Plant biology: proteolytic release of damage signals

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Plants protect their wounds against pathogen invasion by releasing damage signals that induce immune responses in neighboring cells. A new study shows that a conserved bioactive peptide is released from its cytoplasmic precursor upon wounding by a metacaspase that is activated by calcium influx into the injured cell.

Wounds inflicted to plants are common in nature and agriculture. Insects, nematodes and other animals, but also wind, hail and other mechanics collectively inflict wounds to plants on a daily basis. Plants protect these sites against pathogen invasion by mounting defense responses. Signals from damaged cells must be released to warn the neighboring cells to induce immune responses. An exciting new study by Tim Hander, Álvaro Fernández-Fernández and colleagues led by Simon Stael [1], reveals how such danger signal is released from the damaged cell.

The signals that damaged cells release to warn their healthy neighbors are damage-associated molecular patterns (DAMPs). One of the universally conserved DAMP signals in plants is the plant elicitor peptide Pep1 [2]. Pep1 is perceived on the cell surface of unchallenged cells by Pep1 receptor PEPR, leading to the induction of immune responses [3, 4].

However, ever since the discovery of Pep1, its release has been a mystery. The 2.3 kDa Pep1 peptide originates from the C-terminus of a 10.4 kDa precursor protein (PROPEP1) that lacks an N-terminal secretion signal peptide. This PROPEP1 precursor indeed accumulates in the cytoplasm, tethered to the vacuolar membrane [5]. So although Pep1 is extracellularly perceived, it originates from a cytoplasmic precursor protein. This subcellular conflict raises an intriguing question regarding the mechanism. A second question relates to the protease that releases Pep1 from its precursor. Plants produce >600 proteases [6], so there is a challenge to identify the protease releasing Pep1 from its precursor. The third question that follows naturally is how PROPEP1 processing is spatial-temporally controlled upon wounding. These three questions have been answered in one unifying mechanism involving the calcium-induced metacaspase-4 (MC4, **Figure 1**).

The authors first used an assay to monitor Pep1 cleavage using a PROPEP1-YFP-fusion protein. PROPEP1-YFP cleavage occurs within minutes upon wounding. This processing could be blocked with metacaspase inhibitor Z-VRPR-fmk, and not with inhibitors of other proteases. Processing of PROPEP1-YFP was also blocked with chelators of divalent metal ions. These two observations let the authors to focus on MC4, a ubiquitously expressed metacaspase that can be blocked by Z-VRPR-fmk and requires calcium ions to be active [7].

Metacaspases are structurally and mechanistically related to animal caspases, which regulate programmed cell death in animals [8]. Both caspases and metacaspases autocatalytically activate themselves from their inactive zymogen precursors through an internal cleavage event that can be detected as a shift on a western blot. However, although evolutionary, structurally and mechanistically related to caspases, plant metacaspases have very different substrates and have very different roles in plants, often unrelated to programmed cell death [9]. Most notably, metacaspases cleave after basic (Arg/Lys) residues, unlike the caspases, which cleave after aspartic acid (Asp) residues [10].

The authors provided excellent proof that MC4 cleaves PROPEP1 [1]. MC4 is responsible for PROPEP1 cleavage because: i) no PROPEP1 cleavage is observed in the *mc4* null mutant upon wounding; ii) purified MC4 can cleave purified PROPEP1 *in vitro*; ii) substitution of the Arg residue before the cleavage site in PROPEP1 into Ala (R69A) prevents cleavage by MC4 *in vitro*, which is

consistent with the specificity of MC4; iv) this R69A mutation in PROPEP1 also blocks cleavage of PROPEP1 in plants upon wounding; and v) both Pep1 and MC4 are also co-expressed in nearly every cell and colocalise in the cytoplasm. These collective experiments comprise a robust evidence that a substrate is cleaved by a specific protease.

But wait, there is more. If MC4 and PROPEP1 colocalise, why is PROPEP1 not instantly cleaved? The answer comes from the calcium-dependent activity of MC4. The calcium ion concentration in the cytoplasm is too low for MC4 to be active. Injury, however, releases calcium ions from the apoplast and the vacuole and this activates MC4 [1] (**Figure 1**). The calcium-dependent activation of MC4 was demonstrated by a shift in the molecular weight when the zymogen precursor (zMC4) is cleaved [1]. This MC4 activation mechanism ensures that Pep1 is only released upon wounding. The release of Pep1 from the injured cell is not clarified yet, but Pep1 may diffuse out of the damaged cell or is actively secreted to warn neighboring cells.

The MC4-Pep1 activation mechanism is supported by stunning cell biology work. By combining live imaging of the Yellow Cameleon probe to monitor calcium ion concentrations, with a multiphoton laser to injure a single cell, a quick calcium ion influx was demonstrated in roots [1]. This calcium ion influx coincided with the release of Pep1-YFP from the vacuolar membrane into the cytosol. The release of Pep1-YFP can be blocked with EGTA and Z-VRPR-fmk, consistent with the involvement of MC4 in PROPEP1 cleavage.

Is this all? No, this is just the beginning. MC4 will have more substrates, and PROPEP1 is not only cleaved by MC4. For instance, MC4 is essential for PROPEP1 processing in leaves, but not in roots. Arabidopsis has another eight metacaspases that may redundantly act in PROPEP1 processing. Furthermore, Arabidopsis has eight PROPEPs, most of which can be processed by MC4 [1]. And although Pep1 clearly has bioactivity, Pep1 null mutants have not yet been described, so it remains unclear to what extend Peps are essential for wound signaling, although mutants of Pep1 receptors indicate their importance [3, 4]. In addition to MC4, many other proteins will be regulated by the calcium influx upon wounding. So this work has a wider implication besides Pep1-MC4.

This study also complements six other recent studies on plant proteases involved in the release of bioactive peptides. GRIM REAPER (GRI), an extracellular peptide that induces cell death in Arabidopsis, is released from its precursor by Metacaspase-9 (MC9) [11]. In contrast to MC4, MC9 has calcium-independent activity and is extracellular [11]. IDA (Inflorescence Deficient in Abscission), a peptide regulating floral organ abscission in Arabidopsis, is processed into its bioactive peptide by multiple subtilases, implicated from the use of subtilase inhibitor EPI1 [12]. The release of RALF23 (rapid alkalinisation factor-23), a peptide triggering immunity in Arabidopsis, and GLV1 (GOLVEN1), a peptide preventing root curling, requires subtilase SBT6.1 (also called Site-1-Protease, S1P) [13,14]. However, precursor cleavage by SBT6.1 does not release the bioactive peptide, implicating other proteases. Systemin, a DAMP peptide of tomato, is released from its prosystemin precursor by subtilisin-like phytaspases *in vitro* [15], but the *in vivo* processing and its phytaspase dependency remain to be demonstrated. Likewise, RAE2 (regulator of awn elongation), a cysteine-rich peptide regulating awn development in rice, is processed by SLP1 (subtilase-like protease-1) *in vitro* [16], but also here the dependency on SLP1 *in vivo* remains to be demonstrated. Notably, all these bioactive peptides are thought to be processed extracellularly, mostly by subtilases, but the spatiotemporal control over this process remains to be clarified.

Together with these other studies, the Pep1-MC4 study shows there is a fascinating world of proteases releasing bioactive peptides. Pep1 and MC4 are conserved across the plant Kingdom, so this mechanism is likely to extend beyond the model plant Arabidopsis into your garden. So the next time you mow the grass or cut the hedge, think what happens in that damp universe.

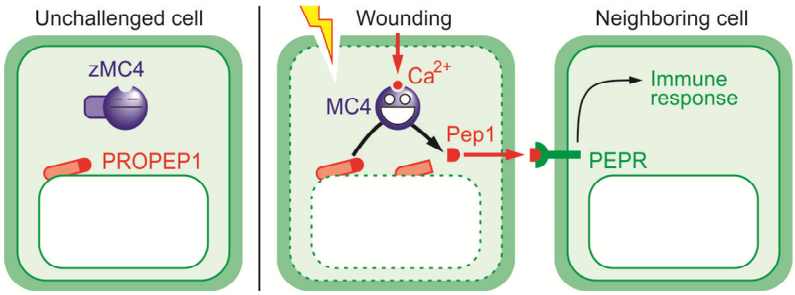

Figure 1 Mechanism underlying the wound-induced release of a damage signal.
In unchallenged cells, cytoplasmic metacaspase-4 is inactive (zymogen, zMC4) and PROPEP1 is tethered to the cytoplasmic side of the vacuolar membrane. Wounding causes an influx of calcium ions that causes autocatalytic processing of zMC4, and the generated MC4 cleaves PROPEP1 to release the Pep1 peptide elicitor. Pep1 is released from the damaged cell to trigger immune responses in neighboring cells through the Pep1 receptor PEPR.

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