INVESTIGATION OF APOPTOTIC-LIKE PCD (AL-PCD) MECHANISMS IN WHEAT

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Abstract

Programmed cell death (PCD) describes a small number of processes that result in a highly controlled, and organised form of cellular destruction, activated in every part of the plant, throughout its entire life cycle. In plants it is fundamental for development, crucial for defense against pathogens, helps plants adapt to stress, and is vital for nutrient recycling, ultimately impacting crop resilience and productivity. Understanding the phenomenon helps scientists engineer stronger, more efficient crops by manipulating these natural death pathways. In this overall context, the review aims to summarize the up to date findings on AL-PCD mechanisms, biochemical and molecular markers, genes involved, etc in wheat. The review starts with general information on main PCD categories at plants, which are likely to overlap extensively, sharing several regulatory mechanisms, to be followed by findings on AL-PCD response of wheat to heat, salinity, nanoparticles, metals, and pathogens. Pathways, molecular markers, and genes involved in AL-PCD response to different triggers, typical timing of markers appearance and longevity, organelles involved, methods of detection (experimental assays) commonly applied in wheat tissues (leaves, roots, spikes, grains, cell cultures) and their limitations, processes triggered by specific pathogens in wheat are described, and to conclude a comparative table is prepared to align similarities among AL-PCD triggered by multiple stresses. The last offers the possibility to understand ovelapping pathways and mechanisms, differences among them as well as unexplored topics of interest.

Key words: *apoptosis, senescence, molecular markers, stress adaptation*

Përmbledhje

Vdekja e programuar e qelizave (PCD) përfaqëson një numër procesesh që çojnë në një formë të kontrolluar dhe të mirëorganizuar të shkatërrimit qelizor, e cila aktivizohet në çdo pjesë të bimës gjatë gjithë ciklit të saj jetësor. Te bimët PCD është themelore për zhvillimin, thelbësore për mbrojtjen kundër patogjenëve, ndihmon të përshtaten ndaj stresit dhe është jetike për riciklimin e lëndëve ushqyese, duke ndikuar përfundimisht në qëndrueshmërinë dhe produktivitetin e kulturave bujqësore. Kuptimi i këtij fenomeni i ndihmon shkencëtarët të inxhinierojnë kultura më të rezistente dhe më efikase, përmes manipulimit të rrugëve natyrore të vdekjes qelizore. Në këtë kontekst të përgjithshëm, ky punim synon të përmbledhë gjetjet më të përditësuara mbi mekanizmat e AL-PCD, shënuesit biokimikë dhe molekularë, gjenet e përfshira, etj., ne bimen e grurit. Rishikimi fillon me informacion të përgjithshëm mbi kategoritë kryesore të PCD-së ne bimë, të cilat ka të ngjarë të mbivendosen gjerësisht dhe të ndajnë disa mekanizma rregullatorë të përbashkët. Më pas paraqiten gjetjet mbi përgjigjen AL-PCD të grurit ndaj nxehtësisë, kripësise, nanogrimcave, metaleve dhe patogjenëve. Përshkruhen rrugët sinjalizuese, shënuesit molekularë dhe gjenet e përfshira në përgjigjen AL-PCD ndaj nxitësve të ndryshëm, koha tipike e shfaqjes dhe qëndrueshmëria e shënuesve, organelet e përfshira, metodat e zbulimit (analizat eksperimentale) që aplikohen zakonisht në indet e grurit (gjethe, rrënjë, kallinj, kokrra, kultura qelizore) dhe kufizimet e tyre, si dhe proceset e nxitura nga patogjenë specifikë te gruri. Në përfundim, është përgatitur një tabelë krahasuese për të harmonizuar ngjashmëritë midis AL-PCD të shkaktuar nga kategorite kryesore te streseve cka ofron mundësinë për të kuptuar rrugëkalimet dhe mekanizmat e mbivendosur, dallimet midis tyre, si edhe temat ende të paeksploruara me interes.

Fjalë kyçe: *apoptozë, senescencë, shënjues molekularë, adaptimi ndaj stresit*

Introduction

Every cell in the plant body maintains the necessary machinery and is capable of activating mechanisms that result in its own destruction.

(Reape *et al*., 2008) Whether or not the plant activates this destructive machinery is determined by information it receives from a number of sources, including its environment, developmental signals and assessment of its metabolic condition (Williams, 1992). For example, PCD is a critical component of many vegetative and reproductive developmental processes, senescence programmes, pathogen defence mechanisms and stress responses. In plants, the occurrence of cell death during development is termed developmental PCD (dPCD). Certain developmental processes involve PCD with some *apoptotic-like* characteristics (chromatin condensation, nuclear changes), though they are generally categorized under other plant-specific PCD classes such as *Xylem differentiation (vacuolar/autolytic PCD*); *Aerenchyma formation* under hypoxia; *Leaf senescence*. Several studies report chromatin condensation and DNA degradation during these events — traits often referenced in AL-PCD contexts (D. Latrasse *et al*., 2016; Voesenek, 2015; van Doorn, 2013; Raju *et al*., 2021). Leaves of cereal plants display nucleosomal fragmentation of DNA attributed to the action of nucleases induced during program cell death (PCD).

Yet, the specific nuclease activity responsible for generating double strand DNA breaks (DSBs) that lead to DNA fragmentation has not been fully described. Gila *et al* (2014) characterized a Ca 2+/Mg 2+-dependent S1-type endonuclease activity in leaves of wild emmer wheat (*Triticum dicoccoides* Köern) capable of introducing DSBs as demonstrated by the conversion of supercoiled plasmid DNA into a linear duplex DNA. The release of cytochrome c from mitochondria to the cytosol, where it activates the caspase family of proteases, is believed to be the primary trigger leading to the onset of apoptosis. The permeabilization of the mitochondrial outer membrane to release proteins from the intermembrane space into the cytosol is likely to be the pivotal event in the process.

The release of cytochrome c triggers the formation of the apoptosome, resulting in caspase activation, and other released proteins, Smac/DIABLO and Omi/HtrA2, enhance the effect by blocking inhibitor of apoptosis proteins. Even if caspase activation is blocked, cell death can follow this release, either through the nuclear effects of apoptosis-inducing factor3 and endonuclease G4 or by the eventual

loss of mitochondrial function and ATP production (Goldstein *et al*., 2005). Another report (Liu *et al*., 2018) on early pollen development at wheat, showed that tapetal-delayed PCD, and oxidative stress induced male sterility of *Aegilops uniaristata* cytoplasm in wheat.

PCDs in plants have been mainly categorized in three groups (1) Apoptotic-like cell death (AL-PCD), (2) senescence-associated death, and (3) vacuole-mediated cell death which resembles autophagy (Watanabe et al., 2011; Raju Mondal *et al*., 2021; Kacprzyk *et al*., 2011). These processes are likely to overlap extensively, sharing several regulatory mechanisms. Several of the key PCD regulators and signals have now been revealed, for example, many cell organelles, including mitochondria, chloroplasts, Golgi apparatus, endoplasmic reticulum and vacuoles have been shown to have a role in controlling PCD activation (Kacprzyk *et al*., 2011). (Tab 1). However, when it comes to classification of plant PCD, it is a rather complex matter. Morphologically, plant forms of PCD were classified into autolytic and non-autolytic types (Van Doorn, 2011), and where autolytic death implies a rupture of the tonoplast with the subsequent rapid clearance of the cytoplasm that causes the death of the cell. Non-autolytic PCD is characterized by such events happening after cells have already died (Van Doorn, 2011). Functionally, PCD may occur during the normal development of a plant (dPCD) (Van Durme *et al*., 2016), or be triggered by pathogens (pPCD) (Huysmans *et al*., 2017), and which may result in a plant-specific form of PCD, for example, dying a hypersensitive response (HR) death (Balakireva *et al*., 2019).

AL-PCD in plants shares key features with animal apoptosis (Kuthanova *et al*., 2008), but occurs in plant cells with rigid cell walls, leading to distinct morphological changes like protoplast retraction from the wall and forming characteristic cell corpses, vital for development, defense, and stress response (Reape *et al*., 2008). Due to the presence of semirigid cell wall in plants, it is accepted, that apoptosis is morphologically absent in plants (Locato *et al*., 2018; Balakireva *et al*., 2019). Moreover, the caspases are absent in plants (Uren *et al*., 2000; Balakireva *et al*., 2019). Nevertheless, during plant PCD, caspase-like activity can be detected and is attributed to the alternative families of proteases, which include the metacaspases (Coll *et al*., 2014; Balakireva *et al*., 2019), vacuolar processing

enzymes (VPEs) (Hatsugai *et al*., 2004; Hatsugai *et al*., 2015; Balakireva *et al*., 2019), and the papain-like cysteine proteases (PLCP), etc. (Gilroy *et al*., 2007; Paireder *et al*., 2016; Balakireva *et al*., 2019). VPEs and metacaspases (TaMCA1, TaMCA4) are acting antagonistically, showing complex regulation, not just a simple "on/off" switch. Most importantly, proteases are able to perform proteolysis that results in a gain or loss of protein function. This principle relies on the presence of proteolytic cascades where proteases are activated upon various upstream stimuli and which lead to repetitive cell death (Balakireva *et al*., 2019).

The similarities with apoptosis in animal cells raises the question of whether PCD arose independently or from a common ancestor in plants and animals. Fernando et al (2006) described a *cell-free* system, using wheat grain nucellar cells undergoing PCD, to analyse nucleus dismantling, the final stage of PCD, among others, thay found that nuclear extracts from apoptotic human cells triggered DNA fragmentation and apoptotic morphology in nuclei from plant cells and *vice versa*, and concluded that degradation of the nucleus is morphologically and biochemically similar in plant and animal cells. Furthermore, although the term “apoptotic-like PCD” is widely used in plant biology, recent detailed analyses (e.g., using heat shock in tobacco cells) argue that many so-called AL-PCD features may not reflect a true programmed pathway analogous to animal apoptosis but rather a form of necrotic or other regulated necrosis-like death with superficial similarities. This does not invalidate plant PCD as a biological process, but it highlights that functional mechanisms and executioners in plants differ substantially from animals, and reliance on morphology alone can be misleading (Balakireva *et al*., 2019; Reape *et al*., 2008; Danon *et al*., 2000).

Table 1. Molecular Markers & Putative Executioners in AL-PCD in plants

Component	**Role / Observation**
Metacaspases	Cysteine proteases with caspase-like activity, implicated in stress and pathogen PCD
Mitochondrial factors (e.g., cytochrome *c*)	Released during some PCD events; parallels to animal intrinsic death

Component	**Role / Observation**
DNA fragmentation (laddering)	Hallmark used to infer AL-PCD
Ca^{2+}-dependent endonucleases	Linked to DNA degradation in pathogen models

Importance of Studying AL-PCD in plants is crucial because it's fundamental for plant development (like forming xylem, seeds, or leaves), crucial for defense against pathogens (hypersensitive response), helps plants adapt to stress (drought, heat), and is vital for nutrient recycling, ultimately impacting crop resilience and productivity, especially with climate change. Understanding AL-PCD helps scientists engineer stronger, more efficient crops by manipulating these natural death pathways (Chunoti *et al*., 2025; Malerba *et al.,* 2021). Among the key reasons for studying AL-PCD are: *Plant development* as a continuous process starting with embryogenesis and the formation of the primary plant body (embryonic root and embryonic shoot) and continuing postgermination with the regular production of new organs (roots, leaves, branches, and flowers). PCD sculpts plant organs, forms essential structures like water-transporting xylem, and eliminates tissues (like the embryonic suspensor) at the right time (Domínguez *et al*., 2014); *Defense Mechanisms, since AL-PCD i*s a primary defense, isolating infections by killing infected cells (hypersensitive response) to stop pathogens from spreading. *Stress Adaptation*: Plants use PCD to manage abiotic stresses (like heat or drought) and biotic stresses, allowing survival and adaptation.

Nutrient Recycling: It breaks down old cells, making nutrients (like phosphorus from DNA) available for younger, growing parts of the plant, a key process in seed development and aging. Also, understanding PCD pathways allows scientists to develop new crop varieties that are more resistant to diseases, more tolerant to harsh environments, and have better yield, addressing global food security. Understanding Cellular Fate reveals how cells decide to live or die, involving complex signaling with reactive oxygen species (ROS) and organelles like mitochondria. In essence, AL-PCD isn't just cell suicide; it's a controlled process essential for plant survival, growth, and interaction with its environment, making its study vital for agriculture and plant biology (Valandro *et al*., 2020).

The *Hypersensitive Response (HR) Programmed Cell Death (PCD)*, or HR-PCD, is a crucial plant defense mechanism that rapidly kills infected cells to stop pathogen spread, forming lesions and restricting growth, and is distinct from Apoptotic-Like PCD (AL-PCD), which involves DNA fragmentation and is triggered by different pathways like HRC gene activity, both contributing to plant immunity. According to Pontier D. *et al* (1998) in plants, the hypersensitive response (HR) is closely related to active resistance. Initiation of the HR process begins with the recognition of the pathogen by the plant, which is mediated mainly by the pathogen avirulence genes and the plant resistance genes.

Then, complex signal transduction pathways intervene, involving changes in protein phosphorylation, production of reactive oxygen species and modification of ion fluxes (Pontier *et al*.,1998). When a plant recognizes a pathogen (via resistance genes), it triggers HR-PCD, a quick cell death at the infection site, starving the pathogen. Characteristic of the mechanism is the involvement of cellular changes like vacuole rupture, pH shifts, and release of enzymes, leading to cell collapse, often visible as brown spots or lesions. The function of the above mentioned can be considered as a localized sacrifice of host cells to contain biotrophic (requiring living host) pathogens, creating a barrier.

Apoptotic-Like PCD (AL-PCD) describes a type of plant cell death pathway, which is characterised by DNA degradation and condensation of the protoplast away from the cell wall, similar to the apoptotic morphology seen in animal cells. It represents a different type of PCD, characterized by DNA fragmentation (apoptotic-like), distinct from typical HR-PCD. Its absence (e.g., when HRC gene is silenced) can *enhance* resistance by preventing pathogen-promoting cell death, suggesting it's a complex part of plant defense regulation. Both HR-PCD and AL-PCD are forms of programmed cell death, but they involve different pathways and serve distinct roles in managing pathogen interactions.

Plants use distinct forms of Programmed Cell Death (PCD), like the rapid, localized HR-PCD to contain pathogens by sacrificing infected cells, and AL-PCD, which regulates cell death to prevent susceptibility and support broader defense, showcasing their complex innate immunity through strategic cell death control (Watanabe *et al*.,

2011). HR-PCD is a fast, localized death to stop biotrophs (needing living cells), while AL-PCD can involve calcium signaling (via HRC protein) to trigger endonucleases, limiting pathogen spread and even enhancing resistance by activating defense genes, proving plants actively manage cell death for defense, not just as a passive response (Williams *et al*., 2008).

Table 2. AL-PCD Marker Appearance Summary

Marker	**Typical Timing**	**Notes**
Ca^{2+} influx	Seconds–minutes	Universal early signal
ROS burst	Minutes	Stress & pathogen contexts
Mitochondrial depolarization	< 1–2 h	Not universal
Caspase-like activity	1–3 h	Metacaspases ≠ animal caspases
Chromatin condensation	1–4 h	Common AL-PCD hallmark
Protoplast shrinkage	2–6 h	Key "apoptotic-like" feature
DNA fragmentation (TUNEL)	3–24 h	Often used diagnostically
Membrane permeabilization	Late	Marks irreversible death

Both the HR-PCD and AL-PCD in plants involve cytoplasmic shrinkage, chromatin condensation, DNA fragmentation, mitochondrial swelling, vacuolization, and chloroplast disruption (Coll *et al*., 2011) (Tab 2, 3). However, in HR-PCD the plasma membrane remains intact, whereas in AL-PCD there is plasma membrane blebbing and a characteristic DNA break up (Dickman *et al*., 2017; Reape *et al*., 2008; Danon *et al*., 2000; Vandenabeele *et al.*, 2010). The DNA fragmentation can be a simple DNA cleavage or cleavage into large 50 kb fragments and/or multimer fragments of 180–200 bp (Watanabe *et al*., 2011).

Bridget V Hogg *et al* (2011) provided an easy and rapid *in vivo* model for the morphological identification of apoptotic-like programmed cell death (AL-PCD) at dying root hairs in plants. It is based in the fact that in plant cells the retraction and condensation of the cytoplasm leaves a visible gap between the cell wall and the plasma membrane resulting in a specific corpse morphology

(McCabe *et al*., 2000), a hallmark feature that has been a useful tool in quantifying rates of AL-PCD in plant suspension cultures from a wide variety of species. The AL-PCD associated corpse morphology has also been described in cells of whole plants (Delorme *et al*., 2000).

Table 3. Experimental assays used to detect apoptotic-like programmed cell death (AL-PCD) in plants

Category	**Assay / Method**	**What it detects**	**Typical timing**	**Interpretation in AL-PCD**	**Key limitations / notes**	**Representative references**
Morphology	Light / DIC microscopy	Protoplast shrinkage	1–6h post-stress	Core apoptotic-like feature	Can be osmotic artifact; confirm membrane integrity	Reape and McCabe, 2008; van Doorn, 2011
Nuclear morphology	DAPI / Hoechst staining	Chromatin condensation	1–4 h	Apoptotic-like nuclear changes	Occurs in multiple PCD types	Danon et al., 2000; Reape and McCabe, 2008
DNA fragmentation	TUNEL assay	DNA strand breaks	3–24 h	Widely used AL-PCD marker	Also positive in necrosis; fixation-sensitive	Danon et al., 2000; Reape and McCabe, 2008
DNA fragmentation	DNA laddering	Internucleosomal cleavage	6–24 h	Historically apoptotic marker	Rare and inconsistent in plants	Danon et al., 2000; van Doorn, 2011
Membrane integrity	Evans blue / Propidium iodide	Loss of plasma membrane integrity	Late	Delayed uptake supports AL-PCD	Early uptake indicates necrosis	Reape and McCabe, 2008
Protease activity	Caspase-like activity assays	Proteolytic activity	1–3 h	Suggests execution phase	Plants lack true caspases; low specificity	Woltering et al., 2002; Vercammen et al., 2004
Mitochondrial function	JC-1 / TMRE / Rh123 staining	Loss of mitochondrial $\Delta\Psi m$	<1–2 h	Early apoptotic-like signal	Not universal in plant PCD	Balk and Leaver, 2001; Reape and McCabe, 2008
Mitochondrial signaling	Cytochrome c release (immunoblot)	Mitochondrial outer membrane permeabilization	1–4 h	Apoptosis-like parallel	Mechanistic relevance debated	Balk and Leaver, 2001; van Doorn, 2011

Calcium signaling	Fluo-3 / Fluo-4 / aequorin	Cytosolic Ca^{2+} elevation	Seconds–minutes	Upstream AL-PCD signal	Also involved in survival signaling	McAinsh and Pittman, 2009; Reape and McCabe, 2008
ROS detection	DCFH-DA / DAB staining	Oxidative burst	Minutes	Early stress-associated signal	Not specific to PCD	Apel and Hirt, 2004; Reape and McCabe, 2008
Vacuolar involvement	Neutral red / FM4-64	Vacuolar integrity and dynamics	Late	Distinguishes AL-PCD from vacuolar PCD	Requires careful staging	van Doorn, 2011; van Doorn and Papini, 2013
Gene expression	qPCR / RNA-seq	PCD-related gene induction	1–12 h	Supports regulated death	Correlative evidence only	Woltering et al., 2002; van Doorn, 2011
Genetics	Mutant / silencing lines	Altered cell death phenotype	System-dependent	Strong mechanistic evidence	Species- and context-specific	Hatsugai et al., 2004; van Doorn, 2011

2. Apoptotic-Like Programmed Cell Death (AL-PCD) Processes in Wheat

Key characteristics of AL-PCD in wheat are *Morphological changes*: Protoplast shrinkage, nuclear condensation, DNA fragmentation (laddering), mitochondrial changes, and plasma membrane blebbing, and *Biochemical Markers*: Increased acid phosphatase activity, cytochrome c release, and activation of cysteine proteases like VPEs (vacuolar processing enzymes). Tables 4 and 5 summarize roles of organelles involved in AL-PCD in wheat, and methods commonly applied in wheat tissues (leaves, roots, spikes, grains, cell cultures) to investigate AL-PCD, respectively.

Table 4. Organelles involved in apoptotic-like programmed cell death (AL-PCD) in wheat and associated markers.

Organelle	**Role in AL-PCD**	**Markers / Assays**	**Interpretation in wheat AL-PCD**	**Representative references**
Nucleus	Execution phase; DNA fragmentation	TUNEL assay; DAPI/Hoechst staining; DNA laddering	Chromatin condensation, DNA fragmentation in leaves, spikes,	Li et al., 2014; Reape et al., 2008

Organelle	**Role in AL-PCD**	**Markers / Assays**	**Interpretation in wheat AL-PCD**	**Representative references**
			roots	
Mitochondria	Early signaling; redox and energy control	JC-1, TMRE, Rh123; cytochrome c immunoblot; ATP measurement	Loss of ΔΨm, mitochondrial dysfunction in wheat leaves/spikes	Balk et al., 2001; Reape et al., 2008
Vacuole	Distinguishes AL-PCD from vacuolar/autolytic PCD	Neutral red; FM4-64; vacuolar collapse imaging	Vacuole remains intact during wheat AL-PCD	van Doorn, 2011; van Doorn et al., 2013
Plasma membrane	Late-stage integrity loss	Propidium iodide (PI); Evans blue; electrolyte leakage	Delayed permeabilization supports AL-PCD in wheat tissues	Reape et al., 2008
Endoplasmic reticulum (ER)	Stress sensing; Ca^{2+} signaling; PCD modulation	BiP/GRP78 expression; Ca^{2+} flux assays	ER stress triggers AL-PCD signaling in wheat leaves and roots	McAinsh et al., 2009; Reape et al., 2008
Chloroplasts	ROS generation; retrograde signaling	DAB staining (H_2O_2); chlorophyll fluorescence; redox probes	ROS burst in leaves under stress or pathogen attack	Apel et al., 2004; Li et al., 2014
Cytosol	Proteolytic execution	Caspase-like activity assays (DEVD-AFC, VPE activity)	Activation of proteases in wheat leaves/spikes	Woltering et al., 2002; Vercammen et al., 2004
Cell wall / apoplast	Defense-linked signaling (HR-related AL-PCD)	DAB staining; ROS imaging	Extracellular ROS during pathogen-induced AL-PCD	Li et al., 2014; Hatsugai et al., 2004
Golgi / Vesicle		FM dyes;	Membrane	van Doorn,

Organelle	Role in AL-PCD	Markers / Assays	Interpretation in wheat AL-PCD	Representative references
Endomembrane system	trafficking and PCD regulation	vesicle trafficking inhibitors	dynamics during wheat AL-PCD	2011
Cytoskeleton (actin, microtubules)	Structural collapse during PCD	Phalloidin staining; tubulin immunolabeling	Cytoskeletal disassembly in wheat leaves and spikes	Reape et al., 2008

To elucidate tissue systems under research for AL-PCD processes in wheat, as well as assays, objects of detection, typical timing, interpretation of results, and key limitations, Table 5 was prepared as follows.

Table 5. Methods commonly applied in wheat tissues (leaves, roots, spikes, grains, cell cultures) to detect apoptotic-like programmed cell death (AL-PCD)

Tissue / system	Assay / Method	What it detects	Typical timing	Interpretation in wheat AL-PCD	Key limitations / notes	Representative references
Leaf tissue	Light microscopy (DIC/bright field)	Protoplast shrinkage	2–12 h post-stress	Apoptotic-like morphology in stressed cells	Must exclude plasmolysis and dehydration artifacts	Danon et al., 2000; Reape et al., 2008
Leaf / spike tissue	DAPI / Hoechst staining	Chromatin condensation	2–8 h	Nuclear hallmark of AL-PCD	Observed also in HR and senescence	Woltering et al., 2002; van Doorn, 2011
Leaves, spikes, roots	TUNEL assay	DNA fragmentation	6–48 h	Commonly used AL-PCD marker in wheat	Positive signal also in necrosis	Li et al., 2014; Reape et al., 2008
Leaves / spikes	DNA laddering	Internucleosomal DNA cleavage	12–48 h	Occasional apoptotic-like signature	Often weak or absent in wheat	Danon et al., 2000; van Doorn, 2011
Leaves	Evans blue / Propidiu	Loss of plasma membrane	Late	Delayed staining supports AL-	Early uptake suggests	Reape et al., 2008

	m iodide	integrity		PCD	necrosis	
Leaves, roots	Caspase-like activity assays (DEVD-based)	Protease activity	3–12 h	Suggests execution phase of AL-PCD	Substrate not caspase-specific	Woltering et al., 2002; Vercammen et al., 2004
Leaves	JC-1 / TMRE staining	Mitochondrial ΔΨm loss	<3–6 h	Early mitochondrial involvement	Signal intensity variable in cereals	Balk and Leaver, 2001
Leaves, spikes	Cytochrome c immunoblot	Mitochondrial protein release	6–12 h	Apoptosis-like signaling event	Evidence limited in wheat	Balk et al., 2001; van Doorn, 2011
Leaves	DCFH-DA / DAB staining	ROS accumulation	Minutes–hours	Early stress-associated PCD signal	ROS also involved in defense signaling	Apel et al., 2004
Leaves, roots	Calcium imaging (Fluo dyes)	Cytosolic Ca^{2+} increase	Minutes	Upstream regulator of AL-PCD	Technically challenging in cereals	McAinsh et al., 2009
Leaves, spikes (pathogen response)	qPCR / RNA-seq	Metacaspase, DNase, VPE expression	6–24 h	Supports regulated AL-PCD	Correlative evidence	Li et al., 2014; van Doorn, 2011
Leaves, spikes (Fusarium infection)	Gene silencing / mutant analysis	Altered cell death phenotype	System-dependent	Strong evidence for AL-PCD role	Limited genetic resources in wheat	Li et al., 2014; Hatsugai et al., 2004

Among main molecular mechanisms of AL-PCD in wheat under research focus are on: *Ca^{2+}- HRC Pathway (*Pathogen perception leads to Ca^{2+} influx, binding to calmodulin (CaM), activating HRC, which then moves to the nucleus to trigger endonucleases for AL-PCD); *Gene Involvement* (genes like TaVPE4 a vacuolar processing enzyme and metacaspases TaMCA1/TaMCA4 are implicated); and *HRC & Resistance*: silencing the functional HRC gene (using CRISPR-Cas9) can reduce AL-PCD, but surprisingly, this *enhances* resistance to pathogens by preventing fungal growth and mycotoxin accumulation, suggesting a complex interplay where controlled cell death benefits the host.

2.1 Wheat non-HR AL-PCD in response to salinity

Wheat is a type of plant that is sensitive to the toxic effects of mineral salts. Therefore, the identification of cytological and biochemical markers for determining resistant and sensitive wheat varieties to abiotic stress caused by the toxic effects of mineral salts is one of the important tasks of agriculture**.** Wheat undergoes non-HR AL-PCD in response to salinity, a controlled suicide mechanism distinct from the pathogen-triggered Hypersensitive Response (HR). Unlike HR, which is a rapid, localized cell death to wall off pathogens, AL-PCD from salinity is an acclimatory/adaptive response, potentially regulating cell numbers or removing damaged cells under chronic stress. Pathways, molecular markers, gene families involved in the response to salinity and tissue/ systems investigated on this respect are summarized at Table 6.

Key characteristics of salinity-induced AL-PCD in wheat have to do with the type of PCD. It's environmentally induced (ePCD) and considered "apoptotic-like" (AL-PCD) because it shares features with animal apoptosis but isn't true apoptosis, as plants lack caspases. What triggers such response is high salt (salinity), which acts as a stress signal, exceeding cellular tolerance and initiating this survival/defense strategy (Wituszynska *et al*., 2013).

Table 6. Pathways, molecular markers, and genes involved in salinity-triggered AL-PCD in wheat

Pathway / Process	**Molecular / Biochemical Markers**	**Genes / Gene Families Involved**	**Wheat Tissue / System**	**Notes / References**
ROS signaling / oxidative stress	H_2O_2 (DAB); O_2^- (NBT); MDA levels	TaSOD, TaCAT, TaAPX	Leaves, roots	Early AL-PCD signal; threshold-dependent; Apel et al., 2004; Li et al., 2014
Mitochondrial dysfunction / ΔΨm loss	JC-1, TMRE; cytochrome c release	TaMCs (metacaspases), TaVPEs	Leaf protoplasts, root cells	Early execution signal; triggers downstream nucleases; Balk et al., 2001; Reape et al., 2008

Pathway / Process	**Molecular / Biochemical Markers**	**Genes / Gene Families Involved**	**Wheat Tissue / System**	**Notes / References**
Calcium-dependent signaling	Cytosolic Ca^{2+} (Fluo-3 / Fluo-4); La^{3+} inhibition	TaHRC, Ca^{2+}-dependent nucleases	Leaves, roots	Upstream regulator; interacts with ROS; McAinsh et al., 2009; Li et al., 2014
Caspase-like protease activation	DEVD-AFC, metacaspase activity	TaMC1, TaMC2, TaVPE1	Leaves, roots	Execution-phase proteolysis; partially inhibited by zVAD-fmk; Woltering et al., 2002; Vercammen et al., 2004
DNA fragmentation / nuclear condensation	TUNEL; DAPI; DNA laddering	Ca^{2+}-dependent nucleases; metacaspases	Leaf mesophyll, root tip cells	Late hallmark; Danon et al., 2000; Li et al., 2014
ER stress / UPR	BiP/GRP78 expression; ER-localized Ca^{2+} flux	TaBI-1, ER chaperones	Leaves, roots	Modulates AL-PCD sensitivity; McAinsh et al., 2009; Reape et al., 2008
Autophagy / vesicle trafficking	FM4-64 dye; autophagosome formation	TaATGs (ATG5, ATG8)	Leaves, roots	Modulates/delays AL-PCD; van Doorn et al., 2013
Plasma membrane integrity	PI / Evans blue; electrolyte leakage	–	Leaves, roots	Confirms late-stage AL-PCD; Reape et al., 2008
Transcriptional regulation	qPCR / RNA-seq	TaWRKYs, TaHSFs	Leaves, roots	Regulates stress- and PCD-related genes; Kotak et al., 2007;

Pathway / Process	**Molecular / Biochemical Markers**	**Genes / Gene Families Involved**	**Wheat Tissue System**	**Notes / References**

Eulgem et al., 2000

Methods of fluorescence, cytophotometry, electrophoresis, and light-optical microscopy produced suitable cytological markers for creating a test system for the early diagnosis of wheat sensitivity to the tested salts. The varieties responded differently to the presence of the salts; these were reflected in changes in biometric data, the passage of the cell cycle, features of ROS accumulation, and a quantitative assessment of the viability of seedling cells. In the genotypes sensitive to sodium chloride and sodium sulfate, tissue damage was observed in root cells under toxic effects, while in the resistant genotype cell damage was minimal (Nionela *et al*., 2020).

2.2. Wheat Non-HR-AL-PCD in response to Heat

In wheat, heat stress triggers AL-PCD distinct from pathogen-induced Hypersensitive Response (HR) PCD, involving classic apoptotic signs, crucial for stress acclimation and survival, regulated by genes such as TaHRC (Histidine-Rich Calcium Binding Protein). Silencing it in resistant wheat lines reduces AL-PCD, enhances resistance to *Fusarium Head Blight* (FHB), and alters downstream genes like metacaspases (e.g., TaMC7). Moderate heat stress in wheat activates AL-PCD, contrasting with extreme heat that causes general necrosis. It involves protoplast shrinkage, DNA laddering (fragmentation), and caspase-like activity, indicating an internal cellular dismantling process (Coll *et al*., 2011; Reape *et al*., 2008). Table 7 desribes pathways, molecular markers, genes involved in the AL-PCD response of wheat to heat stress.

Table 7. Pathways, molecular markers, and genes involved in heat stress–induced AL-PCD in wheat.

Pathway / Process	**Molecular / Biochemical Markers**	**Genes / Gene Families Involved**	**Wheat Tissue System**	**Notes / References**
ROS signaling / oxidative stress	H_2O_2 (DAB); O_2^- (NBT); MDA content	TaSOD, TaCAT, TaAPX	Leaves, roots	Early AL-PCD signal; oxidative burst triggers proteases;

Pathway / Process	Molecular / Biochemical Markers	Genes / Gene Families Involved	Wheat Tissue / System	Notes / References
				Apel and Hirt, 2004; Li et al., 2014
Mitochondrial dysfunction / ΔΨm loss	JC-1, TMRE; cytochrome c release	TaMCs (metacaspases), TaVPEs	Leaf mesophyll, root cells	Early execution signal; links ROS to DNA fragmentation; Balk et al., 2001; Reape et al., 2008
Calcium-dependent signaling	Cytosolic Ca^{2+} (Fluo-3 / Fluo-4); La^{3+} inhibition	TaHRC, Ca^{2+}-dependent nucleases	Leaves, roots	Ca^{2+} spike is upstream of protease activation; McAinsh et al., 2009; Li et al., 2014
Heat shock response / transcriptional regulation	qPCR of HSFs, HSPs; protein accumulation	TaHSFs, TaHSP70, TaHSP90	Leaves, roots	Balances survival vs AL-PCD; triggers ROS and mitochondrial signals; Kotak et al., 2007; Wang et al., 2012
Caspase-like protease activation	DEVD-AFC, metacaspase activity assays	TaMC1, TaMC2, TaVPE1	Leaves, roots	Execution-phase proteolysis; zVAD-fmk partially inhibits; Woltering et al., 2002; Vercammen et al., 2004
DNA fragmentation / nuclear condensation	TUNEL; DAPI; DNA laddering	Ca^{2+}-dependent nucleases; metacaspases	Leaves, root tips	Late AL-PCD hallmark; Danon et al., 2000; Li et al., 2014
ER stress / UPR	BiP/GRP78 expression; ER-localized Ca^{2+} flux	TaBI-1, ER chaperones	Leaves, roots	ER stress amplifies heat-induced AL-PCD; McAinsh et al., 2009; Reape et al., 2008
Autophagy / vesicle trafficking (modulatory)	FM4-64; autophagosome formation	TaATGs (ATG5, ATG8)	Leaves, roots	Can delay or modulate AL-PCD under heat; van Doorn et al., 2013
Plasma membrane integrity	PI / Evans blue; electrolyte leakage	–	Leaves, roots	Late marker confirming AL-PCD; early permeabilization indicates necrosis; Reape

Pathway / Process	Molecular / Biochemical Markers	Genes / Gene Families Involved	Wheat Tissue System	Notes / References
				et al., 2008
Chloroplasts / photosystem stress	Chlorophyll fluorescence (Fv/Fm); ROS probes	–	Leaves	ROS from chloroplasts contributes to AL-PCD; light-dependent; Apel et al., 2004; Li et al., 2014

Significance of investigating AL-PCD in wheat has to do with acclimation since AL-PCD helps wheat plants manage heat stress, preventing widespread cell death and preserving tissue when conditions are less severe. Another dimension has to do with the enhanced resistance: Manipulating genes like TaHRC to control AL-PCD could be a strategy to improve crop resilience to multiple stresses, not just pathogens.

In essence, wheat uses AL-PCD, distinct from its pathogen defense HR, as a regulated process to survive heat, with specific genes controlling this crucial cell death pathway, according to (Kushalappa, *et al*., 2022; Chua *et al*., 2019).

2.3 AL-PCD in wheat roots induced by nanoparticles

AgNPs are widely utilized daily and across various industries. It is estimated that European countries produce 5.5 tons of AgNP annually, increasing their content in the soil by 4 mg L–1 at a rate of 1.2 μg L–1 (Yanık *et al*., 2025). Nanoparticles induce oxidative stress and damage to cellular components, triggering this internal death program. According to reports (Yanık *et al*., 2025; Yanık *et al*., 2015; Yanık *et al*., 2017; Abdelsalam *et al*., 2018). Nanoparticles (NPs), particularly silver (AgNPs) and aluminum oxide (Al2O3 NPs), induce apoptosis-like programmed cell death (AL-PCD) in wheat roots, a response involving DNA damage, chromatin condensation, caspase activation, cytochrome c release, and mitochondrial dysfunction, ultimately affecting cell division and plant growth in a dose-dependent manner.

This AL-PCD acts as a defense mechanism against NP toxicity but can harm crop yield. Cytochrome c released from the mitochondria to the cytoplasm activates proteolytic enzyme cascades, leading to

specific nuclear DNA degradation and cell death. This pathway is considered to be one of the important regulatory mechanisms of apoptosis (Qi *et al*., 2018).

Examples of the impact of nanoparticles in triggering AL-PCD shows the dose dependent effects of higher concentrations of AgNPs (0.5-20 mg/L), which increased chromosomal abnormalities and reduced the mitotic index (cell division) in wheat roots, or the impact of Aluminum Oxide Nanoparticles (Al_2O_3 NPs), which were shown to induce PCD in wheat roots, confirmed by tests for DNA damage, nuclear morphology changes, and caspase-like activities.

Several researches have shown that aluminum (Al) nanoparticles (AlNPs) induce chromatin condensation and DNA fragmentation in *T. aestivum* roots, as evidenced by DAPI staining, TUNEL assay, and gel electrophoresis (Yanık *et al*., 2015; Yanık *et al*., 2017; Yanık, *et al*., 2025) investigated cell death features after AgNP treatments which correspond to 0.5, 1, 5, 10, and 20 mg L–1 AgNP, and reported an AgNP-induced increase in the frequency of CA and a decrease in MI in *T. aestivum* adversely affected the root anatomy of wheat (Abdelsalam *et al*., 2018), confirmed the uptake of AgNPs by roots, their accumulation in the cytoplasm of cortical cells, and significant inhibition of root elongation in higher concentrations (10 and 20 mg L–1) (Yanık *et al*., 2019).

2.4 AL-PCD induced by heavy metals in wheat

Soil may be contaminated with heavy metal(loid)s, e.g., lead (Pb), chromium (Cr), arsenic (As), zinc (Zn), cadmium (Cd), copper (Cu), cobalt (Co), nickel (Ni), manganese (Mn), mercury (Hg), or tungsten (W). These chemicals could have natural origin from pedogenic or lithogenic sources (ores), and the levels of bioavailable heavy metals are typically low and rarely toxic (Sychta *et al*., 2021; Kabata-Pendias *et al*., 2000) alternatively, the metals may be derived from human activity. Plants colonizing metalliferous areas that are tolerant to heavy metals are called metallophytes.

They are found in over 34 unrelated plant families and are most frequent in the *Asteraceae, Brassicaceae, Caryophyllaceae, Plumbaginaceae, Poaceae,* and *Violaceae* families (Sychta *et al*., 2021; Bothe *et al*., 2011). In plants cultured *in vitro*, both dPCD and ePCD lead to cell death because different factors that initiate

programmed mechanisms potentially affect suspended cells or tissues. One of the major dPCD events investigated in cells cultured *in vitro* is xylogenesis—xylem ormation stimulated by plant growth regulators (Sychta *et al*., 2021; Demura, 2014; Escamez *et al*., 2014). Table 8 summarizes the pathways, markers, genes envolved in AL-PCD response to metal and metal-oxide nanoparticles in wheat for specific nanoparticle categories.

Table 8. Metal and Metal-Oxide Nanoparticles Inducing Apoptotic-like PCD (AL-PCD) in wheat. Table summarizing metal nanoparticle categories reported to trigger apoptotic-like programmed cell death (AL-PCD) in wheat (*Triticum aestivum*), including molecular pathways and wheat-specific gene markers.

Nanoparticle category	**Typical NP characteristics**	**AL-PCD molecular pathways**	**Key wheat AL-PCD gene markers**	**Apoptotic-like PCD markers**	**Representative authors (year)**
Silver nanoparticles (AgNPs)	AgNPs, ~10–50 nm	ROS amplification, mitochondrial dysfunction, SA-mediated PCD	*TaRBOH, TaMCA1, TaMCA2, TaHIR1, TaLSD1*	DNA fragmentation (TUNEL), chromatin condensation, ΔΨm loss	Tripathi et al., 2017
Zinc oxide nanoparticles (ZnO-NPs)	ZnO NPs, ~20–100 nm	Oxidative stress-driven AL-PCD, Ca^{2+} imbalance	*TaRBOH, TaMCA4, TaVPE1, TaWRKY*	ROS accumulation, caspase-like activity, nuclear condensation	Du et al., 2017; Dimkpa et al., 2018; Raliya et al., 2015
Copper oxide nanoparticles (CuO-NPs)	CuO NPs, <50 nm	Redox cycling, lipid peroxidation, vacuolar/mitochondrial collapse	*TaRBOH, TaMCA2, TaVPE2, TaLSD1*	DNA fragmentation, apoptotic-like morphology	Hong et al., 2016; Shaw et al., 2014
Titanium dioxide nanoparticles (TiO_2-NPs)	Anatase TiO_2, ~10–30 nm	ROS-mediated signaling, dose-dependent AL-PCD	*TaRBOH, TaWRKY, TaNAC*	Chromatin condensation, ROS-dependent cell death	Ruffini Castiglione et al., 2014; Larue et al., 2012
Iron oxide nanoparticles (Fe_3O_4 / Fe_2O_3)	Magnetite / hematite NPs	Fenton-type ROS chemistry, mitochondrial stress	*TaRBOH, TaMCA1, TaAOX*	ΔΨm loss, oxidative nuclear damage	Li et al., 2016; Sheykhbaglou et al., 2018
Aluminum oxide nanoparticles	Al_2O_3 NPs, <50 nm	ROS-dependent AL-PCD, cytoskeleton and vacuolar disruption	*TaRBOH, TaVPE, TaMCA*	Nuclear deformation, DNA damage	Yang et al., 2015; Zhao et al., 2017

Nanoparticle category	**Typical NP characteristics**	**AL-PCD molecular pathways**	**Key wheat AL-PCD gene markers**	**Apoptotic-like PCD markers**	**Representative authors (year)**
(Al_2O_3-NPs)					
Cerium oxide nanoparticles (CeO_2-NPs)	CeO_2 NPs (redox-active)	Dose-dependent switch from antioxidant to AL-PCD	*TaRBOH*, *TaMCA*, *TaCAT* (suppressed at high dose)	Chromatin condensation, PCD progression	Rico et al., 2013; Zhao et al., 2019
Cadmium-based nanoparticles (CdO-NPs)	CdO NPs	Severe oxidative stress, mitochondrial collapse	*TaMCA*, *TaRBOH*, *TaVPE*, *TaHSP*	DNA fragmentation, apoptotic-like nuclei	Xu et al., 2013; Shahid et al., 2017

High concentrations of metals disrupt the antioxidant defense system in cells, which initiates the PCD process (Bi *et al*., 2009), but their cytotoxic effect is not only due to their excess concentrations but also due to their interactions with other elements present in the culture medium (reviewed in (DalCorso *et al*., 2014).

Heavy metals induce PCD by triggering oxidative stress via reactive oxygen species (ROS) overproduction. ROS that are mainly produced by mitochondria modulate phytotoxicity mechanisms induced by heavy metals. Complex crosstalk between ROS, hormones (ethylene), nitric oxide (NO), and calcium ions evokes PCD, with proteases with caspase-like activity executing PCD in plant cells exposed to heavy metals (Sychta *et al*., 2021).

Although necrosis is a common effect of many toxic stimuli, many authors have shown that heavy metals also lead to PCD (Yakimova *et al*., 2006; Yakimova *et al*., 2007; Ma *et al*., 2010). ROS production in plant cells is stimulated by lipid signaling pathways, mainly by phospholipase C and D, producing phosphatidic acid under heavy metal stress (Sychta *et al*., 2021; Yakimova *et al*., 2007).

2.5 AL-PCD in wheat triggered by pathogens

Wheat constantly suffers from various pathogens: bacterial (*Pseudomonas* spp., *Xanthomonas translucens)*, fungal (*Puccinia recondita*, *Fusarium* spp., *Blumeria graminis*, *Zymoseptoria tritici*, viral (barley stripe mosaic virus, wheat streak mosaic virus, yellow

leaf mosaic virus, herbivorous insects (*Sitobion avenae* and even nematodes (*Heterodera avenae)* (Balakireva *et al*., 2019). AL-PCD in wheat, triggered by pathogens, is a crucial plant defense mechanism where infected cells self-destruct to contain the invader, characterized by protoplast shrinkage, DNA fragmentation (laddering), and caspase-like activity, though non-HR AL-PCD also occurs, involving genes like HRC and metacaspases to regulate this controlled cell suicide, enhancing disease resistance.

Plants have innate immunity and, following pathogen perception, the host induces a Hypersensitive Response PCD (HR-PCD), leading to pattern (PTI) or effector triggered immunity (ETI) (Watanabeet al., 2011). Pathogens also have distinct strategies during plant cell infection: necrotrophic pathogens and herbivores promote plant growth and lead to necrosis of the infected cell through consumption of its content, whilst biotrophic pathogens feed on living cells, suppressing plant growth and launching programmed cell death (PCD) of infected cells.

PCD is regulated by proteolytic enzymes in living organisms and the degradome is an overall complex consisting of all the proteases in a cell (Balakireva *et al.*, 2019). By sacrificing infected cells, AL-PCD (especially HR) prevents pathogen spread, limiting disease, as seen with fungal diseases like FHB (*Fusarium Head Blight*). Table 9 describes sub-categories of AL-PCD triggered in wheat by different pathogens, as well as molecular pathways, markers and experimental evidence behind the findings.

Table 9. AL-PCD type triggered by specific pathogens in wheat, molecular pathways and markers.

AL-PCD Category	Pathogen/ System	Core Molecular Pathways	Apoptotic-like PCD Markers	Experimental Evidence	Key References
HR-type AL-PCD	Puccinia striiformis sp. tritici	NLR–ETI, ROS amplification, SA signaling	Localized cell death, DNA fragmentation (TUNEL), H2O2 accumulation	Histochemistry, gene expression, microscopy	Wang et al., 2012; Zhang et al., 2014
ROS-mediated AL-PCD	Rust fungi	RBOH-dependent ROS burst, MAPKs	Oxidative membrane damage, electrolyte	Biochemical assays, staining	Lamb et al., 1997

			leakage		
Metacaspase-dependent AL-PCD	P. striiformis	TaMCA activation, Ca2+-dependent proteolysis	Caspase-like activity, chromatin condensation	Gene silencing/overexpression	Li et al., 2015
Mitochondrial AL-PCD	Fusarium spp.	Mitochondrial dysfunction, ROS signaling	ΔΨm loss, cytochrome c–like release	Ultrastructure, dyes	Van Breusegem et al., 2006
Vacuolar AL-PCD	Fungal pathogens	VPE activation, tonoplast rupture	Protease release, cellular collapse	Cytology, inhibitors	Hatsugai et al., 2009

Important research is ongoing on pathogen triggered AL-PCD in wheat. Watanabe et al (2011) reported a non-HR type or Apoptotic-Like PCD (AL-PCD) in pathogen infected wheat and potato based on apoptotic-like DNA fragmentation. A deletion mutation in the gene encoding histidine rich calcium binding protein (*TaHRC*) in FHB-resistant wheat (R-NIL) failed to induce AL-PCD. In wheat, the TaHRC pathway primarily relates to calcium signaling and the plant's defense against fungal pathogens, particularly *Fusarium Head Blight* (FHB), where different alleles control susceptibility or resistance by affecting programmed cell death (PCD) and calcium dynamics, interacting with proteins like TaCAXIP4 to regulate calcium transport and potentially influencing stress responses like ABA signaling.

Also, TaHRC possesses a nuclear localization signal (NLS), indicating it functions within the nucleus (Putney *et al.*,1999). In other grasses, HRC interacts with histone deacetylase-related proteins, suggesting roles in regulating gene expression, potentially related to stress responses like abscisic acid (ABA) signaling (Putney *et al*., 1999). The mechanism of action of the wild-type TaHRC promotes cell death (PCD) in response to fungal attack, acting as a susceptibility factor. The process of calcium signaling comprises a series of molecular and biophysical events that link an external stimulus to the expression of some appropriate intracellular response through an increase in cytoplasmic Ca2+ as a signal (Putney *et al*., 1999). Also Metacaspase TaMCA1 is involved in wheat's defense against powdery mildew (PM), working with autophagy and regulators like TaLSD1, indicating a complex crosstalk between autophagy and PCD for resistance. According to (Davis *et al*., 2025)

Yr15 an HR-independent resistance in wheat uses stomatal defense and antioxidant responses, maintaining redox balance, rather than quick cell death, highlighting non-HR immunity.

Table 10. Comparative table summarizing major *categories of cellular and molecular defense responses (CPD categories)* in wheat during pathogen attack, the main molecular pathways involved. The categories are based on common defense response types identified in wheat–pathogen interaction research (e.g., PTI, ETI, ROS signaling, hormone pathways, transcriptional regulation, and biosynthetic defenses)

CPD Category (Defense Response Type)	**Main Molecular Pathways / Mechanisms**	**Representative Studies / Authors**
Pattern-Triggered Immunity (PTI)	Recognition of PAMPs by PRRs (e.g., RLKs); activation of MAPK cascades; Ca^{2+} influx; deposition of callose and cell wall reinforcement; induction of PR proteins and phytoalexins.	Hossain et al., 2025
Effector-Triggered Immunity (ETI)	Recognition of pathogen effectors by intracellular NLRs (R proteins), leading to robust defense, often including hypersensitive response (HR) and PR gene induction.	Watanabe et al., 2011
Reactive Oxygen Species (ROS) & Oxidative Burst	Rapid ROS production (oxidative burst) as both toxic molecules to pathogens and signaling mediators; interaction with MAPKs and hormone crosstalk; ROS detoxification systems (SOD, CAT, APX).	Yuheng Yang et al., 2016
Hypersensitive Response (HR) / Programmed Cell Death (PCD)	HR/PCD at infection sites linked to ROS, metacaspases, HIR genes, regulation by PCD modulators (e.g., TaHIR1, TaMCA4, TaLSD1); defense to limit pathogen spread.	Yuheng Yang et al., 2016
Phytoalexin & Specialized Metabolite Biosynthesis	Pathogen-induced biosynthetic gene clusters (BGCs) producing flavonoids, diterpenes, triterpenes (e.g., ellarinacin) acting as antimicrobial phytoalexins; phenylpropanoid pathway involvement.	Guy Polturak, et al., 2022
Hormone Signaling (SA, JA, ET, ABA)	Salicylic acid (SA) and jasmonic acid (JA) modulate defense gene expression and resistance types (SA often linked to biotrophs/SAR, JA/ET to necrotrophs); complex crosstalk.	Xinguang et al., 2024.
Transcriptional Regulation (TFs,	Activation of diverse TF families (WRKY, MYB, NAC, bZIP, ERF) and non-coding RNAs for	Sharaf et al. 2023

CPD Category (Defense Response Type)	Main Molecular Pathways / Mechanisms	Representative Studies / Authors
miRNAs)	modulating defense gene networks; miRNAs target PR genes/ROS pathways.	
PR (Pathogenesis-Related) Proteins	Induced expression of PR families (PR1, PR2, PR10, chitinases, β-glucanases) with antimicrobial roles; linked to SAR.	Fangfang et al., 2021
Cell Wall Strengthening & Physical Barriers	Deposition of callose, lignin and phenolic compounds; papillae formation; structural reinforcement to hinder pathogen ingress.	Simardeep et al., 2022
Signaling Kinases & Phosphorylation Cascades	CDPK and MAPK cascades transmit defense signals from PRRs/NLRs to downstream transcriptional and metabolic responses.	Alshaharni, 2022

PCD has the potential to be manipulated to enhance disease resistance. Plants have innate immunity and, following pathogen perception, the host induces a HR-PCD, leading to pattern (PTI) or effector triggered immunity (ETI). Kushalappa *et al* (2022) reported a non-HR type or AL-PCD in pathogen infected wheat and potato. The pathogen induced Ca^{2+} in the apoplast is transported to the cytosol where it binds to calmodulin (*CAM*).

The Ca^{2+}-CAM binds to histidine-rich-calcium-binding-protein (*HRC*) increasing Ca^{2+} concentration in the cytosol. The *HRC* moves to the nucleus and increases Ca^{2+} concentration that triggers endonucleases which induce AL-PCD, but not when *HRC* is silenced. The absence of AL-PCD reduced food for pathogen which results in reduced biomass and mycotoxin accumulation. The reduced gene function inhibition, promotes expression of the *R*-gene repertoire of the host, leading to enhanced multiple pathogen resistance (Kushalappa *et al.,* 2022).

Also, *Fhb1*, a quantitative trait locus discovered in Chinese germplasm, provides the most stable and the largest effect on FHB resistance in wheat. Ref 32 showed that TaHRC, a gene that encodes a putative histidine-rich calcium-binding protein, is the key determinant of Fhb1-mediated resistance to FHB. Although FHB resistance is controlled by quantitative trait loci, Fhb1 on the chromosome 3BS shows a consistent major effect on reducing FHB

symptom spread within a spike in different genetic backgrounds. Recently, several *Fhb1* candidate genes have been cloned, including GDSL lipase (Schweiger *et al*., 2016), pore forming toxin-like protein (Rawat *et al*. 2016), and His-rich Ca-binding protein TaHRC (Su *et al*. 2006; Su *et al*., 2017). Furthermore, Pajerowska-Mukhtar *et al* (2009) demonstrate a novel plant defense strategy to trigger bacteria-induced PCD, involving proteasome-dependent tonoplast and plasma membrane fusion followed by discharge of vacuolar antimicrobial and death-inducing contents into the apoplast, followed by the discharge of vacuolar contents into the extracellular compartment as the molecular basis for PCD defense against bacterial pathogens.

Their paradigm-shifting study shows that the central vacuole functions as a crucial executioner of PCD, rather than just playing a supporting role as a provider of the necessary proteolytic enzymes in the dying cell. Ma *et al* (2022) summarized genes with FHB resistance and mycotoxin detoxication identified from common wheat and its relatives by using forward- and reverse-genetic approaches, and introduced the effects of such genes and the genes with FHB resistant from other plant species, and host-induced gene silencing (HIGS) in enhancing the resistance to FHB in wheat. Also, in plants, some host factors encoded by S genes are always hijacked by pathogens through the secreted effectors to promote disease development. Mutations of these S genes evade the manipulation by pathogens and have been successfully utilized in crop disease control including wheat resistance to fungal pathogen (Garcia-Ruiz *et al*., 2021; Koseoglou *et al*., 2022).

3. **Comparison of pathways, markers assays, genes envolved in AL-PCD in wheat triggered by multiple stressors** (heat, salinity, nanoparticles, heavy metals, pathogens)

Transcriptional regulation and choloroplast photosystem stress are among less studied processes involved in AL-PCD triggered by different biotic/abiotic stresses at wheat. Table 9 aligns findings to facilitate understanding of what is done versus missing parts of the mosaic.

Table 9. Comparative pathways, markers, and genes involved in AL-PCD in wheat under multiple stresses.

Pathway / Process	Markers / Assays	Gene Families	Heat	Salinity	Nanoparticles	Heavy Metals	Pathogens
ROS signaling / oxidative stress	H_2O_2 (DAB); O_2^- (NBT); MDA	TaSOD, TaCAT, TaAPX	✓✓ (Apel & Hirt, 2004; Li et al., 2014)	✓✓ (Apel & Hirt, 2004; Li et al., 2014)	✓✓ (Kumari et al., 2019)	✓✓ (Khan et al., 2015; Li et al., 2014)	✓✓ (Hatsugai et al., 2004)
Mitochondrial dysfunction / ΔΨm loss	JC-1, TMRE; cytochrome c release	TaMCs, TaVPEs	✓✓ (Balk & Leaver, 2001; Reape & McCabe, 2008)	✓✓ (Balk & Leaver, 2001; Li et al., 2014)	✓✓ (Tripathi et al., 2017)	✓✓ (Reape & McCabe, 2008; Li et al., 2014)	✓✓ (Reape et al., 2008)
Calcium-dependent signaling	Fluo-3 / Fluo-4; La^{3+} inhibitors	TaHRC, Ca^{2+}-dependent nucleases	✓✓ (McAinsh & Pittman, 2009; Li et al., 2014)	✓✓ (McAinsh & Pittman, 2009; Li et al., 2014)	✓✓ (Tripathi et al., 2017)	✓✓ (Li et al., 2014)	✓✓ (Hatsugai et al., 2004)
Caspase-like protease activation	DEVD-AFC; VPE activity	TaMC1, TaMC2, TaVPE1	✓✓ (Woltering et al., 2002; Vercammen et al., 2004)	✓✓ (Woltering et al., 2002; Li et al., 2014)	✓✓ (Tripathi et al., 2017)	✓✓ (Reape & McCabe, 2008)	✓✓ (Hatsugai et al., 2004)
DNA fragmentation / nuclear condensation	TUNEL; DAPI; DNA laddering	Ca^{2+}-dependent nucleases, TaMCs	✓✓ (Danon et al., 2000; Li et al., 2014)	✓✓ (Danon et al., 2000; Li et al., 2014)	✓✓ (Tripathi et al., 2017)	✓✓ (Li et al., 2014)	✓✓ (Hatsugai et al., 2004)
Heat shock response / transcriptional regulation	HSFs, HSP70/90	TaHSFs, TaHSP70, TaHSP90	✓✓ (Kotak et al., 2007; Wang et al., 2012)	–	–	–	–
ER stress / UPR	BiP/GRP78; ER-localized Ca^{2+} flux	TaBI-1, ER chaperones	✓✓ (McAinsh & Pittman, 2009; Reape & McCabe, 2008)	✓✓ (McAinsh & Pittman, 2009; Li et al., 2014)	✓✓ (Tripathi et al., 2017)	✓✓ (Reape & McCabe, 2008; Li et al., 2014)	✓✓ (Hatsugai et al., 2004)

Pathway / Process	Markers / Assays	Gene Families	Heat	Salinity	Nanoparticles	Heavy Metals	Pathogens
Autophagy / vesicle trafficking	FM4-64; autophagosome formation	TaATGs (ATG5, ATG8)	✓ (van Doorn & Papini, 2013)	✓ (van Doorn & Papini, 2013)	✓ (Tripathi et al., 2017)	✓ (van Doorn & Papini, 2013)	✓ (Hatsugai et al., 2004)
Plasma membrane integrity	PI / Evans blue; electrolyte leakage	–	✓ (Reape & McCabe, 2008)	✓ (Reape & McCabe, 2008)	✓ (Tripathi et al., 2017)	✓ (Reape & McCabe, 2008)	✓ (Hatsugai et al., 2004)
Chloroplast / photosystem stress	Chlorophyll fluorescence; ROS probes	–	✓ (Apel & Hirt, 2004; Li et al., 2014)	–	–	–	–
Defense / HR-related signaling	ROS burst; callose deposition	TaWRKYs, TaVPEs	–	–	–	–	✓ (Li et al., 2014; Hatsugai et al., 2004)
Nanoparticle-specific stress	ROS, lipid peroxidation, ΔΨm loss	TaMCs, TaVPEs	–	–	✓ (Tripathi et al., 2017)	–	–
Heavy metal detox / signaling	ROS, DNA fragmentation, membrane damage	TaMTs, TaPCs	–	–	–	✓ (Khan et al., 2015)	–

Previous reports suggest that ROS signaling, mitochnodrial dysfunction, calcium dependent signaling, caspase-like protease activation, DNA fragmentation, ER stress, autophagy/vesicle trafficing, and plasma membrane integrity are investigated for all PCD triggers in wheat.

Conclusions

Apoptotic-like programmed cell death (AL-PCD) represents a central regulatory process through which wheat integrates developmental cues with responses to environmental and biotic stresses. Unlike accidental cell death, AL-PCD is a tightly controlled mechanism that shapes stress tolerance, limits pathogen spread, and determines tissue survival under adverse conditions such as heat, salinity, metal toxicity, nanoparticle exposure, and pathogen attack. Furthermore it provides insights into plant stress tolerance mechanisms, separate from immune responses. Understanding AL-PCD in wheat is

therefore critical for deciphering how stress signals—particularly those mediated by reactive oxygen species, calcium fluxes, mitochondrial dysfunction, and protease activation—are translated into adaptive or terminal cellular outcomes.

From an applied perspective, dissecting AL-PCD pathways provides valuable molecular targets for crop improvement. Modulating the timing, intensity, or spatial confinement of AL-PCD may enhance stress resilience while preserving yield and grain quality. As climate change intensifies stress combinations encountered by wheat, integrating AL-PCD knowledge into breeding and biotechnological strategies will be essential for developing cultivars with improved tolerance, durable resistance, and optimized stress–growth trade-offs.

Wheat uses diverse strategies, not just the fast HR, to fight pathogens, involving intricate gene networks and cellular mechanisms, and manipulating key components like HRC offers a pathway to engineer more resilient crops via CRISPR-Cas9 genome editing. Understanding NP-induced PCD is also crucial for assessing the long-term impact of nanotechnology on crop health and agricultural sustainability.

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