Ripened by Redox: Sulfoxidation of NOR Regulates Tomato Ripening

Fruit ripening is a process unique to plants that makes their fruit more appealing to seed-dispersing animals. Ripening involves a combination of physiological and metabolic reprogramming events that lead to dramatic changes in the color, taste, texture, and aroma of fruits. Tomato (*Solanum lycopersicum*) is an economically important crop and an excellent model organism in which to study ripening due to its simple genetics, wealth of genomic resources, ease of transformation, and short life cycle (Karlova et al., 2014). Fruit ripening is underpinned by large-scale changes in gene expression, controlled by a complex network of transcriptional regulators (Giovannoni et al., 2017).

NON-RIPENING (NOR) is a NAC-domain transcription factor involved in tomato ripening. NOR has long been considered a master regulator of ripening due to the severe nonripening phenotype of *nor* mutants. Recent re-evaluation of natural mutations using genome editing technology has revealed that the *nor* mutation is a gain-of-function allele, with a frameshift mutation resulting in a dominant-negative NOR protein (Wang et al., 2019, 2020). Loss-of-function *nor* mutants were subsequently shown to have a milder phenotype than the dominant-negative mutant (Wang et al., 2019). Nonetheless, it is clear that NOR plays a central role in tomato ripening and although its downstream effects are well established, how the activity of NOR itself might be regulated remains obscure.

Transcription factors are often regulated by post-translation modifications in response to developmental or environmental cues (Skelly et al., 2016). Oxidation of sulfur-containing amino acid residues within proteins can act as reversible redox-based switches to propagate cellular signals. While Cys residues are well-established as targets of redox modifications (Bak et al., 2019), Met residues are also emerging as targets of reversible modification (Rey and Tarrago, 2018). Met sulfoxidation can occur under oxidative conditions and is reversed by Met sulfoxide reductase (Msr) enzymes.

In this issue of *Plant Physiology*, Jiang et al. (2020) report that NOR is regulated by Msr activity to influence tomato ripening. The authors showed that during ripening, hydrogen peroxide levels and global protein oxidation increase, suggesting that modification of protein redox status is related to ripening. By examining the expression of all *Msr* genes during ripening, they observed that transcripts of two of them, *E4* and *SIMsrB2*, accumulated in fruit tissue to a much greater level than those of any other *Msr* genes. Furthermore, *E4* and *SIMsrB2* showed a similar expression pattern to NOR, increasing as ripening progressed. Using various techniques, Jiang et al. (2020) demonstrated that NOR interacts with both *E4* and *SIMsrB2* in vitro and in vivo. Together, these results suggest that NOR might be a substrate of Msr enzymes during tomato ripening.

To test whether NOR is indeed a Msr substrate, the authors oxidized purified recombinant NOR before incubating it with recombinant E4 or *SIMsrB2*. Both Msr enzymes were capable of reducing oxidized NOR, and mass spectrometry analysis identified four Met residues that might be targets of sulfoxidation. The authors also examined the redox status of NOR in tomato fruit during ripening. They generated transgenic p35S:NOR-GFP plants and performed pull-down assays coupled to mass spectrometry. These experiments revealed that the Met at position 138 (Met-138) appears to be the sole site of sulfoxidation in vivo.

The identification of NOR as a potential substrate of E4 and *SIMsrB2* begs the question of how the methionine redox status of NOR affects its function as a transcription factor. To answer this, the authors generated a mutant NOR in which Met-138 was replaced with a Gln to mimic sulfoxidation (NOR-M138Q). This mutant NOR as well as oxidized wild-type NOR were compromised in binding to target DNA sequences in vitro, as shown by electrophoretic mobility shift assays. Furthermore, NOR-M138Q was defective in transcriptional activation of target gene sequences, demonstrated by transient dual luciferase assays in *Nicotiana benthamiana*. Together, these data suggest that sulfoxidation of NOR at Met-138 inhibits its DNA binding and transcriptional activity. The authors analyzed the protein sequence surrounding NOR Met-138 and observed that identical motifs were conserved in different NAC transcription factors from a wide range of plant species. They made mutants mimicking Met sulfoxidation in five of these other transcription factors, and transcriptional activities were inhibited for all proteins tested. This suggests that Met sulfoxidation of NAC transcription factors might be conserved across the plant kingdom as a mechanism to regulate transcriptional activation.

To uncover the effect of NOR Met sulfoxidation in vivo, the authors generated transgenic plants expressing either NOR or NOR-M138Q in the *nor* mutant background. Plants expressing NOR (NOR/*nor*) exhibited almost full rescue of the ripening defects of *nor*, while plants expressing NOR-M138Q (NOR-M138Q/*nor*) showed only partial rescue (Fig. 1). These results suggest that Met sulfoxidation of NOR inhibits tomato ripening. The authors also performed RNA-sequencing to compare the transcriptomes of these transgenic plants.
Compared to nor plants, genes were up- or down-regulated to a much greater extent in the fruit of NOR/nor plants than NOR-M138Q/nor plants, suggesting that mimicking sulfoxidation of NOR compromises its ability to activate target gene expression during ripening. To further test this hypothesis, the authors performed chromatin-immunoprecipitation assays to examine NOR binding at ripening-related gene promoters. These experiments showed that mimicking sulfoxidation at Met-138 decreased NOR binding at target gene promoters. Additionally, the authors showed that NOR directly binds the promoters of both E4 and SlMsrB2 and activates their expression, suggesting a potential mechanism by which NOR might regulate its own activity through a feed-forward signaling loop.

In summary, this exciting work provides evidence for post-translational regulation of NOR and uncovers a role for Met sulfoxidation in regulating tomato ripening. The authors propose a model in which E4 and SlMsrB2 accumulate throughout ripening to maintain NOR activity in an increasingly oxidative cellular environment. Redox signaling appears to be important for fruit ripening (Decros et al., 2019), but until now, no roles for Met sulfoxidation have been reported. This work by Jiang et al. (2020) provides new insights into not only tomato ripening, but also how transcription factors in general might be regulated by Met sulfoxidation. Future work should focus on how NOR sulfoxidation can be engineered to alter ripening in tomato and perhaps in other fruits, with potential for profound economic and societal impacts in the agricultural and food industries.

Michael J. Skelly1,2
ORCID ID: 0000-0002-9024-0037
University of Edinburgh, Edinburgh, EH8 9YL United Kingdom

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