TARIMSAL BİYOTEKNOLOJİDE İLERİ ARAŞTIRMALAR

Editör: Doç.Dr. Raziye IŞIK KALPAR

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İÇİNDEKİLER

THE USE OF NANOTECHNOLOGY IN PLANT BIOTECHNOLOGY: NEW HORIZONS IN FUTURE AGRICULTURE

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1. INTRODUCTION

Agriculture worldwide is transforming significantly due to rapidly changing environmental and economic conditions (Toksha et al. 2021). Sustainable development goals have emphasized considerably the challenge of feeding a growing global population, and the Food and Agriculture Organisation (FAO) estimates that food production will need to increase by 70% by 2050 to meet projected demand (Ranganathan et al. 2018). Agriculture significantly contributes to human health, environmental sustainability, adequate nutrition, and economic well-being. However, global agriculture today faces numerous challenges, including climate change, soil degradation, dwindling water resources, management of agricultural chemicals, the continuous spread of plant pathogens and diseases and a rapidly growing

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global population (Dhakate et al. 2022; Kumari et al. 2023). Though historically significant, conventional agricultural practices often fall short of addressing these multifaceted challenges effectively (Tripathi et al. 2018). This highlights the urgent need for advanced technologies to transform agriculture into a resilient and resource-efficient system.

Nanotechnology, the science and engineering of manipulating materials at the atomic and molecular scale has emerged as a transformative tool across multiple disciplines, including medicine, energy, and environmental sciences (Elzein 2024). Nanotechnology has emerged as a pivotal field where various scientific disciplines intersect, offering innovative solutions to enhance crop productivity. Recognized by the European Commission as a key enabling technology, it represents a promising frontier in agricultural research, driving advancements in agricultural practices and sustainability (Fincheira et al. 2020). Nanoparticles (NPs) ranging in size from 1 to 100 nm exhibit enhanced catalytic reactivity, biological activity, thermal conductivity, nonlinear optical performance, and chemical stability compared to their bulk counterparts, owing to their high surface area-to-volume ratios. Due to these characteristics, NPs are utilized across a wide array of technical processes, such as the characterization, fabrication, and regulation of materials, to develop new substances and advance applications in fields like chemistry, biology, biomedicine, pharmaceuticals, agriculture, electronics, and bioengineering (Ray and Bandyopadhyay 2021). The integration of nanotechnology into plant biotechnology represents a frontier of scientific discovery with great potential to revolutionize agriculture (Ahmar et al. 2021). Plant biotechnology has opened new horizons in science and technology, playing an important role in improving crop yield and quality, preserving valuable plant genetics and germplasm, producing secondary metabolites and developing disease-resistant and novel plant varieties. Furthermore, advanced research is delving into transgenic plants and the development of pharmaceuticals derived from plants, such as vaccines, therapeutic proteins, and plant-based antibodies, often known as plantibodies (Bhatia and Sharma, 2015). These advancements underscore the transformative potential of plant biotechnology in addressing global challenges in medicine, agriculture, and industry, attracting the attention of researchers to develop new ideas, tools, processes, and strategies (Das et al. 2023).

Nanotechnology plays numerous beneficial roles, including facilitating biomolecule delivery (Demirer et al. 2019), enabling genetic transformation (Johnson et al. 2021), regulating various growth stages in plant tissue cultures (Feizi 2023), enhancing somaclonal variation (Gunasena et al. 2024), stimulating the production of secondary metabolites (Selvakesavan et al. 2023), promoting plant growth and development (Hassanisaad et al. 2022), and improving resistance to both abiotic and biotic stresses (Kokina and Plaksenkova 2022). Nanoparticle-mediated biomolecule delivery has a high potential to improve transformation efficiency in plant biotechnology and overcome the shortcomings of conventional biomolecule delivery techniques (Wu et al. 2023). Due to their extremely small size, extensive surface area, and abundant binding sites, NPs are highly effective nanocarriers for bioactive molecules such as plasmid DNA and double-stranded RNA (Zhao et al. 2020; Zhang et al. 2021). Engineered NPs promote improved plant growth and development and increase resistance to biotic stresses when utilized as fertilizers or pesticides (Das et al. 2023). The advancement and utilization of nanosensors in precision agriculture have significantly enhanced the ability to monitor and measure crop growth, soil conditions, disease presence, agrochemical usage and absorption, as well as environmental pollution (Chen et al. 2016). These innovations have greatly improved human control over soil and plant health, ensured quality control, and strengthened safety measures, thereby making substantial contributions to sustainable agriculture and environmental stewardship (He et al. 2018; Shang et al. 2019). Therefore, nanotechnology not only helps reduce uncertainty but also enables the coordination of agricultural management strategies, serving as an alternative to traditional technologies. In many cases, agro-nanotech innovations provide short-term technological solutions to the challenges encountered in modern industrial agriculture (Shang et al. 2019). Thus, it offers a sustainable approach for plant biotechnology (Lowry et al. 2019; Zhao et al. 2020; Das et al. 2023; Gunasena et al. 2024). However, despite their remarkable potential and wide-ranging applications, these technologies have also sparked significant concerns regarding their potential environmental and biological risks (Dikshit et al. 2021). Given the great potential of these technologies and the increasing awareness of their long-term ecological and physiological impacts, it is paramount to exercise caution in their use (Bommasani et al. 2021).

This chapter presents a comprehensive examination of nanotechnology's advancements and diverse applications in plant biotechnology, particularly emphasizing its potential to transform the agricultural sector. It examines how nanoscale innovations address critical challenges such as crop improvement, stress resistance, resource efficiency, and sustainability, paving the way for a new era of precision agriculture.

2. NPs UPTAKE AND IMPROVING PLANT GROWTH

NPs entering plants through various routes, mainly roots and leaves, interact with plants and promote changes in morphological and physiological states (Khan et al. 2019). The processes of uptake, transport and accumulation of NPs in plants are contingent upon several factors such as the application method, the concentration of the NPs and their physical and chemical properties, including size, shape, and surface charge and the physiology and cell structure of the plant (Fincheira et al. 2020; Fiol et al. 2021). Plants possess distinct barriers that control the entry of NPs. The polymers forming the cell wall play a critical role in this process, as their composition and properties influence the solubility and permeability of NPs, depending on the NPs' characteristics (Fincheira et al. 2020). The waxy cuticle, primarily composed of wax, cutin, and pectin, serves as the main natural barrier preventing NPs from entering leaves while also protecting them from water loss during growth (Wang et al. 2023). NPs sprayed to plant leaves are taken up by cells through endocytosis via stomata or cuticles on the leaf surface. Stomatal pores, which are in the micrometer range, enable the entry of larger NPs, while those smaller than 5 nm can penetrate through the cuticular pathway (Avellan et al. 2019; Chandrashekar et al. 2023). The entrance of NPs to plant mesophyll tissue is influenced not only by their size but also by their shape and charge. Variations in NP shapes result in differing interface properties, which alter the surface area and contact angle with the plant surface, influencing the efficiency of NP absorption (Sun et al. 2021; Zhu et al. 2021). For instance, Zhang et al. (2022) demonstrated that both positively and negatively charged rod-shaped gold NPs were more readily absorbed and internalized by Arabidopsis leaves compared to spherical NPs of the same size. However, experiments have shown that positively charged NPs exhibit stronger adsorption to leaves than negatively charged ones, primarily because of the electrostatic attraction between the positively charged NPs and the negatively charged plant cell walls. This also facilitates the extracellular transport of positively charged NPs (Azeem et al. 2021). NPs present in soil initially interact with plant roots by adsorbing onto the root surface, where the release of negatively charged compounds like organic acids and mucilage makes positively charged NPs more likely to accumulate and be absorbed. While the root epidermis resembles the leaf surface in composition and function, it is less developed at the root tip and hair regions, allowing direct contact and entry of NPs. With its small pores, the semi-permeable root cell wall restricts larger particles but permits smaller NPs to pass through, particularly in areas lacking an exodermis, enabling access to the central column or xylem (Rajput et al. 2020;

Wang et al. 2023). Transport of NPs occurs in two ways, apoplastic and symplastic. Studies indicate that NPs smaller than 50 nm are typically transported within plants via the symplastic pathway, whereas those ranging from 50 to 200 nm are predominantly transported through the apoplastic pathway (Raliya et al. 2017; Ali et al. 2021; Saritha et al. 2022). The apoplastic pathway facilitates the radial movement of NPs, whereas the symplastic pathway enables intracellular movement (Pérez-de-Luque 2017; Sanzari et al. 2019). These two pathways allow NPs to reach different parts of the plant and accumulate in specific locations.

NPs contribute significantly to seed germination, plant growth and quality improvement by boosting nutritional content, photosynthetic activity and metabolism (Chandrashekar et al. 2023; Wang et al. 2023). Several researchers have determined that different NPs (e.g. CuO, SiO_2, ZnO, TiO_2) on plant growth and development by increasing biomass (Dimkpa et al. 2012), root length (Cañas et al. 2008), leaf length (Jasim et al. 2017), chlorophyll amount (Dai et al. 2020) and soluble carbohydrates (Bala et al. 2019). With NP-treated seeds, notable increases in germination rates and total yield have been attained (Ali et al. 2021). Moderate dosages and regulated concentrations of NPs significantly increase seed germination and support healthy plant growth (Singh et al. 2024). They (Hashmi et al. 2024). Higher germination rates have been seen in seeds treated with NPs, most likely as a result of increased synthesis of phytohormones that serve as germination promoters (Gholami et al. 2022). Foliar application of metal NPs significantly enhances chlorophyll content in plants. This boost allows plants to synthesize more light-harvesting complexes, facilitating increased light absorption and improved photosynthesis. Because of its photocatalytic properties and ability to initiate an oxidation-reduction reaction, which aids in the charge transfer between light-harvesting complexes II and TiO_2 NPs, TiO_2 is the most researched NP (Shafea et al. 2017; Ali et al. 2023). The application of TiO_2 NPs enhances photosynthetic pigments and gas exchange properties by stimulating enzyme activities involved in CO_2 fixation and chlorophyll synthesis (Mohammadi et al. 2016; Faraji and Sepehri 2020). However, some studies have reported reductions in plant growth, chlorophyll content, photosynthetic rates, leaf stomatal conductance, intercellular CO_2 concentration, and transpiration rates. Additionally, NPs have been found to inhibit the expression of genes associated with chlorophyll synthesis and photosystem structure (Wang et al. 2016; Yan et al. 2020).

Nanofertilizers can be described as nanomaterials or NPs that deliver essential or beneficial nutrients to plants at the nanoscale, helping to promote plant growth and enhance production (El-Ghamry 2018). Nanofertilizers have demonstrated considerable potential for transforming agriculture, influencing a range of processes such as seed germination, plant growth, crop productivity, nutritional content, disease resistance and stress management (Kumar et al. 2018; Zahedi et al. 2020; Dorjee et al. 2023; El-Sayed et al. 2023). While conventional fertilizers provide the essential nutrients required for plant growth, they can also give rise to environmental issues. These fertilizers, which contain essential nutrients such as nitrogen, phosphorus and potassium, facilitate the replenishment of nutrients that are deficient in the soil. However, given that the excessive use of fertilizers causes environmental damage, it is important to ensure that fertilizers are used in a controlled and efficient manner (Havlin et al. 2005; El-Saadony et al. 2022). The use of nanofertilizers at lower doses than conventional fertilizers has the additional benefit of reducing environmental impacts, due to their controlled release feature. It has been demonstrated that these fertilizers enhance the uptake of nutrients by plants and facilitate their stress resistance at enzymatic and hormonal levels (Mukherjee et al. 2016). However, nanofertilizers cannot address all the issues about fertilization, as some challenges persist, such as leaching and the bulk delivery of nutrients. Moreover, enhancing the efficacy of nutrient utilization remains a crucial objective (Haydar et al. 2024). Slow and controlled release nanofertilizers represent a significant advancement over regular NPS, offering a more efficient solution to the abovementioned problems (Fig 1).

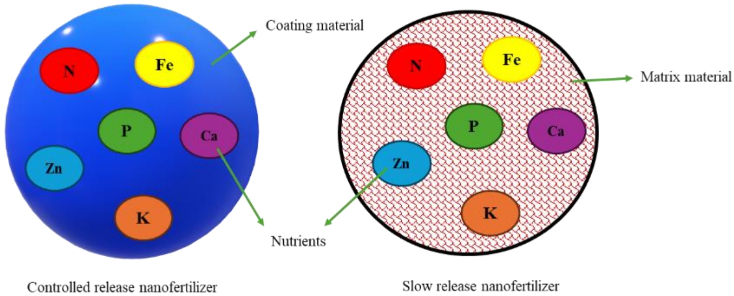

Fig 1. Controlled and slow release nanofertilizer

Slow release nanofertilizers primarily consist of nanocomposite materials such as hydroxyapatite, hydrogels, chitosan, and alginate, where the polymer matrix retains nutrients and releases them in response to environmental factors like pH, temperature, and soil moisture. In contrast, controlled release nanofertilizers feature outer coatings that meticulously regulate nutrient discharge, ensuring controlled and gradual release while minimizing leaching and nutrient over-application (Chouhan et al. 2022; Elsayed et al. 2022). These fertilizers are produced through encapsulation or coating techniques, and their nutrient release can be monitored using advanced analytical methods, including atomic absorption spectroscopy (AAS), inductively coupled plasma (ICP) techniques, and sensor technologies, enabling precise and sustainable nutrient management (Haydar et al. 2024). The utilization of NPs loaded nanofertilizers has been shown to support plant growth to a certain extent; however, the direct absorption of these nanomaterials by plants is often limited. Alternatively, they serve as nanocarriers, facilitating a continuous and regulated delivery of nutrients. This approach enhances nutrient uptake by plants while minimizing the environmental damage caused by traditional fertilizers (Adisa et al.

2019). Nanocarriers may be exemplified by chitosan NPs and nanozeolites. Chitosan NPs offer several significant advantages in comparison to bulk chitosan. The combination of chitosan's surface and interface effect, superior physicochemical properties, high solubility in aqueous media, environmentally friendly structure and bioactive properties with those of NPs results in a unique set of advantages. Consequently, chitosan NPs are frequently employed as nanocarriers for the loading of NPK substances, enabling the controlled and sustained release of NPK fertilisers. This optimises the efficacy of fertilisers and supports the adoption of sustainable agricultural practices (Zhang et al. 2022).

3. NANOSENSORS AND DISEASE-PEST CONTROL

Nanotechnology-based sensors (nanosensors) are playing a revolutionary role in the agricultural sector, offering significant advantages over conventional plant monitoring methods (Chaudhary et al. 2019). Nanosensors provide highly sensitive, stable and reproducible detection down to the single molecule level due to their unique nano-interface (Wang et al. 2004; Hu et al. 2016). These sensors not only detect the signals they receive but also amplify them, providing more accurate and reliable data. This feature of nanosensors enables the biochemical signals emitted by plants to be converted into digital information by directly connected electronic devices. This process is both faster and more efficient than traditional detection techniques (Maes and Steppe 2019; Ballesteros et al. 2020). Nanosensors are facilitating the transition from traditional macro-scale plant monitoring systems to micro-scale, automated monitoring and information-gathering systems in agricultural applications. This technological shift offers the opportunity to more accurately monitor and manage the plant growth process. For example, it allows early detection of plant stress, nutrient requirements or responses to environmental changes thus, contributing to improved agricultural productivity and sustainable agricultural practices (Zhang et al. 2022). Different nanosensors have been employed in plants, including fluorescence resonance energy transfer-based nanosensors, nanowire nanosensors, plasmonic nanosensors, carbon-based electrochemical nanosensors and antibody nanosensors (El-Chaghaby and Rashad 2024).

Nanosensors offer innovative solutions for the monitoring of plant growth, the tracking of diseases and pests, and the assessment of stress conditions in agricultural settings (Afsharinejad et al. 2015). In addition, by adding GPS technologies to nanosensors during plant growth, information on climatic changes, control of irrigation systems, soil and water tension can be accessed throughout the plant's life. The aim is to increase product yield and quality through the use of controlled agricultural systems (Humbal and Pathak 2023). Nanosensors offer safe and stable results for the detection of fungal spores due to their small size and portability (Nugaeva et al. 2005). Accurate and timely detection of pathogens helps to apply pesticides at the right time to protect crops from disease (Bergeson 2010). NPs have been used as biomarkers or diagnostic tools for the rapid detection of bacteria, viruses and fungi (Boonham et al. 2008; Yao et al. 2009; Chartuprayoon et al. 2010). It can also be used effectively for the detection of compounds indicative of disease. Nanomaterial-based nanosensors can serve as an alternative to conventional techniques such as gas or liquid chromatography and mass spectrometry for detecting pesticide residues. These sensors offer high sensitivity, low detection limits, and exceptional selectivity in pesticide residue analysis (Liu et al. 2008). Although nanotechnologies in agriculture offer unique advantages over conventional methods, these technologies are still predominantly in the research phase. While studies have been repeatedly validated in the laboratory, examples of successful transition of these technologies from the laboratory to the field are very limited. There are even fewer examples of commercial applications. This suggests that both technological and economic barriers need to be overcome for the widespread availability of nanosensors in the agricultural sector (Zhang et al. 2022).

4. ENVIRONMENTAL STRESS MANAGEMENT

Plants are subjected to various environmental stresses drought, salinity, temperatures, heavy metals, flooding, and UV radiation (Fig 2). These stress conditions frequently result in the overproduction of reactive oxygen species (ROS), negatively impacting plant growth by inducing cellular toxicity. ROS accumulation causes macromolecule breakdown, membrane structure disruption, and subsequent cytotoxicity and genotoxicity, ultimately inhibiting plant growth and development (Qados and Abdul 2015; Prasad et al. 2017). NPs are recognized as an effective and promising tool to regulate plant yield and overcome limitations of agricultural production by enhancing tolerance mechanisms under abiotic stress conditions (Khalid et al., 2022). Studies have demonstrated that NPs have the potential to facilitate plant growth as well as build tolerance to environmental stress (Omar et al., 2019; Amiri et al., 2010). Excessive use of NPs causes significant problems for plants by causing oxidation of biomolecules due to their phytotoxicity effects, leading to damage and perhaps cell death of plant cells (Ruttkay-Nedecky 2017). However, when NPs are used at optimal levels, they function as key regulators of plant growth and development (Wahab et al. 2023).

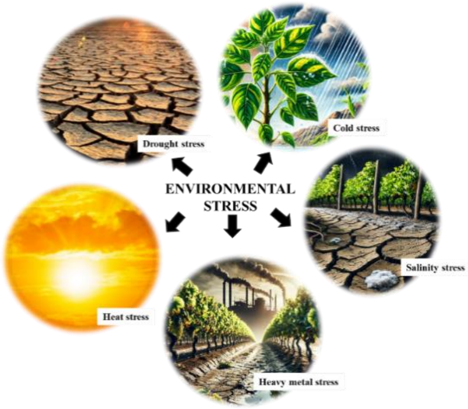

Fig 2. Environmental stress factors

4.1. NPs and Drought Stress

Drought adversely affects agriculture when there is insufficient moisture for the normal development and the life cycle completion of plants. Drought becomes more severe as evaporation rises and precipitation falls (Farooq al. 2012). By 2100, the average temperature is expected to increase by 1.8 to 4.0 °C, and drought is predicted to impact vast regions of the planet, according to the Intergovernmental Panel on Climate Change (IPCC) (Ozturk et al. 2021). The complex nature of drought adversely impacts various physiological parameters in plants, including reduced germination, slower growth rates, decreased levels of plant pigments, diminished yields, disrupted membrane integrity, and significant damage to the photosynthetic machinery (Kumari et al. 2022). Drought affects protein-protein interactions, leading to increased protein aggregation and denaturation, which disrupts the photosynthetic electron transport chain and reduces overall net photosynthesis (Stefanov et al. 2023). Additionally, limited CO_2 uptake due to stomatal closure impairs the activity of CO_2 reduction enzymes. The use of NP-based approaches offers promising solutions to alleviate the harmful impacts of drought stress on plants. NPs alleviate the impact of drought on plants by enhancing the activities of enzymatic and non-enzymatic antioxidants, regulating phytohormone levels, and enhancing various physiological traits (Chandrashekar et al. 2023; Dilnawaz et al. 2023). Research has indicated that using NPs mitigates the adverse effects of drought stress by minimizing the oxidative damage caused by the production of ROS and enhancing the accumulation of osmolytes and osmoprotectants, which support osmotic adjustment (Mustafa et al. 2021; Van Nguyen et al. 2022). NPs participate in signaling pathways, defense mechanisms, metabolic processes and regulatory activities in drought stress conditions (Alabdallah et al. 2021). The efficacy of carbon and metal-based NPs is contingent upon several factors, including application, concentration, surface properties, morphology and plant species (Chandrashekar et al. 2023).

4.2. NPs and Salinity Stress

Salinity stress is a major environmental challenge that disrupts plant growth and development by causing water deficits, osmotic stress, and oxidative damage (Hasanuzzaman and Fujita 2022). Salinity stress gives rise to a multitude of intricate processes, including morphological, physiological, biochemical and molecular changes. As a result of salinity, plants frequently experience oxidative stress, ROS production and nutritional as well as hormonal imbalances (Dilnawaz et al. 2023). High salt concentrations block water uptake, restrict cell expansion and disrupt shoot and root development, resulting in stunted growth and reduced biomass (Zafar et al. 2024). Additionally, they adversely affect chlorophyll synthesis and stability, leading to a decline in chlorophyll content and photosynthetic efficiency in plants (Ali et al. 2023). Nowadays, NP-mediated applications, which attract attention as one of the most promising methods to improve plant growth and performance under salt stress, significantly affect the morphological, physiological and biochemical properties of different plants (Das et al. 2023). NPs improve shoot and root length, plant weight and leaf area by increasing chlorophyll and photosynthetic properties as well as protein, proline content and antioxidant enzyme activities in plants under salinity stress (Zhou et al. 2018; Gulzar et al. 2019). Moreover, NPs regulate transpiration and stomatal conductance in response to salt stress by protecting plants from Na^+ and Cl^- ions, thereby supporting the osmotic balancing processes of plants (Zulfiqar and Ashraf 2021). NPs reduce the harmful effects of salt ions on plants by decreasing Na^+ absorption. This mechanism enhances the defense of plants against salt toxicity by increasing K^+ absorption and decreasing Na^+ levels (Etesami et al. 2021). Depending on the biological functions of the plants, NPs have variable effects on gene expression under salt stress, which alters the expression of several genes involved in different cellular processes and impacts plant growth (Kumar et al. 2013; Ali et al. 2021). One notable application is the seed nanopriming technique, which enhances seed germination and viability by modifying seed metabolism and increasing the expression of aquaporin genes (Shelar et al. 2024). Nanopriming with NPs such as Mn (Ye at al. 2020), MnO (Kasote et al. 2021), CeO_2 (Antony et al. 2021), ZnO and Se (El-Badri et al. 2021) has been identified to play an important role in plant salt stress management by triggering compositional changes and molecular interactions among essential biomolecules. Nanopriming accelerates the hydrolysis of starch into highly soluble sugars and supports embryo development by boosting oxidative respiration (Khan et al. 2023).

4.3. NPs and Heavy Metal Stress

Soil contaminated with heavy metals as a result of rapid industrialization and anthropogenic activities leads to environmental problems and toxic effects on plants (Jorjani and Karakas 2024). The accumulation of heavy metals such as arsenic (As), aluminum (Al), cadmium (Cd), chromium (Cr), lead (Pb), mercury (Hg), and beryllium (Be) leads to morpho-physiological abnormalities such as reduced photosynthesis, plant growth, biomass, and yield (Ahmad et al. 2023); triggers ROS production, disrupting cellular redox homeostasis and causing damage to the plasma membrane, proteins, lipids, and nucleic acids, ultimately impairing metabolic activities (Faizan et al 2023). Therefore, enhancing plant tolerance to heavy metal stress is extremely important. The application of NPs under heavy metal stress aids in reducing heavy metal concentrations in the soil, modulating the expression of heavy metal transporter genes, boosting antioxidant system activity, enhancing physiological processes, and promoting the synthesis of protective compounds (El-Saadony et al. 2022). Several NP types, such as metallic, carbon-based, inorganic nonmetallic NPs, and organic polymeric materials are advanced depending on their use and application. To improve plant development, raise the phytoavailability of pollutants, and remove harmful compounds from contaminated soil, phytoremediation, a plant-based technique for eliminating soil impurities, involves plant-NP interaction (Singh and Lee 2016). TiO_2, nanoscale hydroxyapatite, nZVI, salicylic acid, fullerene, silicon, ZnO, and Ag NPs are commonly applied in nano-phytoremediation to mitigate the harmful effects of various heavy metals (Prakash and Smitha 2023).

5. NP-MEDIATED GENETIC MODIFICATION AND GENE TRANSFER

Plant genetic engineering represents a pivotal technique for enhancing yield, quality, and stress resilience in sustainable agricultural practices. Genetic transformation plays a pivotal role in functional genomics and molecular breeding within plant science research (Steinwand and Ronald 2020). The development of safe and effective transformation methods is critical for genetic engineering and breeding (Jiang et al. 2024). However, current technologies have yet to provide sufficiently effective and species-independent methods to overcome the cell wall barrier, making the genetic transformation of certain plant species still unachievable (Yan et al. 2022). Recently, a new gene transfer system using NPs has been developed, enabling high transformation efficiency without the need for physical or chemical tools. This innovation offers a more efficient and practical solution for plant genetic engineering (Jat et al. 2020).

Nanotechnology-based applications offer reliable and cost-effective techniques for delivering genes and other molecules to plants with high efficiency and low toxicity (Chandrasekaran et al. 2020). NPs facilitate the effective transport of genetic material because of their small size, biocompatibility, and customizable surface properties (Komarova et al. 2023; Zhi et al. 2024). They protect nucleic acids, enhance cellular uptake, and improve transfection efficiency (Ahmar et al. 2021). Advancements in nanotechnology have led to the development of a range of nanocarriers that can be used to deliver plasmid DNA and siRNA into plants. Moreover, nanocarriers can facilitate the delivery of CRISPR-Cas9 gRNA, which was developed as a powerful and flexible genetic editing device to generate targeted DNA double-stranded breaks, making nanocarrier-mediated delivery as an essential tool for plant genetic engineering (Demirer et al. 2021; Kumari et al. 2023).

NPs used in gene transfer are classified based on their core materials: carbon, silicon, metallic, and polymeric NPs. Each type has specific capabilities for carrying genetic material. For instance, carbon nanotubes (CNTs) can transport both RNA and DNA, while metallic NPs can only carry DNA (Bates and Kostarelos 2013; Zhao et al. 2017). Silicon-based NPs deliver DNA and proteins, whereas polymeric NPs facilitate the transfer of encapsulated RNA, DNA, and proteins (Moon et al. 2016; Zhou et al. 2018). The first nanoparticle-based plant genetic engineering studies were concentrated in plant cell cultures. Among these studies, transformation methods using silicon carbide (SiC) have successfully provided DNA delivery in different plants such as tobacco, maize, rice, soya bean and cotton (Sanzari et al. 2019). The delivery of double-stranded RNA molecules to plants loaded with non-toxic and biodegradable materials provided protection against viruses such as Cauliflower Mosaic Virus (CMV) in tobacco leaves (Mitter et al. 2017), indicating that an important method for delivering genetic material to plants without the introduction of transgenes has been developed. Despite the revolutionary potential of nanoparticle (NP)-mediated genetic transformation in plants, there are certain drawbacks (Deng et al. 2019; Landry and Mitter 2019). Nanophytotoxicity, or the detrimental effects of NPs on plant growth and the environment, is one of the primary issues (Cox et al. 2016). Research has shown that the absorption of NPs by plants can result in vascular system blockage and phytotoxicity, which causes oxidative stress and structural damage to plant DNA (Pachapur et al. 2016; Du et al. 2017; Rastogi et al. 2017). On the other hand, the potentially unpredictable effects of nanoscale structures on beneficial insects, undesirable organisms and soil microbiota are of concern for ecosystem balance and biodiversity (Ameen et al. 2021; Khanna et al. 2021; Mubeen et al. 2023). Another challenge in NP-mediated genetic transformation is related to the effective binding of biomolecules to NPs (Saptarshi et al. 2013; Fleischer and Payne 2014). The binding affinity of different biomolecules with NPs varies depending on their structural characteristics, charge, chemical composition and surface area (Ahmar et al. 2021) The high oxidative properties of some types of NPs disrupt normal cell metabolism and interfere with the genetic regulation of plant cells, causing oxidative bursts in transformed cells (Hossain et al. 2015; Du et al. 2017).

6. NP-MEDIATED *IN VITRO* SECONDARY METABOLITE PRODUCTION

In vitro tissue culture techniques, a fundamental application of plant biotechnology, enable the production of cells, tissues, plants, or plant-derived products from whole plants or specific plant parts under sterile conditions in an artificial medium (Thorpe 2007). These methods have enabled secondary metabolite production in a wide range of plants (Hashim et al. 2021). Numerous factors, such as genotype, explant type, physiological state, disinfection techniques, culture medium, plant growth regulators, light, photoperiod and temperature, affect the success of *in vitro* culture (Rahmawati et al. 2022). Secondary metabolites, known for their defensive properties and pharmaceutical potential, are typically synthesized by plants in response to various elicitors or inducer molecules (Hatami et al. 2019). Elicitors are added to enhance secondary metabolite production by triggering plant stress responses. These molecules interact with membrane receptors, activating gene expression related to secondary metabolite synthesis (Nabi et al. 2021). Today, both biotic (yeast, bacteria, fungi, proteins) and abiotic elicitors (heavy metals, salt, UV radiation, jasmonic acid, salicylic acid, ethylene) are widely used to enhance secondary metabolite production and increase cell volume in suspension cultures (Narayani and Srivastava 2017; Twaij et al. 2019; Bhaskar et al. 2021). NPs have emerged as promising abiotic elicitors in plant biotechnology, capable of inducing secondary metabolite biosynthesis. Recent studies have increasingly focused on the nano-eliciting role of NPs in promoting secondary metabolite production in plant cell and tissue cultures, highlighting their potential for innovative applications in this field (Cessur et al. 2023). Although the precise mechanism remains unclear, NPs are thought to enhance metabolite production by inducing ROS and secondary signaling messengers, which regulate transcription in plant secondary metabolism (Prasad et al. 2024). Both ROS and calcium ions play crucial roles as signaling molecules, upregulating transcriptional regulators involved in secondary metabolite biosynthesis (Anjum et al. 2019). The activation or suppression of oxidative stress varies depending on plant species (Hasanuzzaman et al. 2020), tissue type and developmental stage (Vannini et al. 2013), as well as the nature of stressors (Tassi et al. 2017). Studies have shown that NP applications induce oxidative stress by promoting ROS accumulation in plant cells, which in turn triggers direct and indirect modifications in secondary metabolite production (Marslin et al. 2015). The effect of NPs has significant implications for biotechnology, contributing to research aimed at increasing the production of valuable secondary metabolites from medicinal plants (Anjum et al. 2021; Rivero-Montejo et al. 2021).

7. CONCLUSION

NPs offer significant potential for applications in the field of agricultural biotechnology with their superior properties and innovative solutions due to their nanometric dimensions and large surface/volume ratios. These small-sized materials can be successfully used in agricultural biotechnology, with diverse applications including promoting plant growth, managing diseases and pests, enhancing pesticide efficiency, improving soil fertility and sustainability, increasing plant secondary metabolite production, and eliminating the disadvantages of traditional gene transfer techniques. Although significant scientific advances have been made in the use of NPs, thoroughly investigating their potential environmental and health impacts and aligning these technologies with local and global regulatory frameworks are crucial importance to mitigate associated risks. Unquestionably, the significance and application areas of nanotechnology, which is widely recognized as the technology of the future and offers a variety of applications today due to the various approaches and innovations it brings to the solution of issues encountered in the field of agricultural biotechnology, will progressively grow. The full potential of nanotechnology offers in agricultural biotechnology can only be fully utilized and developed into an effective instrument for sustainable agricultural practices when these advancements are realized as a result of thorough and interdisciplinary study.

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THE USE OF MOLECULAR MARKERS IN REPRODUCTION IN SHEEP BREEDING[1]

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1. INTRODUCTION

Reproductive efficiency is one of the most important factors influencing profitability in sheep breeding. Reproductive performance is influenced not only by genetic structure but also by environmental factors. Improving reproductive efficiency through traditional methods may require long processes and high costs. Therefore, the development of molecular biotechnology and genetic sciences in recent years has made it possible to use molecular markers to improve reproductive traits in sheep. Molecular markers are DNA sequences that identify genetic variations, and they enable faster and more accurate determination of the genetic basis of reproductive traits. The most important characteristics related to reproductive performance in sheep include litter size, lambing interval, lambing rate, and twinning. These traits are typically polygenic and are also influenced by environmental factors. However, certain genetic variants play a significant role in some of these traits. For example, genes such as BMP15, GDF9, and BMPR1B are key genes related to fertility in sheep. In addition, genes such as FETUB, HIRA, PTX3, HRG, COIL, SLK, GRM1, RXRG, B4GALNT2, and CLSTN2 also affect ovulation, follicle development, and the number of offspring per birth. Specific mutations in these genes can increa-

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se twinning rates and improve reproductive performance. The identification of mutations in these genes using molecular markers facilitates the selection of animals with high fertility. The use of molecular markers in sheep enables faster and more effective selection for reproductive traits. This approach, known as genomic selection, makes more accurate predictions by using genetic information, unlike traditional methods that rely solely on phenotypic data. As a result, genetically superior individuals can be identified at an earlier age and included in breeding programs. At the same time, individuals with undesirable genetic traits can be eliminated. Thus, the overall reproductive performance of the flock is improved, and the production process is optimized. In conclusion, the application of molecular markers in sheep breeding has the potential to increase productivity. A better understanding of genetic variations and integrating this knowledge into selection programs allows for faster progress. This study compiles research on reproductive-related molecular markers and their potential applications in sheep farming.

2. SHEEP BREEDING

Livestock production, including meat and dairy, provides numerous benefits such as a source of income, social status, and control over weeds, for people involved in this sector. Livestock farming, often a primary livelihood in rural areas, is one of the most important branches of agriculture. Small ruminants, such as sheep, which belong to the suborder of ruminants, have a history that dates back to the Neolithic period. For breeders, sheep are easier to manage, produce, and convert into economic gain compared to other livestock, playing a crucial role in ensuring livelihood and food security for farmers and countries involved in livestock activities. Sheep can live and maintain reproductive performance in challenging environments, such as hot, dry, and humid conditions, depending on their breed-specific characteristics (D'Silva, 2019; Kaymakçı and Taşkın, 1997; Rout and Behera, 2021).

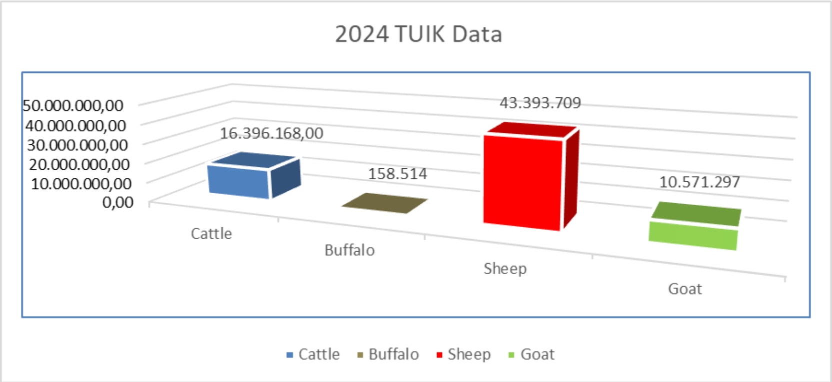

Figure 1. Livestock numbers (TUIK, 2024)

According to the data released by the Turkish Statistical Institute (TUIK) for 2024, the sheep population in Turkey is 43 million 393 thousand. Of the produced red meat, 70.1% comes from cattle, 23.9% from sheep, 5.4% from goats, and 0.6% from buffaloes, indicating that sheep farming plays a significant role in red meat production.

As in all livestock farming, one of the primary principles in sheep farming is the productivity per sheep. Since lamb production is the most important source of profit in sheep farming, it is desirable to increase the number of lambs per ewe. Both genetic and non-genetic factors influence reproductive traits. Non-genetic factors include lambing age, care, nutrition, seasons, climate, and geographical conditions. Genetic factors, on the other hand, involve reproductive data, parameters, and the ability to perform genotyping. Key parameters such as ovulation rate and the number of offspring are crucial for determining economically important sheep with the desired reproductive traits (Assan, 2020; Hameed Ajafar, Hasan Kadhim, and Mohammed Al-Thuwaini, 2022).

In sheep farming, the morphological and physiological characteristics of breeds are not well understood, and there is insufficient data on their genotypes, distributions, and the characteristics they carry (Ertuğrul et al., 2009).

Fertility is an important reproductive ability in female sheep and is one of the essential traits for the sustainability of sheep farming. Ovulation rate, the number of ovulations, and the number of lambs per lambing are key parameters for fertility. Since the heritability of these traits is relatively low, genetic progress and classical breeding processes are extended. As a result, this increases essential input costs, such as labor and feeding, in sheep farming. Modern breeding approaches can increase the rate of genetic gain in breeding programs by shortening the breeding cycle, improving precision in selection, and utilizing genetic diversity more efficiently. To accelerate genetic progress and minimize costs, the integration of available data using molecular techniques such as Marker-Assisted Selection (MAS) is required (Abdoli et al., 2016; Öner et al., 2011; Pramod et al., 2013).

Molecular markers are methods that reveal the differences in DNA sequences within an organism's genome. They play a structural and functional role in understanding the genomic structure of animals, plants, and microbial species (Gürses and Bayraktar, 2014).

In general, a marker must be polymorphic, meaning it should exist in different forms so that chromosomes carrying the mutant gene can be distinguished from chromosomes carrying the normal gene based on the form of the marker. Polymorphism in a marker can be detected in three ways: at the phenotypic level (morphological marker), through differences in proteins (biochemical markers), or by differences in the nucleotide sequence of DNA (molecular markers) (Filiz and Koç, 2011).

Various molecular markers exist, including:

RFLP (Restriction Fragment Length Polymorphism): DNA fragments cut by restriction enzymes are transferred onto a membrane after gel electrophoresis, and hybridization is performed using labeled probes. Polymorphisms are detected based on different DNA fragments caused by mutations. RFLP markers exhibit high polymorphism, are codominant, and have high reproducibility. They are used in genetic diversity determination, gene mapping, phylogenetic, and taxonomy studies (Filiz and Koç, 2011; Young et al., 1992).

SSR (Simple Sequence Repeats): These markers consist of repeating 1–6 base pair sequences in the DNA. Primers are designed using the sequences surrounding the microsatellites. Due to the frequent mutations occurring during DNA replication, changes occur in the primer binding regions. Polymorphisms are detected in population genetics and gene mapping studies after PCR and gel electrophoresis. These markers require small amounts of DNA material, are codominant, abundant in the genome, and highly repeatable (Freudenreich et al., 1997; Matsuoka et al., 2002).

ISSR (Inter Simple Sequence Repeat): Primers that amplify regions between microsatellites consist of repeating nucleotides in 2, 3, 4, or 5 units. The high GC content of the primers results in higher annealing temperatures. Amplified PCR products range from 200 to 2000 base pairs. After electrophoresis in agarose gel, the bands are stained with ethidium bromide. ISSR is a dominant marker with high polymorphism (Joshi et al., 2000; Zietkiewicz et al., 1994).

SNP (Single Nucleotide Polymorphism): This marker identifies differences between individuals by changes in a single base within a specific DNA segment. The human genome, for example, consists of approximately 2.9 billion base pairs, 99.9% of which are identical. The remaining 0.1% variation is known as genetic diversity. SNP frequency greater than 1% in a population is classified as an SNP, while mutations with a frequency lower than 1% are considered as mutations (Heaton et al., 2002).

SNPs are found in exons, introns, and promoter regions. SNPs located in coding regions can alter the structure of proteins. SNPs are a very common type of DNA polymorphism and occur frequently (1/1000 base pairs to 1/100-300 base pairs) (Vignal et al., 2002).

Molecular marker technology is an integral part of Agricultural Biotechnology and is of great importance for the development of agriculture. Molecular breeding is an approach in which tightly linked molecular markers are used to define the desired traits in breeding populations. Marker-Assisted Selection (MAS) is a novel and intelligent breeding technique based on using a molecular marker linked to a target gene or trait to select for both quantitative and qualitative characteristics and to identify high-performance animals (Singh and Upadhyay, 2016).

Sheep are seasonally polyestrous animals, and the physiological events of their reproductive cycle consist of several stages. Ovulation, the number of follicles, and the ovulation rate are crucial parameters that affect the lambing performance of the ewe. Fertility is expressed through Fec genes, and it is genetically regulated by major effect genes and many other genes with smaller effects (Akal and Akdağ, 2018; Tanış, 2021; Türk, Güngör, and Cihangiroğlu, 2022).

However, in addition to these markers, investigating other genes and mutations that affect the reproductive systems across various sheep breeds will further assist in understanding sheep breeding and reproductive systems.

3. REPRODUCTIVE EFFICIENCY IN SHEEP BREEDING: THE ROLE OF MAJOR GENES AND MOLECULAR MARKERS

In sheep farming, reproductive efficiency is one of the most important economic factors and is influenced by both genetic structure and environmental factors. Improving reproductive efficiency using traditional methods is a challenging, time-consuming, and costly process. Recent advancements in molecular biotechnology have mitigated the impact of these negative factors, making the use of molecular markers a viable alternative. Reproductive efficiency in sheep encompasses key traits such as follicular development, ovulation, the number of lambs born per litter, and multiple births. These traits are influenced by major genes such as BMP15, GDF9, and BMPR1B, which play a significant role in reproductive performance.

In addition to these, several genes that affect reproduction in sheep have been elucidated through research. Genes such as FETUB, HIRA, PTX3, HRG, COIL, SLK, GRM1, RXRG, B4GALNT2, and CLSTN2 impact fertility, litter size, and follicular rate, and are increasingly used in breeding programs to improve reproductive traits (Davis, 2005; Hameed Ajafar, Hasan Kadhim, and Mohammed Al-Thuwaini, 2022).

The primary aim of this seminar paper is to conduct a literature review on the application of molecular markers related to reproduction in sheep farming.

4. MAJOR GENES AFFECTING REPRODUCTION IN SHEEP

Reproductive traits in sheep have low heritability, which makes them difficult to select for using phenotypic methods. To address this challenge in sheep farming, genes that influence ovulation rate and litter size have been identified through studies using molecular markers. Among these, the GDF9, BMP15, and BMPR1B genes and their mutations have been shown to be the major genes affecting fertility, sterility, and traits such as lambing rate and ovulation rate (Davis, 2005).

4.1. BMP15 (Bone Morphogenetic Protein 15) GENE

This gene, also known as FecX, is part of the TGF-β (Transforming Growth Factor Beta) family and is one of the key genes responsible for reproduction in sheep. It is expressed in oocytes and promotes the development and growth of granulosa and cumulus cells (Davis, 2005; Wang et al., 2023). Located on the X chromosome (NCBI Reference Sequence NC_056080), it is 6,681 base pairs long and consists of two exons and 393 amino acids (Figure 2.1).

In sheep worldwide, eight different mutations have been identified in the BMP15 gene, which are: FecXI, FecXG, FecXI, FecXH, FecXB, FecXL, FecXR, FecXGr, and FecXO. Heterozygous sheep carrying one allele of this gene show an increase in lambing rate. However, homozygous individuals exhibit infertility due to dysfunction in ovarian function and a lack of follicular development (Aymaz, Özdil, and Yaman, 2024; Galloway et al., 2002).

A study conducted on Lacaune sheep identified the c.963G>A SNP mutation, resulting in a cysteine-to-tyrosine amino acid change (p. Cys321Tyr). This mutation, known as FecXL, has been associated with high lambing performance due to its effects on ovarian folliculogenesis and ovulation rate control (Bodin et al., 2007).

Another study comparing high-yielding and normal-yielding sheep breeds, such as French Grivette and Polish Olkuska, through a GWAS analysis determined allele frequencies and performed genotyping. In both homozygous breeds, FecX Gr and FecX O mutations were observed, and it was reported that homozygous genotypes (FecX Gr/FecX Gr and FecX O/FecX O) were not infertile and could produce offspring (Demars et al., 2013).

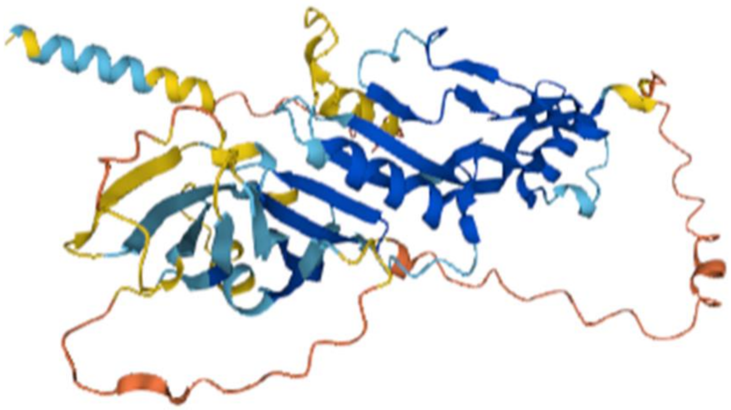

Figure 2. Protein Structure of BMP15 Gene

4.2. GDF9 (Growth Differentiation Factor 9) GENE

The Growth Differentiation Factor 9 (GDF9) gene in sheep encodes a protein belonging to the Transforming Growth Factor Beta (TGF-β) superfamily. It is located on chromosome 5 in sheep (NCBI Reference Sequence: NC_056058.1) and spans 2,727 base pairs, consisting of two exons and 453 amino acids. GDF9 plays a critical role in regulating follicular development and maturation in the ovaries, which is essential for female reproductive systems in sheep and other mammalian species (Davis et al., 2002; Sadighi, Bodensteiner, Beattie, and Galloway, 2002).

The expression of this gene has significant effects on oocyte development. A mutation in the GDF9 gene, known as FecGH, results in a serine-to-phenylalanine amino acid change due to a C>T substitution. Mutations in GDF9 have been shown to have substantial impacts on fertility, particularly increasing twinning rates, which has drawn attention to molecular studies in sheep breeding. Research has shown that mutations in the GDF9 gene, compared to BMP15, result in a greater increase in ovulation rates. Additionally, in Cambridge and Belclare breeds, sheep carrying one allele of FecGH exhibit an approximately

1.4-fold increase in ovulation rate (Çelikeloğlu et al., 2021; Gürsel, 2009).

In studies conducted in Australia and New Zealand, the effects of GDF9 gene mutations on fertility were examined, and the FecG mutation was found to increase follicle development, ovulation rate, and twinning rates (Davis et al., 2002). In Turkey, the effects of the GDF9 gene on fertility were studied in the local Çepni and Of sheep breeds, and five known SNPs (c471C>T (G2), c477G>A (G3), c721G>A (G4), c978A>G (G5), and c994G>A (G6)) were identified. These variants were shown to influence reproductive performance (Figure 2.2).

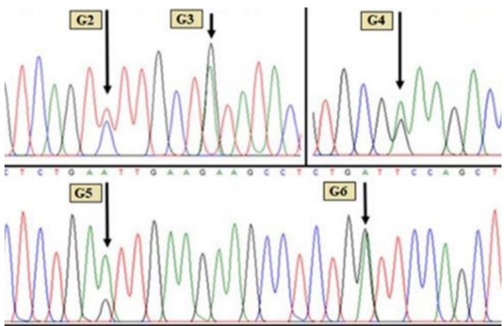

Figure 3. SNPs Investigating the Effects of GDF9 Gene on Fertility (Kirikçi, 2023).

4.3. BMPR-1B (Bone Morphogenetic Protein Receptor 1B) GENE

The first discovered gene affecting reproduction in sheep is the **Booroola Gene**, also known as **FecB** or **BMPR-1B**. It was named after mutations identified in the Booroola breed of sheep in Australia. This gene is located on chromosome 6 and spans 3,234 base pairs, consisting of 12 exons and 11 introns. It encodes the bone morphogenetic protein receptor 1B, which is expressed in the ovaries. The mutation of this gene in sheep causes heterozygous individuals to experience 1.5 times the ovulation rate and an additional lamb per birth. Homozygous individuals experience a threefold increase in ovulation rate and an average of 1.5 additional lambs per birth (Bodin et al., 2002; Davis, 2005).

In studies conducted on native breeds in Turkey, such as Ramlıç and Dağlıç sheep, the BMPR1B gene's effect on lambing rate was examined. In Ramlıç sheep, four SNPs in the BMPR1B gene (g.49496G>A, c.1658A>C, c.2037C>T, c.2053C>T) were found to have a significant impact on lambing rate, while in Dağlıç sheep, five SNPs (c.1487C>A, c.2492C>T, c.2523G>A, c.2880A>G, and c.2763G>A) were associated with significant effects on lambing rate, suggesting that BMPR1B could be used as a genetic marker (Çelikeloğlu et al., 2021).

In Mongolia, mutations in the BMPR-1B, GDF9, BMP15, LEPR, and B4GALNT2 genes have been reported to affect lambing rates in the Ujim sheep breed. Additionally, 13 new mutations were discovered in an F1 population derived from crossing Ujim sheep with Dorper and Suffolk breeds. The c.746A>G mutation in the BMPR-1B gene was found to have a significant effect on lambing rate and could serve as a genetic marker in Marker-Assisted Selection (Figure 2.3) (Ji et al., 2023).

Sheep Population	Mutation	Genotype	Number	Litter Size
Ujimqin	c.746A>G (Gln249Arg)	AA	320	1.18 ± 0.02^{a}
		AG	5	1.60 ± 0.24^{b}
	c.1470G>T (490Thr)	AA	45	1.20 ± 0.05
		AC	155	1.17 ± 0.03
		CC	116	1.21 ± 0.04
Dorper × Ujimqin F1	c.746A>G (Gln249Arg)	AA	300	1.40 ± 0.03^{a}
		AG	4	2.00 ± 0.00^{b}
	c.1470G>T (490Thr)	AA	12	1.25 ± 0.13
		AC	111	1.41 ± 0.05
		CC	181	1.42 ± 0.04
Suffolk × Ujimqin F1	c.1470G>T (490Thr)	AA	14	1.50 ± 0.14
		AC	40	1.63 ± 0.08
		CC	12	1.28 ± 0.06

Note: ^{a,b}: $p < 0.05$.

Figure 4 Effects of BMPR1B mutations on lambing rate in sheep (Ji et al., 2023).

5. OTHER GENES AFFECTING REPRODUCTION IN SHEEP

Initially, the genetic basis of reproduction in sheep was explained by major genes. It was observed that sheep breeds associated with these genes exhibited high fertility. However, due to the relatively small number of sheep possessing these genes, further research has been prompted to investigate and genetically explain variations in certain reproductive systems. Some of these explanations have been linked to biochemical changes in oocytes, embryos, and follicles. These biochemical alterations significantly influence fertility, lambing rate, and the incidence of multiple births.

5.1. FETUB (Fetuin-B) GENE

The FETUB gene is a plasma protein that belongs to the cystatin superfamily of protease inhibitors produced in the liver. In mammals, oocytes are covered by a glycoprotein structure called Zona Pellucida, which can harden around the oocyte, preventing sperm from binding during fertilization. In sheep, the FETUB gene is located on chromosome 1 and consists of seven exons, spanning 14,874 base pairs. It has been shown that FETUB levels significantly affect fertilization rates. FETUB is considered an energy metabolism gene that plays a crucial role in the reproduction of sheep. In studies conducted on various sheep breeds such as Small Tailed Han, Hu, Cele Black, Sunite, and Bamei, the g.421655951C>T SNP was found to significantly influence lambing rate (Dietzel et al., 2013; Kralisch et al., 2017; Ren, He, Wang, and Chu, 2024).

Locus	Genotype	Polytocous genotype frequency	Monotocous genotype frequency	$\chi 2$ (p value)	Allele	Polytocous allele frequency	Monotocous allele frequency	$\chi 2$ (p value)
HRG	GG	0.08	0.06	0.00	G	0.18	0.21	0.24
g.405442728A > G	GA	0.22	0.30		A	0.82	0.79	
	AA	0.70	0.64					
FETUB	CC	0.90	0.90	0.00	C	0.95	0.95	0.92
g.421655951C > T	CT	0.09	0.10		T	0.05	0.05	
	TT	0.01	0.00					
GUCY1A1	GG	0.42	0.97	0.00	G	0.70	0.98	0.00
g.414050897G > C	GC	0.56	0.03		C	0.30	0.02	
	CC	0.02	0.00					

Figure 5. Relationship analysis between FETUB polymorphism and lamb size (Ren et al., 2024).

5.2. HIRA (Histone Cell Cycle Regulator) GENE

The HIRA (Histone Cell Cycle Regulator) gene, located on chromosome 17 of sheep, encodes a histone chaperone that consists of 27 exons and 1,091 amino acids. This gene is involved in various chromatin regulatory processes such as sperm chromatin remodeling and embryonic development. It is essential for embryonic development in vertebrates, and mutations in HIRA can lead to embryonic death. In studies on Small Tailed Han sheep, the g.71874104G>A and g.71833755T>C SNPs were found to be significantly associated with lamb size (Zhou et al., 2018).

Locus	Genotype	Litter Size of the First Parity	Litter Size of the Second Parity
g.71874104G>A	GG	1.97 ± 0.13 (35)	2.24 ± 0.13 (41)
	GA	2.19 ± 0.06 (154)	2.39 ± 0.07 (163)
	AA	2.11 ± 0.06 (141)	2.11 ± 0.07 (159)
g.71833755T>C	TT	2.16 ± 0.06 (193)	2.13 ± 0.06 ^{b} (211)
	CT	2.13 ± 0.07 (115)	2.45 ± 0.08 ^{a} (124)
	CC	2.04 ± 0.15 (27)	2.28 ± 0.15 ^{ab} (32)

Figure 6. Analysis of lamb size for small tailed Han sheep with different g.71874104G>A and g.71833755T>C genotypes (Zhou et al., 2018).

5.3. PTX3 (Pentraxin 3) GENE

The PTX3 gene in sheep encodes a glycoprotein produced by granulosa cells that surround the oocyte. Granulosa cells play a vital role in the growth and development of the oocyte. PTX3 is a member of the pentraxin superfamily and is located on chromosome 1 in sheep (NCBI Reference Sequence NC_056065.1), consisting of three exons. In studies on mice, deletion of the PTX3 gene led to infertility, and in humans, variations in the RS 6788044 SNP were found to affect fertility. In Awassi and Hamdani sheep, the GG and GT genotypes were identified, and the g.22645332G>T SNP was found to be associated with increased lambing rate in GT genotype sheep (Imran and Al-Thuwaini, 2024; May et al., 2010; Salustri et al., 2004).

BREED	NUMBER OF RECORDS	GENOTYPES	DAYS TO LAMBING	LITTER SIZE	LAMB WEIGHT AT BIRTH (KG)
Awassi	74	GG	172[a] ± 7.41	1.41[b] ± 0.11	3.51[b] ± 0.05
	56	GT	159[b] ± 8.30	1.75[a] ± 0.08	3.64[a] ± 0.08
		P value	**.01**	**.01**	**.01**
Hamdani	41	GG	173[a] ± 7.97	1.24[b] ± 0.14	3.67[b] ± 0.06
	29	GT	161[b] ± 8.46	1.62[a] ± 0.09	4.04[a] ± 0.05
		P value	**.03**	**.001**	**.02**

The *P* value with statistical significance is indicated in bold numbers.
[a,b]Significant differences in means are represented by differences in the same column within each classification.

Figure 7. Relationship between PITX3 polymorphism and reproductive performance in Awassi and Hamdani Sheep (Imran and Al-Thuwaini, 2024).

5.4. HRG (Histidine Rich Glycoprotein) Gene

Histidine-rich glycoprotein (HRG) is a 75 kDa glycoprotein synthesized in the liver and circulates in the plasma. It is a versatile molecule that interacts with many gametes. It has been observed that HRG is abundant in follicular fluid, present in the female reproductive tract, and plays a significant role in maintaining pregnancy, promoting lactation, and overall reproduction. The SNP at the g.405442728A>G locus of the HRG gene has been associated with significant effects on lambing performance in the Small Tail Han sheep. The HRG gene is located on chromosome 1 in sheep (NCBI Reference Sequence NC_056054.1), consists of 8 exons, and has a length of 12,630 bp (Nordqvist, Karehed, Stavreus-Evers, & Akerud, 2011; Ren, He, Wang, & Chu, 2024).

Locus	Genotype	Polytocous genotype frequency	Monotocous genotype frequency	χ2 (*p* value)	Allele	Polytocous allele frequency	Monotocous allele frequency	χ2 (*p* value)
HRG g.405442728A > G	GG	0.08	0.06	0.00	G	0.18	0.21	0.24
	GA	0.22	0.30		A	0.82	0.79	
	AA	0.70	0.64					
FETUB g.421655951C > T	CC	0.90	0.90	0.00	C	0.95	0.95	0.92
	CT	0.09	0.10		T	0.05	0.05	
	TT	0.01	0.00					
GUCY1A1 g.414050897G > C	GG	0.42	0.97	0.00	G	0.70	0.98	0.00
	GC	0.56	0.03		C	0.30	0.02	
	CC	0.02	0.00					

Figure 8. Correlation Analysis of HRG Gene Polymorphism and Lamb Size (Ren et al., 2024).

5.5. COIL (Coilin) Gene

Small nuclear ribonucleoproteins, synthesized by the Cajal bodies, play a crucial role in maintaining homeostasis within these bodies. The COIL gene, which regulates the translation process of proteins, impacts cellular pathways by affecting the regulation of the reproductive system. It has been reported that COIL enhances the proliferation and activity of sheep fibroblasts, and its silencing significantly reduces oocyte numbers, fertilization capacity, and the number of offspring, suggesting its importance in fertility and reproduction. In a study conducted on native sheep breeds in the Xinjiang region of China, it was found that this gene could influence ovulation, embryonic development, and the number of lambs born. The study also suggests that COIL could affect reproductive traits in sheep, including ovulation and embryonic development (Gall, Bellini, Wu, & Murphy, 1999; Cao, Wen, Ma, & Liu, 2024).

Population	Trait	Genotype		
		CC	*CG*	*GG*
Hetian duotai hong sheep	Average number of lambs	1.60 ± 0.55^{a}	1.39 ± 0.54^{ab}	1.17 ± 0.40^{b}
Cele black sheep	Average number of lambs	1.65 ± 0.48^{a}	1.39 ± 0.49^{b}	1.50 ± 0.71^{ab}
Duolang sheep	Average number of lambs	1.15 ± 0.37^{b}	1.35 ± 0.49^{b}	1.80 ± 0.45^{a}

^{a,b} Within a row, means sharing different superscript letters differ significantly ($p < 0.05$).

Figure 9. Correlation analysis between COIL genotypes and lamb number (Cao et al., 2024).

5.6. SLK (STE20 Like Kinase) Gene

SLK, a member of the Ste20 serine/threonine protein kinase family, plays a key role in controlling the cell cycle, apoptosis, and many essential cellular processes. It is more highly expressed in reproductive organs such as the uterus and ovaries. SLK is critical for embryonic development, and its suppression can lead to embryonic death. In sheep, this gene is located on chromosome 22 (NCBI Reference Sequence NC_056075.1), consists of 19 exons, and has a length of 61,501 bp. SLK is widely expressed in cell lines and developing mouse embryos, and studies on Suffolk, Duolang, and Hu sheep have shown that the g.27108855 G>A polymorphism in this gene affects reproductive performance and the number of offspring (Tao, Li, Liu, Ma, & Liu, 2024).

Locus	Sheep breeds	Genotype frequency			*p*-Value	Gene frequency		Ho	He	Ne	PIC
		TT	TC	CC		T	C				
g.27107842	Duolang	1	—	—	—	1	—	1	—	1	—
	Suffolk	0.602	0.269	0.129	0.323	0.737	0.263	0.612	0.388	1.634	0.313
	Hu	0.862	0.138	—	0.339	0.931	0.069	0.872	0.128	1.147	0.120
		GG	GA	AA		G	A				
g.27108855	Duolang	0.479	0.260	0.260	0.041	0.609	0.391	0.524	0.476	1.909	0.363
	Suffolk	0.707	0.293	—	0.099	0.853	0.147	0.750	0.250	1.334	0.219
	Hu	0.693	0.253	0.054	0.061	0.819	0.181	0.704	0.296	1.421	0.252

Figure 10. Genetic diversity analysis of SLK gene locus (Tao et al., 2024).

5.7. GRM1 (Glutamate Metabotropic Receptor 1) Gene

The GRM1 gene codes for the metabotropic glutamate receptor type 1, a transmembrane protein expressed on pre-synaptic and post-synaptic neuronal membranes. This protein triggers the release of Ca^{2+} ions inside the cell and regulates neuronal excitation. In sheep, the gene is located on chromosome 8 (NCBI Reference Sequence NC_056061), consists of 10 exons, and has a length of 434,402 bp. It plays a role in follicular development, influencing neural ligand-receptor interactions that affect the release of GnRH and estrus. Studies conducted on Kazakh and Chinese Merino sheep have indicated that this gene is involved in lambing traits and is associated with the control of offspring number (Bhardwaj, Ryan, Wong, & Srivastava, 2015; Rondard & Pin, 2015; Zhu et al., 2022).

Gene locus	Breed	Genotype	litter size	Seasonal reproduction
GRM1	KS	TT	1.013 ± 0.032b	1.001 ± 0.03b
		TC	1.029 ± 0.126b	1.038 ± 0.186b
		CC	1.292 ± 0.283a	1.149 ± 0.261a
	CS	TT	1.091 ± 0.021b	1.031 ± 0.009b
		TC	1.098 ± 0.661b	1.046 ± 0.348b
		CC	1.391 ± 0.164a	1.390 ± 1.101a
	HS	TT	2.018 ± 0.332b	1.767 ± 0.549b
		TC	2.220 ± 0.224a	1.912 ± 0.641a
		CC	2.010 ± 1.010b	1.791 ± 1.149b

Figure 11. SNPs in the GRM1 gene and their relationship to lambing (Zhu et al., 2022).

5.8. RXRG (Retinoid X Receptor Gamma) Gene

The RXRG gene, located on chromosome 1 in sheep (NCBI Reference Sequence NC_056054.1), consists of 11 exons and has a length of 52,605 bp. Retinoic acid, a fat-soluble small molecule, is a major regulator of cell differentiation and tissue morphogenesis. As a member of the steroid/thyroid hormone receptor superfamily, RXRG regulates fetal development and reproduction in humans and animals. In a study involving Luxe, Chinese Simmental, Angus, and Simmental x Mongolia cattle, moderate polymorphism was identified in Luxe cattle associated with twin-bearing traits. Additionally, in studies on Chinese Merino, Hu, and Kazakh sheep, a relationship between the RXRG gene and twin-bearing traits was found, where individuals with the B allele exhibited higher twinning rates (Huang et al., 2008; Zongsheng et al., 2018).

5.9. B4GALNT2 (Beta -1,4- N- Acetyl-Galactosaminyltransferase 2) Gene

B4GALNT2 encodes an enzyme expressed in the kidneys, intestines, uterine tissue, oviducts, and ovaries. This gene plays a vital role in embryo attachment and development during pregnancy. It is associated with high lambing rates, particularly in French Lacaune sheep, where it is considered a key genetic factor alongside BMP15. The FecLL allele of B4GALNT2 has been reported to increase ovulation rates by 1.5 times when one allele is present, and by 3 times when both alleles are present. Studies on Small Tail Han sheep identified two mutations (g.36946470C>T and g.36933082C>T), which significantly impact the number of offspring (Ben Jemaa et al., 2019; Drouilhet et al., 2010; Guo et al., 2018).

Polymorphic Site	Genotype	Litter Size (Means ± S.E.)			
		First Parity (N)	Second Parity (N)	Third Parity (N)	Total (N)
g.36971115C > T	CC	2.18 ± 0.1 (68)	2.37 ± 0.13 (52)	2.7 ± 0.22 (23)	2.43 ± 0.08 (144)
	CT	2.36 ± 0.2 (11)	2.43 ± 0.37 (7)	3 ± 0 (3)	2.60 ± 0.23 (21)
	TT	1 ± 0 (1)	2 ± 0 (1)	2.00 ± 0 (1)	1.67 ± 0.52 (3)
g.36946470C > T	CC	2.17 ± 0.11 (54) ^{b}	2.32 ± 0.16 (38)	2.87 ± 0.27 (15)	2.45 ± 0.10 (107) ^{b}
	CT	2.32 ± 0.18 (22) ^{b}	2.37 ± 0.19 (19)	2.6 ± 0.26 (10)	2.43 ± 0.13 (51) ^{b}
	TT	1.25 ± 0.25 (4) ^{a}	2.33 ± 0.333(3)	2.00 ± 1 (2)	1.58 ± 0.38 (7) ^{a}
g.36933082C > T	CC	2.08 ± 0.09 (64) ^{a}	2.32 ± 0.14 (47)	2.57 ± 0.2 (21)	2.32 ± 0.09 (132) ^{b}
	CT	2.59 ± 0.24 (17) ^{b}	2.5 ± 0.25 (14)	3.29 ± 0.42 (7)	2.79 ± 0.15 (38) ^{a}
	TT	NA	NA	NA	NA
g.36930089T > G	TT	2.11 ± 0.12 (55)	2.32 ± 0.15 (41)	2.8 ± 0.25 (20)	2.41 ± 0.09 (116)
	TG	2.43 ± 0.15 (23)	2.5 ± 0.2 (18)	2.5 ± 0.22 (6)	2.48 ± 0.15 (47)
	GG	1.67 ± 0.33 (3)	2 ± 0 (2)	3.00 ± 1 (2)	2.22 ± 0.35 (7)

Figure 12. Effect of B4GALNT2 gene on lamb number (Guo et al., 2018).

5.10. CLSTN2 (Calsyntenin 2) Gene

CLSTN2, also known as Alcadein-γ, is located on chromosome 1 in sheep (NCBI Reference Sequence: NC_056054). It consists of 17 exons and is 745,380 bp in length, encoding a 955-amino-acid protein. CLSTN2 is involved in various biological processes such as cell growth, differentiation, apoptosis, and tumor formation. It is expressed in tissues like the brain, kidneys, heart, spleen, liver, ovaries, fallopian tubes, and placenta. CLSTN2 plays a significant role in the secretion of gonadotropins and estrogen levels, and influences follicular growth, ovulation, fertility, and the number of offspring after pregnancy. In studies on Shaanbei White Cashmere goats, a 16 bp insertion-deletion variation was identified that affects offspring number, and in Pelibuey sheep, genome-wide association studies have suggested that CLSTN2 may affect the number of lambs born (Hernández-Montiel et al., 2020; Wijayanti et al., 2023).

SNP ID	Chr	Position (bp)	Gene Name	Gene Description	Traits	Signal Pathway
s71757.1	1	51,963,826	*ST6GALNAC3*	ST6 *N*-acetylgalactosaminide alpha-2 6-sialyltransferase 3	MUSWT, LMYP, BONE_WT, BONEP, FATP [36]	Glycosphingolipid biosynthesis
OAR1_155672687.1	1	189,855,910	*ROBO2*	Roundabout guidance receptor 2	MUSWT, LMYP, BONE_WT, BONEP, FATP [36]	Axon guidance
OAR1_204970872.1	1	189,855,910	*DLG1*	Discs large MAGUK scaffold protein 1	ASREP [37], SAOS [43], LMYP, FATP, BONE_WT, MUSWT [36]	Hippo signaling pathway; tight junction; T-cell receptor signaling pathway
s09883.1	1	246,913,454	*CLSTN2*	Calsyntenin 2	ASREP [37], TFEC_1 [44], FATP [36], FCURV [45]	
OAR2_65914681.1	2	61,498,071	*TRPM6*	Transient receptor potential cation channel subfamily M member 6	HCWT [46], MF [39], MFY, PP, MY [38], BW [36]	Mineral absorption
OAR2_95966123.1	2	89,499,669	*COL11A1*	Collagen type XI alpha 1 chain	SCS [46], PP [38], LATRICH2 [47], HCWT [36], MF [38]	Protein digestion and absorption
OAR3_85112203.1	3	80,398,784	*ABCG5*	ATP-binding cassette subfamily G member 5	INTFAT [36], MCLA [48], SL [49]	ABC transporters; fat digestion and absorption; bile secretion; cholesterol metabolism
OAR3_104545117_X.1	3	98,126,615	*HTRA2*	HtrA serine peptidase 2	FECZ [50], MCLA [48], SL [49], INTFAT [36]	Apoptosis
s62827.1	8	49,878,423	*CGA*	Glycoprotein hormones alpha polypeptide	LATRICH2 [47], INTFAT [36], FECGEN [51]	cAMP signaling pathway; GnRH signaling pathway; ovarian steroidogenesis; prolactin signaling pathway; thyroid hormone synthesis; regulation of lipolysis in adipocytes
OAR8_53593379.1	8	49,981,252	*HTR1E*	5-hydroxytryptamine receptor 1E	LATRICH2 [47], INTFAT [36], FECGEN [51]	cAMP signaling pathway; neuroactive ligand–receptor interaction; serotonergic synapse; taste transduction
OAR9_36598045.1	9	34,604,487	*ATP6V1H*	ATPase H^+ transporting V1 subunit H	HCWT, LMA [36]	Oxidative phosphorylation; metabolic pathways; phagosome; mTOR signaling pathway; synaptic vesicle cycle
OAR15_13905772.1	15	13,872,637	*MTMR2*	Myotubularin related protein 2		Inositol phosphate metabolism; metabolic pathways; phosphatidylinositol signaling system
s07255.1	23	47,438,785	*ST8SIA5*	ST8 alpha-*N*-acetyl-neuraminide alpha-2 8-sialyltransferase 5	IGA [51], FATP, LMYP, HCWT, BW, FATWT [36], MFY, MY [38]	Glycosphingolipid biosynthesis; metabolic pathways
s08197.1	25	40,382,673	*GRID1*	Glutamate ionotropic receptor delta type subunit 1	SL, MFDIAM, CVFD_PRI [49]	Neuroactive ligand–receptor interaction

Figure 13. Genome-wide association of SNPs with reproduction traits (Hernández-Montiel et al., 2020; Wijayanti et al., 2023).

6. CONCLUSION

Reproductive performance is a critical aspect of sheep farming, affecting both productivity and profitability. Traditionally, improving reproductive efficiency has been a challenging task, often requiring long periods of time and significant financial investment. However, with advances in molecular biotechnology and genetic sciences, molecular markers have become an essential tool in enhancing reproductive traits in sheep. These markers allow for the identification of genetic variations associated with reproductive characteristics, enabling more accurate and faster selection processes.

By utilizing molecular markers to detect mutations in these fertility-related genes, breeders can select animals with superior reproductive traits more efficiently. This technique allows for genomic selection, a modern breeding approach that leverages genetic information to make more accurate predictions, in contrast to traditional selection methods based solely on phenotypic traits. Through genomic selection, genetically superior animals can be identified earlier in their lives, allowing them to be incorporated into breeding programs sooner. Additionally, undesirable genetic traits can be eliminated from the breeding population, further improving reproductive performance.

The application of molecular markers in sheep farming represents a significant advancement in the industry. It enables faster and more precise breeding decisions, ultimately leading to improved reproductive efficiency and overall productivity in flocks. The integration of genetic knowledge into breeding programs accelerates progress and enhances the ability to optimize production. Consequently, molecular markers play an essential role in advancing sheep breeding practices, with a direct impact on the sustainability and profitability of the industry.

In sheep breeding, reproductive parameters such as follicular development, ovulation, and multiple births are controlled by both major genes and genes with smaller effects. These genes and mutations can be identified using molecular markers, a development in biotechnology, allowing for the selection of genotypes with desired traits within the flock. Marker-assisted selection in sheep breeding and improvement offers many advantages, such as time and cost savings. As a result, farm owners can achieve profitable production. Efforts are being made to identify genes related to reproduction as markers and to apply them in sheep breeding. This study compiles academic works on the definition of major and other genes affecting reproduction in sheep, and their use as molecular markers in different sheep breeds. As a result, in addition to major genes (BMP15, GDF9, and BMPR1B), genes such as FETUB, HIRA, PTX3, HRG, COIL, SLK, GRM1, RXRG, B4GALNT2, and CLSTN2 have been found to influence fertility, infertility, lamb number per litter, follicular development, and its rate in sheep.

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