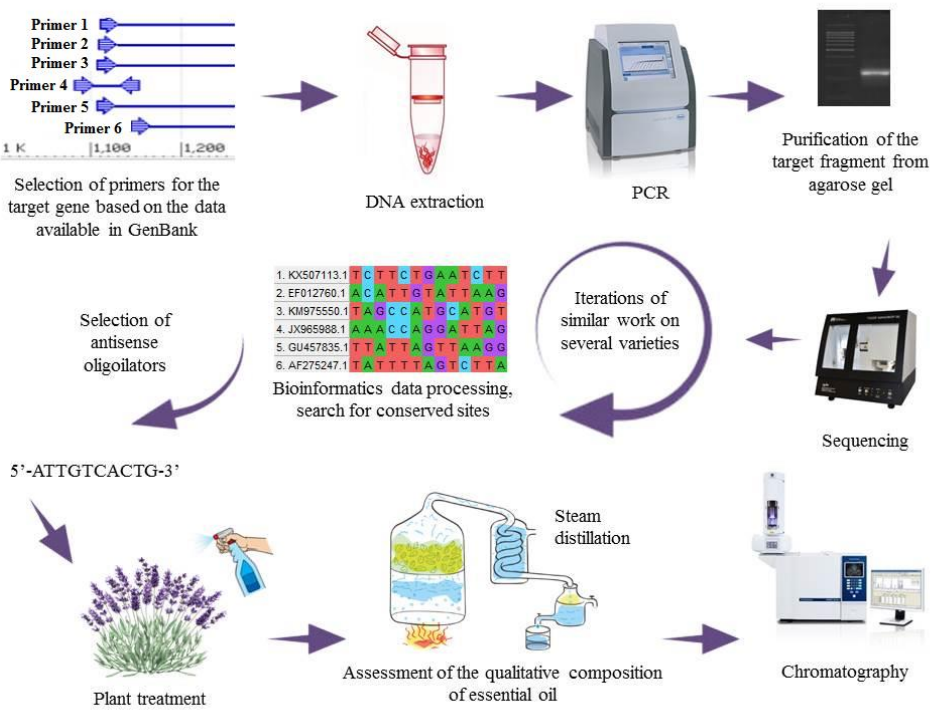

Figure 1. General scheme for the use of antisense oligoilators in controlling plant secondary metabolism of essential oil plants.

The antisense oligoilator LAVAN-11 of the linalool synthase gene of lavender blocks the synthesis of the linalool synthase enzyme responsible for the biosynthesis of linalool from geranyl diphosphate. This in turn blocks the synthesis of linalool, which leads to a change in the trajectory of the secondary metabolism cascade and the conversion of accumulated linalool to linalyl acetate. The fine details of the mechanism of action of antisense oligoilators are currently being studied by our research team. As a rule, oligoilators work according to the general mechanism of action of unmodified ASOs, reducing the expression of the target linalool synthase gene.

It should be noted that the complex application of oligoilators together with oligonucleotide insecticides (olinscides) can give a more significant increase in crop yields. Among the essential oil plants, such as lavender, mint, sage, etc., there are insect pests from the order of Hemiptera [59]. On representatives of families of Hemiptera olinscides show high speed of action and efficiency [33]. Olinscides along with oligoilators 4-5 days before harvesting of essential oil plants make it possible to reduce the content of insect pests in plant raw materials, and also make it possible to accumulate more essential oil in the last days before harvesting. The idea of using unique sequences of antisense oligonucleotides provides the necessary multi-purpose effect without cross-interference, which could reduce the effect of the combined use of olinscides and oligoilators. For each plant-insect pest pair, it is possible to create unique effective pairs of oligoilators and olinscide. Oligoilators can be applied independently or be added to the formula of oligonucleotide insecticides and *vice versa*. Oligoilators and olinscides have a high level of environmental friendliness for the reason that at each trophic level, target and non-target organisms have deoxyribonuclease enzymes that carry out the rapid breakdown of DNA molecules in cells [60]. Thus, antisense oligonucleotides can play an important role in the functioning of agrocenoses, gradually revealing the potential for effective and safe molecular crop management in green agriculture.

The main problem facing the essential oils market is the falsification and use of synthetic aromatic substances that do not have the beneficial properties of natural oils, but only imitate the aromas. This situation has developed due to the increasing demand for oils and the high cost of natural raw materials. To address this, it is necessary to create various technologies that help the agricultural sector to intensify cultivation, namely, to obtain more high-quality raw materials from a smaller area. The coronavirus pandemic and the increase in demand for sustainable, natural products has created a favorable environment for the development and use of such biotechnological techniques.

5. Conclusions

The proposed ASO technology, based on antisense oligoilators and olinscides that increase the concentration of essential oil components and control insect pests, is quite simple and easy to use. The first experiments carried out by our research team have shown that we can, on the one hand, save time, and on the other hand, successfully tackle the this immense project and be able to offer a large number of essential oils with subtle notes in aroma. The rapidly developing and increasingly available next-generation sequencing gives hope that over the next 30 years, data on the genomes of most plants will become available to us, which will open the door to the creation of a biotechnological 'Provence' in every part of the world with land and conditions suitable for the growth of aromatic plants.

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